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The Multifaceted Role of Nanoparticles in Biomedicine: From Early Diagnosis to Precision Therapy By Fiona Catherine Antony Sudharsan

Abstract

Nanotechnology is a branch of science that focuses on the synthesis of nanoparticles, which are materials of dimensions ranging from 1 to 100 nanometers. At the nanoscale, these materials experience quantum confinement effects and a high surface-area-to-volume ratio, which make them possess unique properties including enhanced ability to carry drugs, precise control over light scattering, and flexible modification of their surfaces. These properties support advanced drug delivery systems that enhance bioavailability, minimize systemic toxicity, and enable precise therapeutic effects at the cellular level.

1. Introduction

Nanomaterials have been used by humans for millennia, including in ancient ceramic mixtures and dyeing processes (Joudeh & Linke). A nanometer, a unit of length equivalent to 1×10^{-9} meters, is the unit used to measure nanoparticles. During the American Physical Society's annual meeting in 1959, Nobel Prize laureate Richard Feynman discussed the potential for manipulating matter at the nanoscale in his famous lecture, "There's Plenty of Room at the Bottom" (Pokrajac et al.). In the 1980s, the invention of the scanning tunnelling microscope allowed for the discovery of nanotechnology, which is made up of building blocks called nanoparticles, having a diameter ranging from 1 to 100 nanometers (Utreja et al.).

Owing to their small scale, nanoparticles display unique physicochemical properties that set them apart from the bulk materials (Murthy). As the size of the material decreases, the proportion of surface atoms increases drastically, creating a high surface-area-to-volume ratio (Rudramurthy & Swamy). This ratio allows for enhanced surface reactivity because more atoms are exposed due to the higher surface area. Their small size causes the nanoparticles to behave differently due to their increased surface energy and altered structure (Altammar). As a result, nanoparticles display more reactivity and interactions with biological molecules, and absorb and emit light. They also conduct heat and electricity, and respond to magnetic fields.

Nanoparticles can be classified into various categories based on their chemical composition, dimensions, and shape, such as organic, inorganic, metal-based, metal oxide-based, and carbon-based nanoparticles (Joudeh & Linke)(Khan et al.). Since the effectiveness of a nanoparticle lies in its intrinsic properties, each type of nanoparticle possesses its own individual characteristics that make it perform various functions across a wide array of applications in the field of scientific research. Employing these distinguished characteristics, nanoparticles have become a fundamental part of nanotechnology.

This review discusses the promising potential of various nanoparticulate systems, ranging from metal-based and carbon-based structures to lipid-based and polymeric carriers, in addressing complex clinical challenges by facilitating targeted drug delivery and improving patient-specific therapeutic responses (Waheed et al.).

Nanotechnology when applied in the biomedical field; the term is coined as nanomedicine. Nanomedicine involves the use of nanoparticles for therapeutic applications in medicine. Some properties that make nanomaterials useful in medicine are their biocompatibility, controlled drug release, and surface functionalization. With these properties, applying nanotechnology in medicine has brought significant advancements in preventing, diagnosing, and treating disease. Recently, nanomaterials have been used clinically across diverse research areas, ranging from biosensing and imaging to drug carriers and gene delivery for tumors. They can be effectively used in areas like cancer therapy, drug delivery, tissue engineering, regenerative medicine, biomolecular detection, and also as antimicrobial agents, all thanks to the different properties of nanoparticles (Liu et al.).

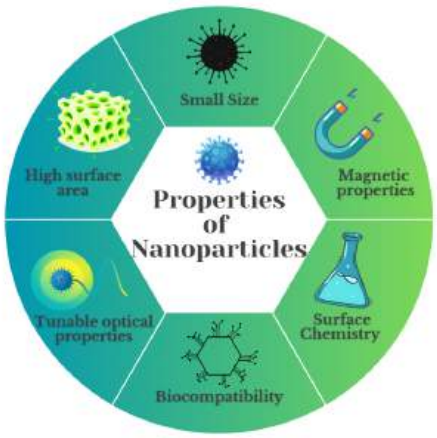

Figure 1: Properties of Nanoparticles at the nanoscale

2. Structure and Classification of Nanoparticles

The structure of nanoparticles consists of a layer on the surface constituting compact molecules, polymers, surfactants and metal ions (Kumari & Sarkar). There exists a chemically distinct layer, added over the core, which is referred to as the shell layer (Baig et al.). The succeeding layer is the core that makes up the middle portion of the nanoparticle and helps to determine the nanoparticle's characteristic properties (Joudeh & Linke).

Nanomaterials can be classified into three categories based on their dimensions. Nanomaterials are classified based on their dimensionality. Zero-dimensional (0D) nanomaterials have all three dimensions in the nanoscale (e.g., quantum dots and nanoparticles). One-dimensional (1D) nanomaterials have one dimension outside the nanoscale (e.g., nanotubes and nanofibers). Two-dimensional (2D) nanomaterials have two dimensions outside the nanoscale (e.g., nanosheets and graphene). Three-dimensional (3D) nanomaterials are materials where none of the dimensions are in the nanoscale (e.g., bulk materials) (Joudeh & Linke).

Nanoparticles can be classified into several categories based on their material composition, including organic, inorganic, metal-based, metal-oxide based, and carbon-based nanoparticles (Joudeh & Linke).

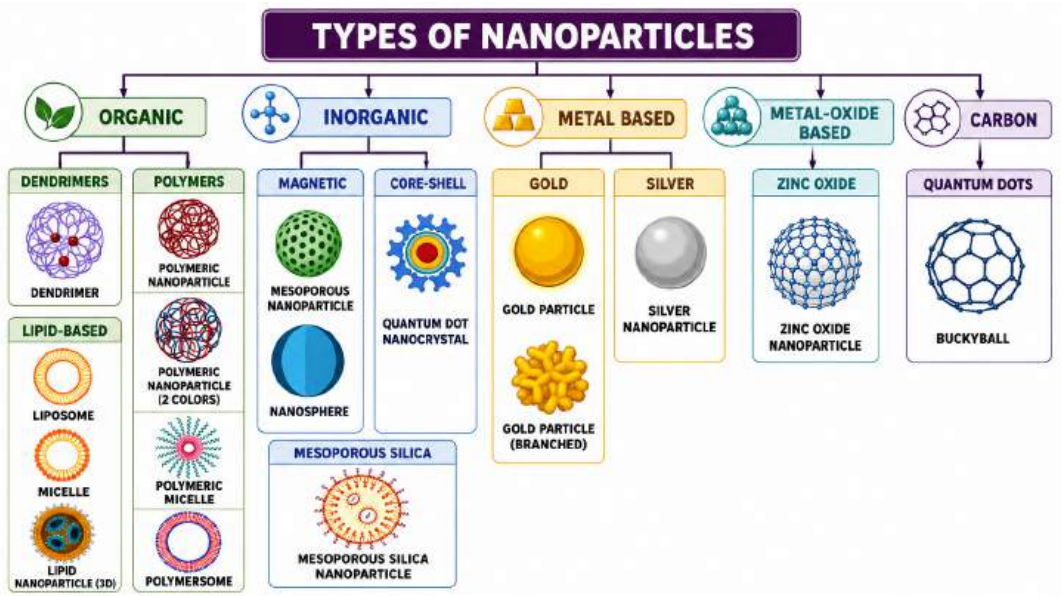

Figure 2: Classification of Nanoparticles

2.1. Organic Nanoparticles

Organic nanoparticles are primarily constituted of organic materials such as polymers, lipids (Joudeh & Linke). They can form hollow nanocapsules because they are heat-sensitive materials and also show sensitivity towards electromagnetic radiation. Some notable examples of organic nanoparticles include liposomes, micelles, dendrimers, ferritin and other lipid-based and polymer-based nanoparticles. They are well known for their environmentally benign and biodegradable nature, non-toxicity, and economic efficiency (Jha et al.). Their sensitivity to thermal and electromagnetic radiation including heat and light makes them potential candidates for targeted drug delivery.

2.1.1. Dendrimers

Dendrimers are globular in shape with high reactivity, water solubility, and consist of internal cavities (Zorab et al.). Dendrimers have a hyperbranched, tree-like structure and are approximately spherical (Algar et al.). Their internal cavities can encapsulate hydrophobic drug molecules (Sun et al.), which can be used to transport hydrophobic molecules to a hydrophilic environment and this feature aids in the delivery of drugs. Additionally, dendrimers are used in the pharmaceutical industry as non-steroidal anti-inflammatory drugs because dendrimers can reduce the toxicity of drugs and increase their efficacy (Chiş et al.).

2.1.2. Polymeric Nanoparticles

One unique property of polymeric nanoparticles is that their size can be controlled from the ranges 1 to 1000 nm, with ranges of approximately 80 nm often cited as optimal for various biomedical applications (Mendes et al.). Their polymeric composition allows for stimuli-responsive behavior, allowing drugs to be released based on environmental factors: pH,

enzymes, and temperature. These nanoparticles also exhibit tunable shape, meaning they can be modified, and surface characteristics, such as surface charge and functional groups (Chen et al.). Polymeric nanoparticles can make surface modifications that aid with reduced immune recognition, extended circulation time, and penetration across biological barriers such as mucus. Furthermore, they can encapsulate a wide range of agents which include hydrophobic and hydrophilic drugs, proteins, peptides, and nucleic acids.

2.1.3. Lipid-Based Nanoparticles

Lipid-based nanoparticles are spherical vesicles composed of ionizable lipids, helper lipids, cholesterol, and polyethylene glycol (PEG)-conjugated lipids, with each component contributing to the functionality of nanoparticles (Sarnelli et al.). Under acidic conditions, ionizable lipids become positively charged to encapsulate negatively charged nucleic acids in order to maintain neutral at physiological pH to reduce toxicity. In our bodies, lipid nanoparticles help with cellular uptake through endocytosis, while PEG-lipids enhance circulation (Mendes et al.). Helper lipids such as cholesterol promote formulation stability and membrane fusion (Mendes et al.). Furthermore, lipid nanoparticles can be used to influence biodistribution, encapsulation efficiency, intracellular delivery, and controlled release of therapeutic cargo, thereby making lipids very successful in drug and gene delivery.

2.2. Inorganic Nanoparticles

The next classification of nanoparticles is inorganic nanoparticles. The term inorganic nanoparticles means that they have no carbon. They are composed of inorganic atoms found with covalent or metallic bonds, and they can be split into further classification as metal-based, metal-oxide-based, and metal salts (Joudeh & Linke). While maintaining their crystallinity, they can become a variety of shapes. Some examples of inorganic compounds used in medicine are magnetic and silica (Luther et al.).

2.2.1 Magnetic Nanoparticles

As magnetic nanoparticles decrease significantly in size, they become single-domain particles which exhibit superparamagnetism, which is when a particle displays magnetism only in the presence of an external magnetic field (Wu et al.). So, once the magnet is removed the external field is withdrawn and the magnets lose their magnetism. On the other hand, in larger particles, coercivity, a measure of how difficult it is to demagnetize a magnetic material, increases as size decreases, but it reaches zero when particles reach a superparamagnetic state (Akbarzadeh et al.). Also, surface effects impact magnetic anisotropy, stability, and interactions with surrounding biological environments (Gavilán et al.). These properties allow the magnetic nanoparticles to be manipulated by external magnetic fields while also maintaining stability in biological systems.

2.2.2 Mesoporous Silica-Based Nanoparticles

Mesoporous silica nanoparticles (MSNs) have a highly ordered honeycomb-like porous structure with pore diameters ranging from 2 to 50 nm, which makes them have exceptionally large surface area and pore volume for the encapsulation of therapeutic agents, making MSNs highly effective drug delivery carriers (Xu et al.). With their rigid honeycomb structure, they have excellent thermal stability, chemical stability, and mechanical strength, along with high biocompatibility (Tang et al.). In addition, silica surfaces allow for functionalization with polymers, targeting ligands, or therapeutic molecules. Also, the porous structure protects the encapsulated drugs from degradation and enables stimuli-responsive drug release (Bharti et al.).

2.3 Core-shell Nanoparticles

Coreshell nanoparticles are not another classification of nanoparticles, but they fall under both organic and inorganic depending on the materials that compose them (Huang et al.). These nanoparticles consist of two regions, one of which is a central core that provides the primary functional properties. The other is a surrounding shell that controls surface interactions, stability, and biological behavior. The core region can cover therapeutic agents through hydrophobic interactions, electrostatic interactions, hydrogen bonding, or covalent attachment. The outer shell is composed of hydrophilic polymers, and it can improve water dispersibility and biocompatibility. Their moldable surface chemistry allows for modification with ligands and functional groups.

2.4 Metal-Based Nanoparticles

Metal-based nanomaterials are made up of transition metals due to their partially filled d orbitals, making them highly redox active, which is important for their catalytic properties. Unlike the organic and inorganic nanoparticles, metal-based ones have high electrical and thermal conductivity. This quality makes it easy for efficient heat transfer and electrochemical detection systems. Additionally, the structure of these particles is crystalline meaning that they will be mechanically stable, which is influential for optical, magnetic, and catalytic properties. Also, metal-based nanoparticles are useful in catalysis due to their catalytic properties (Burlec et al.).

2.4.1 Gold Nanoparticles

Gold nanoparticles are capable of scattering and absorbing visible/near-infrared light (Dreaden et al.). This property can be used to convert light into heat through photothermal cancer therapy, which helps destroy tumor cells (Wang et al.). Also, this property allows for the nanomaterials to absorb or block X-rays – called High X-ray attenuation – which helps produce a greater image contrast against surrounding tissues. Thus, they are useful as contrast agents for imaging techniques such as X-rays and CT scans (Xi et al.). In addition, their strong optical properties make them useful in biosensing, bioimaging, and diagnostic assays (Liu et al.). Due to gold being a non-reactive metal, gold nanoparticles are biocompatible and chemically stable, withstanding oxidation and deterioration.

2.4.2 Silver Nanoparticles

Due to its antimicrobial properties, silver particles inhibit bacterial, viral, and fungal growth, allowing safe use in medical environments (Doolan et al.). This property of silver nanoparticles is attributed to the release of silver ions (Ag+), which interact with microbial cell membranes (Durán et al.). This increases membrane permeability, meaning it allows more materials to pass through the membrane, which causes structural damage. Additionally, silver ions can penetrate microbial cells, where they can interfere with metabolic pathways, disrupt protein function by binding to sulfur-containing groups, inhibit DNA replication, and promote the generation of reactive oxygen species (ROS) (Alshameri & Owais).

2.5 Metal Oxide-Based Nanoparticles

Because of their nanoscale size, they exhibit altered electronic surface energy that gives them higher reactivity and efficiency than their pure metal counterparts. To add to that, they possess physicochemical properties, optical, electrical, magnetic, semiconducting, and catalytic behavior, which can be controlled by modifying their size, shape, crystal structure, and composition (Joudeh & Linke). Many metal oxide nanoparticles have excellent chemical stability and can be combined with polymers, biomolecules, drugs, and targeting ligands, improving their biocompatibility and targeting ability. Some examples are zinc oxide, titanium oxide, iron oxide, and copper oxide.

2.5.1 Zinc Oxide Nanoparticles

Zinc is a trace element which is involved in multiple biological processes, so under certain conditions ZnO can dissolve and release zinc ions (Zn^{2+}). This property allows ZnO nanoparticles to withstand high temperatures and pressures, is biocompatible, and is safe for human skin which is why it's widely used in textiles (Sirelkhatim et al.)(Wang et al.). They are also classified as semiconductors because of their wide band gap and high exciton binding energy. These properties also contribute to their optical properties: fluorescence and near-UV emission, which can be used in bioimaging, fluorescent labeling, and biosensing applications. Furthermore, ZnO nanoparticles have antimicrobial and anticancer properties because of photocatalytic properties which can generate reactive oxygen species (ROS) (Alshameri & Owais).

2.6 Carbon-Based Nanoparticles

Carbon-based nanoparticles are made from carbon, and, as such, they can form long and resistant chains. Some common structures the carbon nanoparticles form are carbon nanotubes, graphene, graphene oxide, fullerenes, nanodiamonds, and carbon dots (Yang et al.). They have high chemical stability, thermal conductivity, and electrical conductivity. Since they directly interact with bacteria causing oxidative stress that damages the cell membrane, leading to cell apoptosis, they exhibit antibacterial properties (Serrano‐Aroca et al.).

2.6.1 Quantum Dots

Due to their luminescence properties, they are used to develop sensitive and selective sensors (Yang et al.). One unique property is that carbon dots can absorb light, and then emit the light in a different color, and the light emitted can change colors by changing size: size-dependent color emission (Algar et al.). Additionally, they demonstrate photo-induced electron transfer properties making them valuable for photocatalytic light energy conversion. They display advanced capabilities for scavenging reactive oxygen species, meaning they can remove or neutralize them before they damage cells (Gao et al.). Moreover, they have low toxicity which makes them environmentally friendly.

Table 1

S.No	Type of Nanoparticle	Examples	Properties
1.	Polymeric Nanoparticles	Polymer chains	Biocompatible, biodegradable, controlled drug release
2.	Dendrimers	Branched polymers	Highly controlled structure, multiple surface functional groups
3.	Lipid-Based Nanoparticles	Lipid bilayer structures	Biocompatible, encapsulation ability, low toxicity
4.	Core-Shell Nanoparticles	Core with outer shell: Gold-silica, Iron oxide-silica	Tunable properties, improved stability, multifunctional
5.	Magnetic Nanoparticles	Fe, Co, Ni-based	Magnetic responsiveness, external control
6.	Mesoporous Silica Nanoparticles	Mesoporous silica nanoparticles	Biocompatible, porous structure, high surface area, chemically stable
7.	Silver Nanoparticles	Silver nanospheres, nanowires	High conductivity, antimicrobial, high surface reactivity
8.	Gold Nanoparticles	Gold nanospheres, nanorods, nanoshells	Biocompatible, excellent optical properties, easy surface modification
9.	Zinc Oxide	ZnO nanospheres, nanorods	Withstands high temperatures and pressure, UV blocking, compatible with skin
10.	Quantum Dots	Semiconductor nanocrystals (Ge et al.)	Size-dependent color emission

3. Applications of Nanoparticles in Biomedicine

3.1 Nanoparticles as Antimicrobial Agents

Nanoparticles possess many unique properties that allow them to serve as antimicrobial agents in biomedicine. They can interact with bacterial cells, penetrate bacterial membranes, and kill bacteria through different bacteriostatic (stops growth) and bactericidal (kills bacteria) mechanisms (Gupta et al.). Nanomaterials overcome antibiotic resistance because some bacteria resist antibiotics using drug efflux pumps that remove drugs from their cells.

Scientists can strongly influence the antimicrobial activity of nanoparticles through surface modification and thereby improve properties such as bacterial targeting, bacterial toxicity, and efficient drug delivery. For example, gold nanoparticles coated with *vancomycin*

showed increased antibacterial activity against *E. coli* and *Vancomycin-resistant enterococci* (VRE) (Wang et al.). Researchers have modified gold nanoparticle surfaces with cationic (positively charged) and hydrophobic (water-repelling) groups.

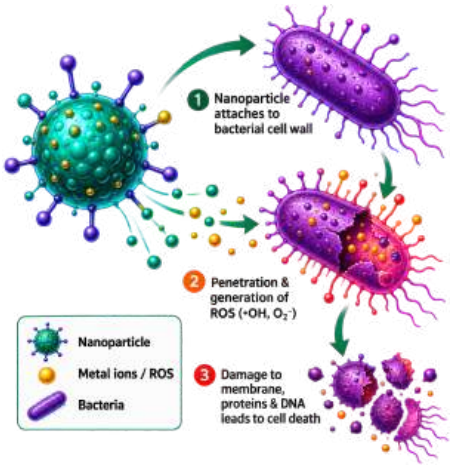

Figure 3: Mechanism of action of Nanoparticles as Antimicrobial Agents

Additionally, silver nanoparticles also work as antimicrobial agents; as the surface area increases, the release of active Ag^+ ions, responsible for the antimicrobial effect, increases. Some drawbacks of this method include the high toxicity to human cells and poor penetration into bacterial biofilms. Thus, researchers alter the surface properties of nanoparticles and increase their magnetic effect. For instance, Silver-coated Superparamagnetic Iron Oxide Nanoparticles (SPIONs) consist of an iron oxide core coated with silver, which is magnetic (Eghbalifam et al.). Owing to the enhanced magnetic behaviour, these NPs possess stronger antimicrobial activity and have better compatibility with human cells (Eghbalifam et al.). They can also efficiently penetrate bacterial biofilms due to the external magnetic fields (Eghbalifam et al.).

3.2 Nanochemistry in Diagnostic Applications

Diagnostic applications consist of the technologies used to test, detect, identify, monitor, or diagnose diseases and other medical conditions. Nanochemistry is highly beneficial in diagnostics due to the increased surface area of nanoparticles, which allows the interaction and binding of a significant number of biomolecules to them. The enhanced conductivity, on the other hand, helps to produce stronger electrochemical signals, and optical and magnetic properties are used for separation and imaging, sensitivity and specificity, detection of disease biomarkers, and low concentrations of target molecules.

One such application is a nanobiosensor, employed to improve diagnostic performance (Chamorro-García & Merkoçi). It combines biological recognition elements (antibodies, enzymes, DNA), a nanomaterial (which enhances signal detection), and a transducer, responsible for translating biological interactions into a quantifiable signal (electrical, electrochemical, optical, mechanical, or magnetic) (Holzinger et al.). The nanomaterial is designed to amplify

signal generation, increase biomolecule binding, and improve sensitivity (Das et al.)(Riseh et al.). The biological recognition element (bioreceptor) acts like a lock-and-key system, and only the correct target fits and binds to the bioreceptor. Examples of bioreceptors include antibodies, DNA or RNA probes, enzymes, aptamers, and cells.

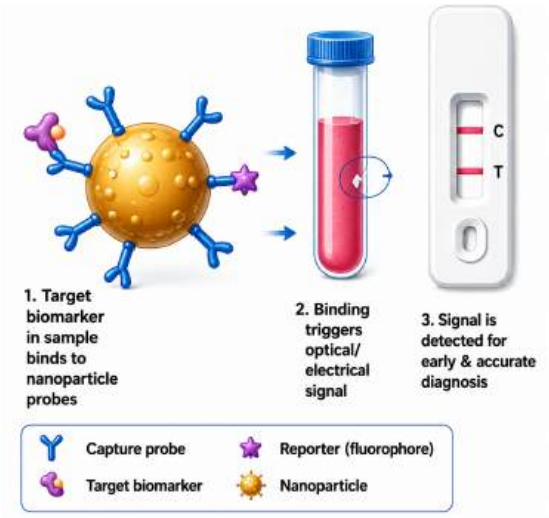

Figure 4: Application of Nanoparticles in Diagnostics as Nanobiosensors

Various types of nanomaterials widely used in nanobiosensors are gold nanoparticles, magnetic nanoparticles, quantum dots, and carbon-based nanomaterials such as graphene. Gold nanoparticles are preferred for their high sensitivity, rapid results, and biocompatibility (Kalogianni)(Yi & Xianyu). Quantum dots are employed for their long-lasting fluorescent signal and their ability to detect multiple biomarkers simultaneously. Magnetic nanoparticles are used for their faster sample preparation and improved diagnostic accuracy (Kalogianni),(Vogel et al.). Lastly, carbon-based nanoparticles are utilized for their sensitivity and rapid signal transmission.

Nanobiosensors support portable diagnostic devices that are used in hospitals, clinics, doctors' offices, remote locations, and at home (Talreja et al.). Their benefits include immediate results, faster treatment decisions, reduced need for laboratory equipment, and lower health care costs. Although biosensors are advantageous, some limitations persist, including difficulty in manufacturing on a large scale, standardization between devices, and long-term stability for clinical validation and effective translation into healthcare systems.

3.3 Nanoscale Technology in Cancer Treatment

Different types of nanoparticles, including polymeric nanoparticles, are used in drug delivery systems to transport anticancer drugs to tumor sites. These drugs can either be present inside the nanoparticle (forming a nanocapsule) or on the surface, forming a nanosphere (Beach et al.). The polymers used conventionally were non-biodegradable such as Polymethyl methacrylate (PMMA), Polyacrylamide, Polystyrene, and Polyacrylates (Chehelgerdi et al.). Their associated drawbacks include difficulty in breaking down and removal from the body, accumulation in tissue, causing toxicity and inflammation (Chehelgerdi et al.). They were,

therefore, replaced by modern polymers which are biodegradable, and superior in terms of safety, drug release control, and biocompatibility. Some polymers used currently for cancer therapy include Polylactic acid (PLA), Poly(lactic-co-glycolic acid) (PLGA), Poly(amino acids), Poly(ε-caprolactone) (PCL), and natural polymers (Chitosan, Alginate, Gelatin, Albumin). They are advantageous in the context that they protect drugs from degrading, increase drug stability, reduce the damage to healthy tissues, provide multiple administration methods (oral and intravenous), and reduce chemotherapy side effects (Sun et al.).

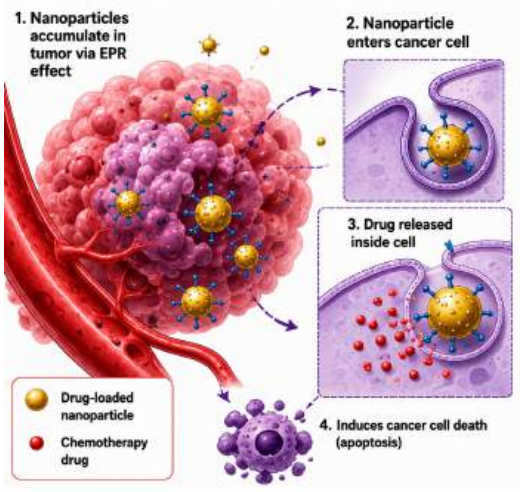

Figure 5: Schematic of Cytotoxicity Mechanism of Nanoparticles in Cancer Cells

Another nanoparticle used in cancer treatment are lipid-based nanoparticles such as liposomes. They were the first developed nanoscale drug delivery system made of phospholipid bilayers. The phospholipids are made up of a hydrophilic core (meaning it interacts favorably with water) and a hydrophobic tail (meaning it does not interact favorably with water). So, the hydrophilic drugs would be stored in the core, and the hydrophobic drugs would be stored in the lipid membranes where the tails are. Some advantages of using lipids are that they protect drugs from degradation, are biocompatible, have low toxicity, are biodegradable, and can deliver multiple drugs in one go (Wang et al.). When liposomes are coated with PEG (polyethylene glycol) they have increased circulation time and they can reduce immune system removal. On the other hand, when liposomes are loaded with doxorubicin, they can be used in cancer therapy. Liposomes respond to pH changes, enzymes, light and redox conditions. This allows them to detect tumors because tumors are usually characterized by a lower pH, increased enzyme activity, and hypoxia, which is the lack of oxygen in body tissues. Some limitations of liposomes is that they have a short shelf life, they can only carry some drugs, they have stability problems, and that they are rapidly removed by immune cells (Wang et al.).

Lastly, dendrimers are also used in cancer treatments as they are a type of highly branched nanoscale polymer. They are made up of three parts: the central core which holds drugs, the branches which are the internal structure, and the surface groups which allow targeting modifications. Advantages of using them include their precise structure, high drug solubility, good drug delivery ability, and they can transport DNA/RNA (Chehelgerdi et al.). Some

examples of them are PAMAM dendrimers which commonly deliver chemotherapy drugs and are used in cancer imaging. They can combine chemotherapy and photothermal therapy. Some limitations to dendrimers are their low stability, poor cellular uptake, and low gene delivery efficiency (Chehelgerdi et al.).

3.4 Nanoparticles for Targeted Drug/Gene Delivery

In gene and drug delivery, nanoparticles can be used to transport drugs and genetic material directly to the targeted cell or tissue. One advantage of them is that they can protect drugs and nucleic acids from degradation before reaching the targeted cell. With their small size, they can circulate easily through the bloodstream and can pierce through tissues that other drugs cannot easily access (Mitchell et al.)(Li et al.). Their high surface area to volume ratio allows large amounts of drugs, genes, or ligands to be attached to a single particle as there are more space around atoms on the outside than on the inside.

During drug delivery, nanoparticles can bind to agents such as small molecule drugs, proteins, peptides, antibodies, and nucleic acid therapeutics. They can improve the solubility of drugs, therefore, increasing their bioavailability (Wang et al.): meaning the rate at which a drug successfully reaches the bloodstream and becomes available to target active sites in the body. Additionally, they can protect drugs from premature metabolism or degradation, meaning it can stop metabolism under strain before it turns into a full disease. They have controlled, sustained, or stimuli responsive drug release, which maintains therapeutic concentration and reduces dosing frequency. When surface modifications are made to nanoparticles, they can selectively bind to receptors that are overexpressed on diseased cells. They can target through passive and active targeting methods. The active method is where ligands on the surface bind to receptors on cancer cells. The passive method is through the enhanced permeability and retention (EPR) effect, in which nanoparticles accumulate because of leaky tumor blood vessels (Chehelgerdi et al.).

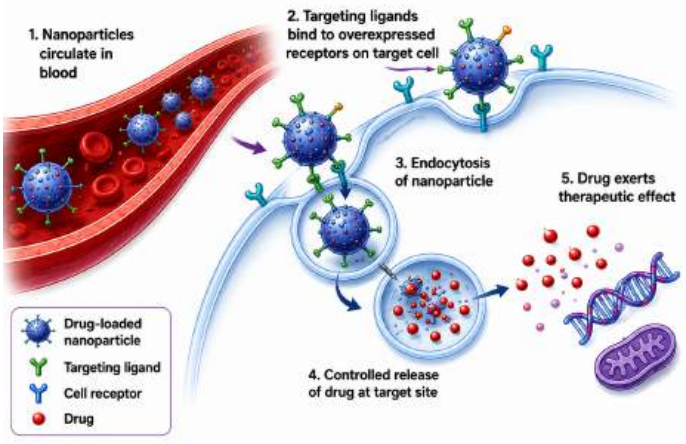

Figure 6: Nanoparticles for Controlled Release and Targeted Drug Delivery

Nanoparticles can also be useful in gene delivery. They can deliver various genetic material such as plasmid DNA, messenger RNA (mRNA), small interfering RNA (siRNA), Micro RNA (miRNA), and CRISPR-Cas gene-editing components. During transport, they can

protect nucleic acids from degrading. They can help cellular uptake through endocytosis and some nanomaterials can help genetic material to reach the cytoplasm or the nucleus before degrading in a process called endosomal escape (Li et al.). The genetic material is very important as they can be used as replacement for inherited disorders, for silencing using siRNA, or editing through CRISPR technologies. Some advantages of nanoparticle delivery are that lower immunogenicity (the ability of a foreign substance to trigger an immune response in the body), reduces the risk of mutagenesis (changing genetic material to create a mutation), and it is easier to produce large scale.

When nanoparticles carry drugs and imaging agents together, they create theranostic platforms (Pellico et al.). These platforms allow clinicians to track distribution of nanoparticles in real time, monitor drug release, assess treatment response, and adjust the therapy based on the imaging results.

On the whole, nanoparticles can target chemotherapy by reducing systemic toxicity. They can also help with the treatment of inherited genetic diseases, as well as, it can help with the delivery of mRNA vaccines and RNA-based therapeutics. They can help with the treatment of infectious diseases through the delivery of antimicrobial agents, and it can help with regenerative medicine and tissue engineering. Lastly, it can help personalize medicine by tailoring it to each specific individual, and it allows for combination therapies which can deliver multiple drugs together (Li et al.).

3.5 Nanoparticles in Medical Imaging

Nanoparticles can improve both optical imaging, using fluorescence microscopy, and magnetic resonance imaging, which is an MRI (Algar et al.). They can provide higher sensitivity, meaning that the imaging technique can detect very small amounts of biomarkers; improve image quality, making it easier for healthcare professionals to distinguish between healthy and unhealthy tissue; and provide more targeted imaging, which improves the detection of disease. Depending on the imaging technique, different nanoparticles and compositions will be used.

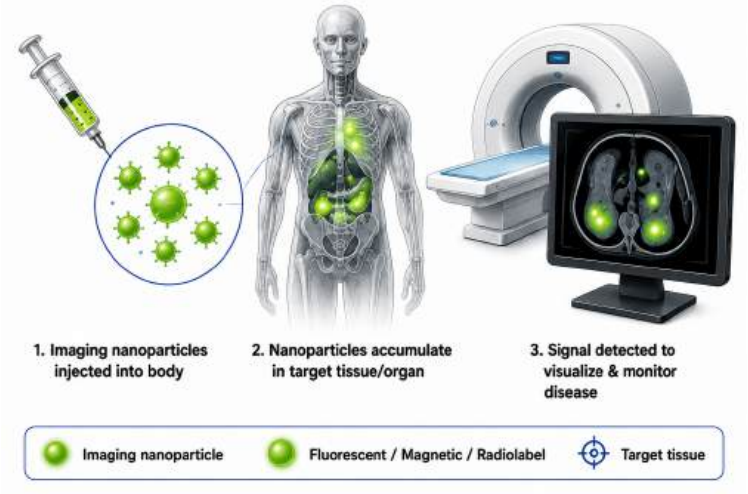

Figure 7: Nanoparticles for Imaging of various diseases

In optical imaging, researchers use organic fluorescent dyes such as fluorescein isothiocyanate (FITC) and rhodamine, which attach to biomolecules that bind to cells. One limitation of this conventional method is the low fluorescence intensity and photobleaching, which happens when fluorescence gradually fades because of damage to the dye molecules during imaging (Ashoka et al.).

On the other hand, there are quantum dots which are used as fluorescent imaging agents. They can emit bright fluorescent light when exposed to ultraviolet (UV) light. The wavelength or color of the light depends on the size of the quantum dot, as mentioned earlier. The fluorescence of quantum dots is much brighter and more resistant to photobleaching than the organic dyes (Refaat et al.). This technique is useful in long-term imaging, for three-dimensional tissue imaging, and high-resolution cellular imaging.

Additionally, quantum dots are insoluble in water, so when using them biomedically, they are coated with a water soluble material (Piao et al.). At the same time, their surfaces can contain many attachment sites: biomolecules, drugs, targeting ligands, and polymers. These attachment sites allow the nanoparticle to perform multiple tasks like imaging and targeting simultaneously (Yukawa et al.). This surface modification using the attachment sites allows quantum dots to bind to specific tissues or cell types. Specifically binding to targeted ligands, increases imaging specificity while reducing binding to healthy tissue (Sun et al.). These surface coatings also help with improving circulation time, increasing retention in the bloodstream, and enhancing targeting efficiency.

Some examples of this technique being used are near infrared quantum dots being used to map lymph nodes in mice and pigs (He et al.). Using NIR allows visualization of lymph nodes up to 1 cm under the skin without surgery. Another instance is peptide coated quantum dots targeting lung endothelial cells in mice (Wei et al.). Similarly, quantum dots were targeted to blood vessels and lymphatic vessels within tumors (Pang et al.). The targeted quantum dots were taken mainly to target cells through endocytosis and minimally taken up in healthy tissue. Furthermore, quantum dots are involved in tumor imaging. Quantum dots, which were covered with polymers and antibodies, were to target prostate-specific membrane antigen (PSMA). After IV injections, these quantum dots will accumulate in tumors and will not accumulate anywhere else demonstrating targeting specificity. This quality of quantum dots helps improve tumor visualization and aids in cancer detection and image-guided therapy (Hasan et al.).

MRI is used to visualize internal structures of our body. So it is commonly used to diagnose diseases in the brain, spine, and our muscular system (Pellico et al.). An MRI works by sending signals from hydrogen atoms, which are present in water and tissues. Using a magnetic field, the hydrogen nuclei align and a radiofrequency pulse changes their alignment. While the hydrogen nuclei return to their original relaxed state, they emit signals that are used to generate images (Lü et al.). There are two relaxation times that MRIs depend on: T1 which is the time required for nuclei to realign with the magnetic field and T2, the time required for transverse magnetization to disappear. These relaxation times are shown differently on scans because they

require different times to happen (Tuguntaev et al.). Contrast agents are used to enhance these images.

One such contrast agent is the superparamagnetic iron oxide nanoparticles (SPIO), which are a T2 agent (Huang et al.). Because of their superparamagnetic properties, they produce stronger magnetic effects than normal contrast agents. SPIO can be modified with antibodies to target cancer cells. One such case is when SPIO nanoparticles were modified with Herceptin to target breast cancer cells. The targeted nanoparticles accumulated in tumors and caused a rapid change in the MRI signal.

Overall, using nanoparticles in MRI helps improve image quality, contrast, and diagnostic accuracy (Lu et al.). It helps visualize the smaller tumors and allows for targeted molecular imaging through surface functionalization. Also, it increases sensitivity in MRI allowing for the detection of disease at earlier stages.

Conclusion and Future Prospects

Nanomaterials are helping advance precision medicine by combining improved imaging with treatments that deliver therapies directly to specific targets, addressing some limitations of conventional contrast agents (Wang et al.).

Although challenges remain, using nanotechnology will be a hope for the future. It is likely in a few years that nanomaterials will be used very commonly in the medical field. Scientists hope to make huge progress in the treatment of chronic diseases which will align with their goals of reducing accumulation of non-biodegradable nanoparticles in the body, which may interact with the biological processes of the cells inside the human body, which is counterproductive. There are some risks as this technology will raise ethical issues and it is likely the technology will be exploited for destroying human lives instead of benefitting them by making biological weapons. In the future, treatments requiring nano injection will likely be expensive, thus mostly wealthy individuals will reap benefits. Despite the nanoparticles demonstrating “health for all”, regrettably it is not possible to achieve this in real life.

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A Comparative Analysis of CRISPR-Cas9 and Gene-Lowering Approaches for the Treatment of Huntington's Disease By Zixuan Zhou

Abstract

Huntington's disease is a severe autosomal dominant inherited disease, which means a person will suffer from this disease even if only one allele is mutated. The disease is caused by CAG trinucleotide repeat expansion in 4th chromosome, HTT gene, and only a little approach could be helpful to treat this disease from now. Today, approximately 5 out of every 100,000 people will suffer from this disease all around the world. This article explores two groundbreaking technologies that emerged at the end of 2025, marking the first time Huntington's disease has ever been successfully treated. Two rivaling approaches are at play: CRISPR-Cas9 editing of the gene responsible for Huntington's disease (HTT), and gene-lowering, which involves the degradation of the mRNA produced by miRNA. In previous research, the gene-lowering method functioned in mutant Huntington mRNA, instead of targeting the gene itself. This paper compares the benefits and shortcomings of these two prevailing methods for curing Huntington's disease. According to this paper, the gene-lowering method is relatively mature, and it is getting increasingly closer to being released in the market as a drug. The CRISPR-Cas 9 method is theoretically more efficient due to its once-and-for-all characteristic, and could be more convenient. However, both treatments are all facing the delivery challenge. This paper provides a comprehensive comparison of two novel approaches for treating Huntington's disease, providing a roadmap for where this research needs improvement, and may progress in the future.

Introduction

Huntington's disease is classified as a neurodegenerative disorder. This means that it will damage some specific areas of the people's central nervous system. The source of Huntington's disease is a mutation of the HTT gene (Tong et al.). This particular gene is located on the 4th chromosome. For many patients, within the HTT gene, the 1st exon will continuously copy a "CAG" mutation into the gene, termed a trinucleotide repeat expansion. In people unaffected with Huntingtons, the CAG sequence within the HTT gene only exists around 6~35 times; however, patients with Huntington's disease can have as many as 40 times the number of CAG insertions (Mühlbäck et al.). The HTT protein is an essential protein for human neurons to survive, grow properly, and assist in intracellular transport within the central nervous system (CNS). However, mutated copies of the HTT protein have an abnormal length, leading to misfolding and build-up within the CNS, where it can become toxic and cause neuronal death (Palaiogeorgou et al.). In summary, the CAG repeat expansion in the HTT gene results in a misfolded protein that accumulates in the CNS, driving the neurodegenerative process characteristic of Huntington's disease.

Huntington's disease has three primary symptoms: movement disability, cognitive dissonance, and psychiatric disorder (some serious psychological problems such as depression,

bipolar) (Mehanna and Jankovic). The impacts to movement can be divided into two parts: involuntary motion disorder and voluntary movement disorder. Involuntary movement can cause patients to exhibit dance-like twitching, which is an apparent symptom of Huntington's disease, and is done unintentionally by the patient. The voluntary movement disorder presented by Huntington's causes patients to lose the ability to move and operate their muscles efficiently, or in some cases, completely lose the ability to move at all. What's more, cognitive impairment can greatly impact the lives of those affected. Patients can begin to exhibit a loss of attention, the ability to change their minds or switch between perspectives in different contexts, and the loss of future planning (Andriessen et al.). Language can also decline as the disease progresses. Besides that, patients might exhibit psychiatric symptoms such as anxiety, depression, and aggression, which greatly impact the patient's quality of life.

Researchers have spent decades trying to elucidate the mechanisms leading to Huntington's disease, to develop therapies that could stop the progression of the disease, or prevent it altogether. The first step was discovering the gene responsible for the disease (*HTT)*, which was finally found in 1993, and represented a breakthrough in the field (Alkanli et al.). Today, tests for the repeat expansion of the *HTT* gene are the most reliable biomarker used to diagnose a patient and are considered a genetic biomarker (Lepinay and Cicchetti). However, there are other biomarkers that can be used to diagnose or predict the onset of Huntington's. Protein-based biomarkers detection of the mutant Huntingtin protein in the blood (Lepinay and Cicchetti), neurofilament light chain (NfL), which can be found in the cerebrospinal fluid (Coppens et al.). Finally, non-invasive imaging such as MRIs and PET scans (Farag et al.), and motor control and clinical assessments could be efficient instruments to measure and present the results (Mestre et al.). Each of these biomarker-based diagnosing methods is carefully considered and employed by physicians when diagnosing patients with Huntington's disease (Byrne et al.). That being said, there are limitations to each of these biomarker-based diagnostic tools, and none is completely efficient. A major obstacle in biomarker development is the lack of standardised, sensitive detection methods validated by rigorous statistical analysis. (Przybyl et al.).

There has been considerable effort put into developing treatments for patients with Huntington's disease, and two have emerged that have relatively high efficiency. The first is gene-lowering therapy (RNA interference) and the second is Gene-editing therapy (CRISPR-Cas approach) (Alkanli et al.; Belgrad et al.). Gene-lowering therapy. uses siRNA or miRNA to target the mRNA of the mutated *HTT* gene, thereby preventing the production of the toxic protein (Fihurka et al.). siRNA (small interfering RNA) and miRNA (microRNA) are two different RNAs that act to identify the mutated *HTT* mRNA gene product and to denature (break down) the mutated mRNA (Alkanli et al.). But this method has an apparent limitation: it is difficult to efficiently deliver siRNA and miRNA through the blood-brain barrier (BBB), a highly selective biological barrier located between blood and CNS, and it has the purpose of blocking any harmful substance in blood from entering the brain (Chen et al.). Additionally, these RNAs can degrade within the intestinal tract and blood due to pH and other factors, potentially limiting

their effectiveness (Vignone et al.). So, this approach is relatively safe because it will not modify DNA, but it faces some problems that are still waiting to be solved.

The second method employed to treat Huntington's Disease is a CRISPR Cas-9 therapeutic strategy. CRISPR-Cas9 is a gene editing tool that acts like a precise pair of scissors that can cut off the mutated part of a gene. By targeting the tandem repeats in the mutated HTT gene, researchers may be able to prevent production of the mutated protein and restore production of the healthy HTT gene protein (Ling et al.). For instance, targeting of caspase activity, targeting of mitochondrial dysfunction, Targeting of Transcriptional dysregulation, and targeting of mHTT (Fields et al.). Researchers will use these targets to target the mutated HTT gene, then, using CRISPR, will induce a double-strand break in the DNA (Alkanli et al.). Through this process, the researchers can effectively target the cells impacted by the mutated HTT gene and use CRISPR technology to repair it.

Both approaches, in theory, hold great promise for treating Huntington's disease. However, each therapeutic strategy takes a different approach, which in turn, presents its own distinct challenges.

Session 1: UniQure (Gene-lowering approaches)

A company called UniQuire, located in Amsterdam, Netherlands, has taken a gene-lowering therapeutic strategy for treating Huntington's Disease. Gene-lowering involves using RNA to find and denature the mRNA (protein-coding product of a gene), and then suppressing its ability to be made into a protein. The miRNA and its possible carriers cannot cross through the Blood-Brain Barrier efficiently, so UniQure tried a new method, using nuclear magnetic resonance as a leading approach, implementing the intrastriatal injection. This injection means directly injecting the drug into the corpus striatum, so the drug can bypass the BBB (Evers et al.). A gene-lowering therapeutic strategy is particularly apt in the case of Huntington's disease, since the mutated HTT gene mRNA transcripts can be well established for a given patient ahead of treatment. This means that the risks could be lower for the patient, given that no DNA will directly be edited, but rather the products of the gene are edited. Although this technology is new for the treatment of Huntington's disease, it has been used before. One of the examples of this technology is RNA interference (RNAi) for cancer diagnosis and therapy (Yoon et al.). In this approach, plasmonic nanomaterials, such as gold or silver, are attached to mRNA and used to kill cancer cells through the process of heating them (given that gold and silver strongly absorb light) through a process called photothermal therapy. This research approach for the treatment of cancer is well underway in Korea, and new materials are in the process of exploration(Alkanli et al.).

Another emerging technology using RNA as a therapeutic is siRNA. First, specially designed dsRNA (double-stranded RNA) is delivered into the human body (Dar et al.). Next, an enzyme named Dicer will cut the dsRNA into several smaller siRNAs (silencing RNAs), each with a size of only around 21~23 nucleotides (nt). The newly formed siRNAs are able to form a complex with a protein called the Argonaute, which, when combined, is called the RISC

complex. The entire RISC complex works as a silencer to cut off or silent target mRNA. Inside cells, the Dicer enzyme will load double-strand miRNA onto Argonaute protein (Pratt and MacRae). Then, RISC will unwind the miRNA, and drop one strand named passenger strand and keep another strand named guide strand (Deerberg et al.). After this, RISC is activated. It will patrol and scan mRNAs in the cell. Once it finds any mRNA that is 100% matched with a guide strand, Argonaute protein will slice the mRNA. If the mRNA is only partially matched, RISC will bind with this mRNA and prevent it from being translated (Iwakawa and Tomari).

While these technologies offer immense promise, they also require additional troubleshooting before they are ready for patient use. The central difficulty is that they require an immense amount of labor to generate the necessary components, because they must be individualized per patient. For example, miRNAs used to treat patient diseases must be produced such that they exactly mimic the structure of a naturally produced miRNA. If these miRNAs are too dissimilar from a naturally occurring mRNA, scientists risk activating the immune system of the patient and causing immune rejection, which can be fatal (Kotowska-Zimmer et al.). Due to existing technical limitations, it is possible to produce isomers, or miRNAs with the same molecular formula that have a different structure, leading to a completely different function within the human body. This has the potential to be very dangerous, so additional research and troubleshooting are necessary to expand these methods so they are ready for patient use.

Section 2: Spark Therapeutics (CRISPR-Cas approach)

The other company working quickly to establish a treatment for Huntington's disease is Spark Therapeutics, located in Philadelphia, PA, USA. Their treatment approach involves directly editing the mutated HTT gene so that the HTT mRNA produced will not have the tandem repeats associated with Huntington's Disease. This approach relies heavily on CRISPR-Cas9.

CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) is the most advanced technology in gene editing at present. The CRISPR-Cas9 system uses a customized gRNA to scan the DNA looking for an exact complement to its sequence. Once found, the Cas9 nuclease induces a double-strand break (DSB) in the DNA at the location of interest. This can be used to achieve the inactivation of genes (gene knockdown), the removal of a gene (gene knockout), and the insertion of a different gene (insertion). While CRISPR-Cas9 is a great candidate for treating Huntington's disease, one major problem exists: how do we get the CRISPR-Cas9 components into the brain? To overcome this limitation, Spark Therapeutics has exerted two approaches. First, they developed new Cas9 proteins with smaller size. Traditional Cas9 is oversized and hard to go across the BBB, but the new Cas9 protein named SaCas9from staphylococcus aureus can be packed in single AAV perfectly, which is more efficient to be delivered (Dai et al.). Second, they exerted a directed evolution on AAVs that will be used to deliver drugs for the purpose of letting AAV spread wider in patients' brains. They selected AAV kinds in Non-Human Primates (NHP) and found some AAVs that are efficient in infecting

humans such as AAV Ep+. Those new AAV kinds can cover a larger scale of treatment in single injection, which allows the therapy to become more efficient(Tecedor et al.).

CRISPR-Cas9 therapy has had immense success for treating life-threatening genetic diseases. First, Casgevy was the first ever FDA approved treatment for sickle cell disease in December of 2023. It is the therapy for reversing sickle cell disease and ß-thalassemia (Goswami et al.). Scientists used CRISPR to cause an HPFH3 genotype (cut off HBD and HBB). HBB is an essential gene, mutations of which will cause ß-thalassemia, which prevents the hemoglobin to progress from its neonatal form to its adult form, and sickle cell disease. Some people are born with the HPFH3 genotype, but some aren't, and the deletion of HBB and HBD is totally harmless (Goswami et al.).

Second, scientists used CRISPR to cure Duchenne Muscular Dystrophy, which is a genetic muscular issue (Happi Mbakam et al.). Humans contain a gene named the DMD gene. It is one of the biggest genes in the human body, and it produces a kind of protein named dystrophin in order to increase the strength of muscle cells. Some people don't have this gene, so when they contract their muscles, their muscle cells break. Most of them will die due to heart or breathing problems by 18-30 years old (Erkut and Yokota). So scientists used CRISPR to cut the problematic genetic part, then let the body repair it by itself. The best outcome was that 60% of the dystrophin expression ability was restored. Although the result and efficiency of this approach are not that stable, it provides us with huge development and direction guidelines (Chemello et al.).

Regarding this approach, scientists have already gotten some results for the treatment of HTT, but are also facing some problems. One major result is the CRISPR-Cas13d technology (Hart et al.). This technology will only cut RNA, instead of DNA. Scientists finished gRNA and off-target tests, HTT patients' cell tests, and rat tests; and the results were all better than they thought (Morelli et al.). What's more, Cas13d can stay in body and work for a long period, so the cost will be lower (Treichel and Bazzini).

The second strategy utilizes the combination of PAM (Protospacer Adjacent Motif) and SNPs (single nucleotide polymorphisms) to reach allele-specified editing. The PAM sequence is a kind of identification tool. When Cas9 protein is sliding on DNA, PAM will be used to identify a kind of sequence such as NGG (N represents any kind of nucleotide from A, G, C, T) (Klanschnig et al.). Only when it identifies the sequence successfully, the Cas9 protein will start working (Silverstein et al.). For example, mHTT and the normal HTT gene are considerably homogeneous, only some special SNPs tend to occur between the two alleles. Due to the strict needs on a specific PAM sequence (e.g. NGG) of SpCas9 to activate DNA modification (Mojica et al.), our alleles from both parents, one of the SNPs in an allele can produce a PAM, but another can't (Zhuang et al.). The Spark Therapeutic then tested more than 8000 patients and confirmed some SNP points that can produce the PAM. They picked some SNP points, and made sure that the points would only produce one kind in the mHTT gene and another in the normal HTT gene, and finally, they located the kinds that will occur in mHTT and then cut off the whole mHTT gene sequence (Alkanli et al.; Feng et al.). However, this kind of technology is limited to

be used on a considerably large scale due to the limitation of specificity. The general version of Cas9 protein,SaCas9, could cover a large variety of SNPs and can be used to treat a larger range of patients. But the SaCas9 has a backwardness that is its oversize and difficulty of delivery. Newly developed Cas9, SpCas9, is smaller and can be delivered into the brian more efficiently, but it loses parts of ability to cover patients as wide as SaCas9 do (Ran et al.; Yang et al.).

Spark Therapeutics has successfully constructed a large-scale map for PAM-altering SNPs (PAS), which covers thousands of patients (Fang et al.). Besides that, Spark Therapeutics operated an experiment successfully that the precision of PAS altering could be extremely high because there was no off-target effect (Shin et al.). They aim to cover around 60% to 70% of Europeans about their PAS points, and create a one-and-done therapy for patients (Fang et al.).

Section 3: Assessing Huntington's Disease Treatment Modalities

There are several commonalities shared between UniQuire and Spark Therapeutics' approaches to treating Huntington's disease. For instance, both approaches aim to reduce the amount of hTT mRNA in the brain, thereby reducing the accumulation of the toxic protein within the brain, leading to the development of Huntington's disease. Secondly, both companies took a similar approach to increase target specificity and precision. In UniQuire's gene-lowering approach, target specificity relies on the natural mechanism of base pairing to target the hTT mRNA for degradation (Yao et al.). Similarly, the CRISPR-Cas9 approach used by Spark Therapeutics uses a gRNA to find complementary *in vivo* DNA to target for direct editing. Both approaches rely on homology and complementarity to achieve the goal of reducing hTT protein buildup in vivo.

In addition to the mechanisms of action, sharing many core commonalities with respect to their biological function, there are shared delivery and mechanistic challenges. The BBB is one of the biggest hurdles to treating diseases located in the central nervous system. Spark Therapeutics' CRISPR-Cas9 approach faces many challenges: in normal cells, delivery may disrupt cell integrity (Aussel et al.). When carriers attempt to enter cells, the cell membrane can sometimes be disrupted, resulting in decreased efficacy. However, both Spark Therapeutics and UniQuire have developed a similar design strategy in which the BBB is surpassed altogether. Through this approach, MRI technology is used to drill small holes into the skull and insert thin hollow tubes, called cannula, into the precise regions of the brain where the HTT gene is expressed. Next, the treatments are delivered into the thin cannula. In a Nature article published in October, 2025, the scientist Kostyk remarks that "it's not a minor procedure" (Dolgin). So, while this method may allow researchers to edit brain tissue, it is not a simple or trivial pursuit. Furthermore, the cost is a widely known concern of both of the two therapeutic strategies. High cost could be a huge obstacle for patients to use the two methods.

There are remarkable differences between these two approaches as well. Both therapeutic approaches act on different molecular targets. UniQure's gene-lowering approach targets mRNA. Some argue that targeting the mRNA instead of the DNA is a safer strategy because its effects are reversible, reducing concerns about irreversible and risky outcomes that come with

permanently changing the DNA sequence using CRISPR-Cas9 (Bhati et al.; Ghosh and Tabrizi). Spark Therapeutics, on the other hand, uses gRNAs to target particular sequences and induce mutations using CRISPR-Cas9. This approach may be more convenient, as only a single treatment may cure, or at least significantly reduce the disease burden (Bao et al.). However, it is extremely risky because the process is irreversible. If off-target effects occur, patients may suffer unknown and potentially severe mutations, particularly given that no longitudinal studies have been done to monitor long-term side effects of CRISPR-Cas9 treatments, given the novelty of the technology.

Moreover, the durability of the two approaches also differs. Gene-lowering approaches provide reversible therapy, so durability is limited—sometimes relatively short—depending on the condition and technique used. For example, uniQure's AMT-130 offers effects lasting nearly 30 months, or over two years, per administration (Mwape et al.). On the other hand, CRISPR-based treatments offer nearly permanent durability. By modifying DNA, which is stably replicated, the effects persist (Cohn et al.). This is convenient and comfortable for patients, avoiding repeated MRI cannula brain injections. However, this permanence also means that if modifications go wrong, the resulting issues may be difficult to correct. Although there are only two methods on the way from now, they give patients all around the world large hope, encourage patients to keep conflicting with Huntington's disease and never give up.

Conclusion

A disease once thought to be nearly impossible to treat, Huntington's disease now has two promising treatment approaches. Once thought of as a near impossibility, the treatment of the first ever patient with Huntington's disease in late 2025 marks an incredible turning point in our ability to treat neurological diseases (Dolgin).

Promising results have been seen through two different approaches: targeting DNA or mRNA. While each presents its own challenges and benefits, additional research is necessary to fully elucidate the safety and efficacy of these treatments over time. In both cases, immune responses, off-target effects, and delivery methods must be carefully monitored to ensure the best patient outcomes. Advancements in this technology will not only benefit CRISPR and gene-lowering technologies but will also impact all forms of in vivo drug delivery. Furthermore, the need for accuracy can better ensure patient safety and guarantee that each treatment produces a positive effect. These two technologies still have a long way to go, but clarifying the direction of development will make technological progress more efficient and clearer.

The development of the drugs for the treatment of Huntintgon's disease could be an extremely conducive deal. Thousands of families are suffering from Huntington's disease every single year. The success of this sort of technology could be life-saving for all patients of Huntington's disease in the world.

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The Efficacy of DRE in Inducing Apoptosis in Cancer Cell Lines By Rohini Saha

Abstract

The study of *Taraxacum officinale*, a plant from the Asteraceae family, has increased extensively in laboratory research within the past decade, particularly in clinical trials focused on cancer cells. Both the plant's anti-cancerous and anti-metastatic attributes are associated with its potential as a natural anti-oxidant*. Current findings present in the literature review indicate a lack of standardization into sensitivity levels of cancer types when induced with dandelion root extract, as well as the extent to which it works best. The present study was formed to explore how various cancer types influence the cytotoxicity levels of DRE, through both cell viability and IC50 values in in-vitro studies. To resolve this matter, the study utilized a two part methodology design: (1) a PRISMA-guided systematic review (2) Standard clinical meta-analysis. Results of the study indicate a clear pattern of hematological* cancer types having higher levels of cytotoxicity when treated with DRE. The findings from this study can shape approaches to future experiments testing the efficacy of DRE as a potential cancer therapy (for organizations such as the AACR) and expedite the process of finding innocuous* alternatives for treating malignant cancers.

Introduction

Across generations of medical history, various dandelion extracts have been commonly utilized in traditional medicine to counteract inflammatory conditions and digestive diseases (Yang, 2015). In a study concerning human melanoma* cells, researchers from the University of Windsor discovered that "it is fast-acting, nontoxic, and therefore specific in its targeting of human melanoma cancer cells" (S J Chatterjee, 2010). To put into context, Chatterjee's study was significant in the shift from DRE's original categorization as a traditional medicine to a modern pharmaceutical, and it sparked a new research trajectory for cancer biology. This pioneering study specifically identified DRE as a natural source of anti-oxidants* due to the presence of several phenolic* compounds, allowing it to be anticarcinogenic*, antiangiogenic*, and antinociceptive* (S J Chatterjee, 2010). Natural anti-oxidants are favoured over synthetics due to their efficacy in safety and health, bioavailability*, as well as reduced side effects to name but a few. Additionally, its success as a natural medicine has increased its study in experiments concerning other malignant* cancers. Another study discovered that DRE has a tendency to selectively induce cell death in cancer cells while leaving normal colon mucosal epithelial cells relatively unaffected (Ovadje, 2016). However, as discovered by researchers at Tarbiat Modares University, "chemotherapeutic drugs cannot distinguish normal cells against cancerous cells"(Mollaei et al, 2021). Therefore, unlike modern means of chemotherapy that target both benign* and malignant* cells, DRE only targets malignant* cells. This indicates an advantage over synthetics and highlights the potential of dandelion root as a component for future plant drug therapies. The success of DRE as a natural anti-oxidant source and its success in selectively targeting only malignant* cells proves researching DRE in its potential in selective cancers.

Literature Review

Acquisition of Sources

For the purposes of conducting this study, only peer-reviewed papers, public organization information, and public health service studies were utilized to form a credible, holistic view of the subject matter. In databases Google Scholar and Pubmed, crucial keywords such as "dandelion root extract", "cytotoxicity", "chemotherapies", "oxidative stress", and "in vivo*" were required to gather the following sources.

Multiple Pathway signalling

The original focus of DRE as a therapeutic agent roots back to its success with oxidative stress. A team of researchers gave DRE tea to a group of rats (for a duration of 4 weeks) and regular tap water to a control group to evaluate its effect on heart function and oxidative status in the rat cells (Radoman, 2023). They predicted that rat heart performance would improve and oxidative balance would restore, due to the existing knowledge of DRE's composition of anti-oxidant compounds. They utilized the Langendorff technique, a method for isolating heart cells to examine cardiac functioning, as well as oxidative stress biomarkers that would arise from the blood samples taken. They ultimately determined there were both positive and negative effects on oxidative stress, but found no effects on heart function. In most modern chemotherapies such as Doxorubicin, organisms treated are at risk of permanent heart failure. However, Radoman's study established DRE to have no special effect on heart functioning, therefore providing high assurance of organ safety after treatment. Additionally, DRE maintains a form of homeostasis to protect the heart tissue by utilizing anti-oxidant pathways and "signalling" the heart to remain in oxidative balance. Ensurance of oxidative balance ensures that free radicals do not outnumber the antioxidants, thus avoiding cellular damage (heart functioning in this case). This suggests DRE has a high ability in ensuring functioning organs are unaffected and demonstrates the success of the treatment.

Another study carried out by a team of researchers based in China aimed to identify whether DRE could protect against liver injuries induced by massive alcohol consumption, by using mice cells (in vivo*). They relied on biochemical indicators of liver functions such as ALT, AST and TG to analyze the histology of the liver tissues, the sequencing of the gut microbiota, and identification of the metabolic changes using serums. The team relied on 4 groups of mice that consisted of a control group, an alcohol group, and 2 groups with either high or low dosages of DRE. Findings of the effects of alcohol may suggest the solvent type of producing the extract having a major effect on end results, which were the overall improvement of liver tissue structures and restoration of the microbiota diversity in the gut (X. Wu, 2024). These findings suggest that DRE plays not only an important, but crucial role in the protection against oxidative stress. DRE also has the ability to interrelate with detoxifying signal pathways, by "signalling" the liver to repair itself. Yale Medicine defines hepatotoxicity as the following:

> "a condition characterized by damage to the liver caused by exposure to harmful substances, such as certain medications, toxins, or chemicals. This damage can lead to impaired liver function and, in severe cases, liver failure".

This provides the crucial indicator of maintaining liver function. As the liver functions in drug metabolism, if DRE were to further amplify the liver injury, it would pose serious issues in how it might affect various other organs were it to be made a therapeutic treatment. Therefore, the success of DRE in in vivo* rat models demonstrates its success in being lethal to only the toxic tissues.

Both Radoman and Wu demonstrate DRE and its influence on signalling pathways: oxidative balance and detoxification. DRE ensues the body remains in balance when undergoing stress signals, and provides evidence in helping benign organs. However, while Radoman found no effect of heart functioning, Wu's study demonstrated improvements made to the harmed liver tissue. Therefore, DRE serves multiple purposes that ultimately benefit the model treated. The results of these studies also prove the concentration levels at which DRE can be administered without causing harm to the organism's organs.

Selective toxicity

DRE extracts have also been proven to selectively target cancerous cells over benign cells. As in a study conducted by Ovadje and her team, normal cells (NCM460) were refractive towards DRE treatments when exposed to the "same doses and time points as colon cancer cells" (Ovadje, 2016). Additionally, the team compared its selective nature to that of FOLFOX, a strong chemotherapy regimen, and identified that while FOLFOX killed regular cells, DRE did not. As a result of a scratch wound healing assay on colon cells, results find that the normal NCM460 cells were able to migrate into the wounded area. This specific assay is a test to analyze cell migration, which ties into metastasis and the invasive behavior of cancerous cells (by mimicking a body's ability to heal in an in vitro* sample). Results suggest that DRE selectively blocks cancerous cells' ability to spread to other areas of the body, since HT-29 and HCT116 were inhibited by the root (Ovadje, 2016). Selectivity of the DRE ensures no side effects on regular cells, which improves its potential as an additive in pharmaceuticals. This pioneering study demonstrates DRE's ability to selectively target only those malignant* cancer cells and further reduces its metastatic abilities, proving to not only kill the toxic cells but also halt its spread into other functioning organs. By comparing DRE's efficacy to that of a standard chemotherapy (FOLFOX), DRE resolves the crucial issue of harming regular functioning cells. Therefore, by avoiding its spread into other tissues, DRE has a high potential as an alternative therapy/additive since it selectively kills those cancer cells directly harming the host.

DRE thus has demonstrated abilities in protecting healthy tissues and repairing functioning organs, but also selectively targets ONLY malignant* cells. In treatments, its further benefits include eliminating typical metastatic behaviors and suppressing the spread. As Radoman and Wu identified, DRE protects the healthy tissues, while Ovadje highlighted DRE's

benefit of preserving normal cells while comparing it to modern chemotherapies (FOLFOX) (Radoman, 2023; X. Wu, 2024; Ovadje, 2016). Therefore, DRE has a high potential as a therapeutic agent or additive in chemotherapies.

DRE combinations

DRE extracts combined with other drugs or substances have proven to amplify apoptosis effects. A study evaluating the root's ability to induce apoptosis in drug-resistant melanoma cell lines treated two human melanoma cell cell lines, G631 and A375, with DRE (at a 60% confluence). Additionally, Metformin (an anti type || diabetes drug) was treated upon the G631 cells at 4mM. The team determined the concentration based on findings from previous studies (Isakovic et al.) which varied from 0.5 to 10 mM when treated on glioma/human breast carcinoma cells. The team identified that metformin increases DRE's effectiveness on resistant cells, who would require higher dosages of the extract (Chatterjee, 2011). Therefore, combinations of the root extract with the drug increased DRE's efficacy and decreased cell viability in the samples. By combining other substances with the root extract, DRE's ability to induce toxicity in cells increases. This proves to be substantial when assessing the cytotoxicity levels when treated with just the treatments itself (DRE vs Metformin), versus when both are combined. Additionally, by combining the drug with the extract, cell viability substantially decreased, a result that would have otherwise required higher concentration of the plant extract.

Another study suggests similar results when combining DRE with a different drug. Findings from a team who combined ATRA (All-trans Retinoic Acid), a derivative of vitamin A, with DRE identified additive cytotoxic & anti-metastatic effects on breast cancer cells. They analyzed several gene expressions and the production of respective proteins (KAI1, p53, IL-1β, etc) to confirm how the treatment behaves (Rezaie, 2023). Essentially, the combination of the extract with the derivative implies a correlation between cytotoxic activity and signified fewer side effects than regular chemotherapeutic treatments. By analyzing the gene expressions specifically, the researchers arrived at the conclusion that cytotoxicity of the malignant* breast cancer cells increased while the side effects decreased.

Comparing back to Mollaei, since "chemotherapeutic drugs cannot distinguish [between] normal cells [versus] cancerous cells", both Rezaie and Chatterjee prove that combinations of DRE with other drugs effectively amplify cytotoxicity while being selectively toxic to malignant* cells (Ovadje, 2016). Therefore, cytotoxicity is induced in the addition of substances such as ATRA and metformin and suggests valuable research in the field of apoptosis inducing combinations. The lack of significant research completed suggests further experiments be done using various other drugs, to weaken resistant cells and make them more susceptible to DRE's phenolic compounds.

Summary of Literature

Based on experiments with in-vivo rat models, DRE has proven to signal multiple pathways to maintain homeostasis. DRE has no special effect on heart function, demonstrating

the protection of vital organs (Radoman, 2023) and contains the ability to repair damaged liver tissues (X. Wu, 2024). Additionally, DRE has been proven to selectively induce toxicity to only harmful cancerous cells, while preserving regular functioning cells, unlike modern chemotherapies that harm both. Lastly, combinations of DRE with modern drugs amplify DRE's proven cytotoxicity effects when compared with research that tested DRE and the drug(s) individually. Therefore, DRE has great potential as a new, natural therapeutic drug that overrides the side effects present in modern chemotherapies while aiding functioning organs and selectively attacking the harmful cells.

Gap Statement

As demonstrated by the literature review, DRE has demonstrated proof as a selectively toxic instrument to be a new, pioneering substance capable of not only inducing malignant* specific toxicity but also reducing usual meta-static behaviors of such cancers. However, several of the studies focused only on individual cancers and DRE's effects upon each rather than analyzing the agent's effects holistically. Additionally, there are no present studies analyzing where DRE is more effective. General results show that DRE is suitable as a cancer therapy (in in-vivo since both the model rats were not eradicated by the treatment) but no study goes to the extent of comparing the treatment with each of the cancer types. Therefore, this directs the focus of this research and arises the question, "How do various cancer types influence the cytotoxicity levels of DRE, through cell viability* and IC50 values across in vitro* studies focused on human malignant* cells?".

Methodology

Outline

The goal of this study is to identify whether there is a difference in cytotoxic levels between various cancer types when treated with DRE. The results of this study will help guide future research focused on developing anti-cancerous therapeutic treatments and direct their focus to a specific branch of cancer. According to The Institute of Cancer Research (ICR), a lot of cancers share "genetic weaknesses that can be attacked using the same targeted treatments " but drug therapies may not always be "equally effective in people with different tumour types " (ICR, 2019). Therefore, the present findings DRE inducing apoptosis have no comparisons amongst each other, and require looking into what types of cancers DRE affects the most.

For this study, data was extracted following a PRISMA guided systematic review and a meta-analysis was utilized. A meta-analysis specifically was used because "the results of individual studies are often insufficient to provide confident answers", especially when new medical studies are releasing "increasingly large amounts of new information" (Lee, 2018). Critical variables including (1) Cancer cell line (2) IC50 values (3) Cell viability % , as well as a categorization of each cell line in its retrospective cancer subtype were studied and collected upon the systematic review. The independent variable "cancer cell line" (and later categorization

of its subtype) was chosen because they provide a stable source of biological information and allow researchers to categorize the specific cancer weaknesses in various tissues (Masters, 2000). The dependent variable of "IC50 values" was selected because they are a standardized metric for "indicat[ing] how much drug is needed to inhibit a biological process by half" in pharmaceutical research (Ayakul et al 2021). The second dependent variable "cell viability" was included as an alternative measure of DRE's efficacy, for studies that were unable to provide IC50 values. By determining the effectiveness of DRE through the numerical potency value and comparing the values from cell line to cell line, a clear pattern depicting the relationship between the two variables will be visible. The null hypothesis is written as the following: The cell lines/cancer type had no effect on DRE's efficacy on human malignant* cancerous cells (in-vitro). The assumed hypothesis for this study was the following: Blood-based cancers are more sensitive to DRE compared to other types of cancer when treated with DRE therapies. The foundation for this hypothesis was based upon Pandey and Ovadje's study on leukemia cells, since they discovered that "DRE acts through the extrinsic pathway of apoptosis" (from mitochondrial destabilization) (Ovadje, 2012). Therefore, since blood-based cells are marked for death more than others, hematological* cancers would likely demonstrate lower IC50 values and lower cell viability. As determined from both the literature review and the content of the selected studies, the chosen variables are easily extractable from the available literature.

Step 1: Data Extraction

The variables that were collected were plant part specification, extraction method, cell line, represented cancer type, and cytotoxicity* outcome (represented as either % cell viability or IC50 value). To categorize & allow for comparison of cell viability percentages and IC50 values, a standardization approach was used that ensured comparability. All variables were inputted from published data and a master spreadsheet was created to track the variety of academic literature used. Several databases were initially used to collect the experimental data and compose the following: Embase, PubMed, GoogleScholar, MEDLINE, Web of Science, and Scopus. After the initial collection, a total of 45 studies containing experimental data were inputted into the master sheet. Later after inclusion/exclusion criteria, 18 studies were chosen to carry out the means of this study.

Step 2: Ensuring Homogeneity

Since the method meta-analysis requires an "apples to apples" comparison, it is crucial for the comparison groups to remain the same (UCLA Advanced Research Computing). As previously mentioned, a standardization approach was needed to ensure that percent cell viability and IC50 values were comparable. Therefore, a potency tier categorization was created to categorize the effectiveness of DRE on a scale that took quantitative measures to conclude the qualitative result (the amount of dosage required for DRE to work). Refer to the framework below for further explanations.

Table 1: Potency Tier Framework

Potency tier	Concentration Range (μg/mL)	Cell viability %	Explanation
High Potency	< 100 μg/mL	< 50%	Very effective & low dosages indicate high potential
Medium Potency	100 - 500 μg/mL	50% - 70%	Effective with limits
Low Potency	> 500 μg/mL	> 75%	Needs high dosage, low effective level

The National Cancer Institute (NCI) generalizes that an extract is potent if the IC50 value falls under the 100 μg/mL (exposure time 48-72 hours) (Suffness and Pezzuto, 1990). The extract would have to kill at least half of the cancerous cells in order to be categorized as a highly potent substance. For experiments requiring a dosage over 500 μg/mL, the substance can be categorized as "non-toxic" for particular in-vitro studies (Atjanasuppat et al 2009). The potency tier framework was designed based on these statistical values from peer-published studies. The explanation was created from the conclusions of these studies.

Step 3: Meta-analysis

This study included both a homogeneity test and a random effects model to assess the numerical data within each selected source. In the homogeneity test, Cochrane's Q value was collected because "revised Cochrane Risk of Bias (RoB-2) tool is widely regarded as the gold standard" (Dagner, 2025). In specific words, Cochrane's Q measured the amount of disagreement between the studies. This statistic would prove whether the experiments were similar enough (in terms of variables, outcome and bias) to be heterogeneous. The I^2 value was also taken to get a numerical value of heterogeneity. Since the database is compiled of studies with various experiment procedures/methods, the results would likely not be homogenous. Therefore, a secondary test was conducted (random effects model) to determine the IC50 comparisons between each study (by finding results were heterogeneous). The findings of this result took the mean IC50 value . For studies that listed ranges of IC50 values tested, the average of the range was inputted and the average was calculated for T-score and P-value.

Step 4: Analysis of Results

Each of the final 18 studies were ranked using the potency tier table (refer to Table 1). The potency table revealed a more qualitative result of cytotoxicity* and demonstrated which tumors had higher/lower potency levels. Accompanied by the potency tier table results, the results from the random effects model were utilized to create a bar graph depicting levels of cytotoxicity*/IC50 concentration (y-axis) and tumor type (x-axis) in two categories (hematological/solid). Based on both results, a generalizable conclusion was drawn upon the most sensitive cancer subtype.

Scope & Inclusion

During the data collection phase, there were a series of inclusion/exlusion criteria set in place. First, all collected data ranged from the last two decades (2000-2026) since the study of DRE in clinical cancer settings only persisted recently. Second, all unit conversions were conducted to ensure a standard comparison of IC50 values. For example, some IC50 values were reported in nM/mL or mg/mL and required converting to μg/mL for fair analysis. Additionally, all in-vivo cell models of organisms were excluded from the study, due to variances in internal structure (and thus response to treatment) and the lack of human-studied in-vivo experiments. All in-vitro experiments pertaining to human malignant* cells were included. Additionally, studies that only tested DRE itself (not a combination of DRE & other drugs) were included to ensure all recorded cytotoxicity* outcomes were caused by the extract.

Tests/Results/Findings

Homogeneity Test

Table 2: Homogeneity Results

Cochran's Q value	412.5
Degrees of Freedom	9
P-value	$p < 0.001$
I^2	99.9%

The Cochran's Q value was much larger than the degree of freedom 9 (10 filtered studies [those only contained IC50 values] - 1), meaning the differences between the studies of IC50 values are not by chance. The degree of freedom was 9. The p value came to be < 0.001, which is far less than 0.05 and thus rejects the homogeneity tests. The studies are not homogeneous and demonstrate that DRE doesn't affect each cancer in the same way. Since the studies proved to be heterogenous, a random effects model is needed to identify the reasoning behind such a large difference.

Random Effects Model

Table 3: Random Effect Model

Mean Blood	Mean Solid	T-Score	P-value
713.3 μg/mL	2594 μg/mL	-1880.7/495.27 = 5.2	0.002

The mean blood and mean solid columns represent the average concentration required to kill at least 50% of the cancerous cells to demonstrate DRE's effectiveness upon cancer subtype. Since 713.3 μg/mL was significantly lower than 2594 μg/mL, DRE is proven to be more

effective against blood cancers. Since the T-value score of 5.2 is high, this demonstrates a clear distinction between hematological and solid tumors (T-value looks into the variation in data). The final calculated P-value score of 0.002 indicates the results were statistically significant and since it is far below 0.05, the result is statistically significant. This means that the likelihood this difference between mean blood concentration and mean solid concentration occurred by chance is very low.

Results

All 18 studies were tried by the potency tier categorization. For full results and specific assignments of potency, refer to Appendix B. The general results of the framework had revealed the following:

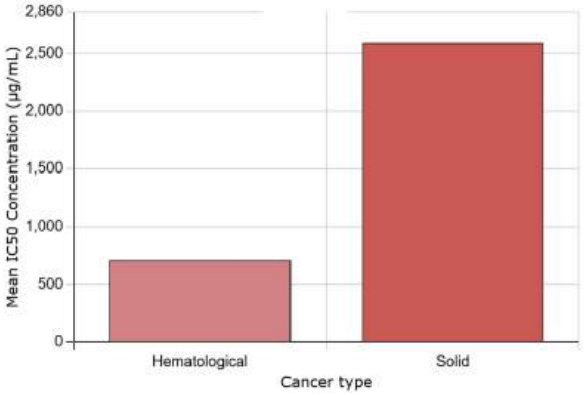
Table 4: Results of Potency

High	Medium	Low
3	8	7

The findings demonstrate 11 out of 18 studies to be of high and medium potency. Referring to Appendix B, most of the moderate and high potencies also fell under the hematological* category (for cancer cell line).

All 18 of the studies that passed inclusion criteria were tested for the independent variables (refer to Appendix B for full table) and yielded IC50 values/percentage inhibition* (using mean values from random effects model). After assessing each cancer type to its IC50 value, the following results were confirmed:

Figure 1: Cancer Type vs IC50 value



As Figure 1 demonstrates clearly, hematological cancers had significantly (4x lower) lower mean IC50 concentrations. This means that low dosages of DRE are effective against blood cancers, whereas solid tumors require far higher concentrations. These findings along with those suggested by the potency table both conclude blood cancers to be more sensitive amongst all cancer types.

Findings

Across all the human malignant* cell studies that were treated with dandelion root extracts, hematological cancers were the most sensitive to DRE and demonstrated the lowest IC50 average values. The null hypothesis was thus rejected because there is a significant effect in DRE's efficacy with human malignant* cancer cells. The original hypothesis was correct and supported by the results of this study since solid tumors require at least 3 times as high of DRE concentration when compared to hematological tumors. Ultimately, this suggests pharmaceutical research concerning DRE as a potential anti-cancer treatment to focus on hematological* cancers.

Discussion/Analysis

The gap between the average IC50 values for blood tumors (713.3 μg/mL) and the average IC50 values for solid tumors (2594 μg/mL) was high in significance [$p = 0.002$]. This results means that DRE is more effective for hematological cancers compared to solid tumors. As Ovadje and Murtaza had suggested in their studies, DRE triggered apoptosis far more in hematological* cancers (leukemia and lymphoma from their studies) (Ovadje, 2012; Murtaza, 2023). The findings of this study aligned with their results, since the blood (subtype cancer) needed far less of DRE concentration to achieve apoptosis of at least 50% of the treated malignant* cells. On the other hand, research by Zhu (who studied colon cancerous cells) suggests higher IC5O values from his findings, thus demonstrating that solid tumors need at least three times as much concentration to achieve the same effect for blood-based cancers.

Finally, a similar pattern in "selectivity" was found in both this study and Ovadje's study on DRE vs. FOLFOX. While Ovadje discovered DRE to selectively target malignant* colon cells over normal cells, this study identified a similar selective nature since DRE only affected certain cancer types far more than others (hematological > solid). This shows that DRE is capable of being selective and targeting some cells over others. Knowledge of this is critical for researchers attempting to find combinations that will yield the best results (high cytotoxicity* and selectively malignant*) and suggests combining DRE treatment to only hematological cancers versus FOLFOX (and other chemotherapies) to a vast span of cancers.

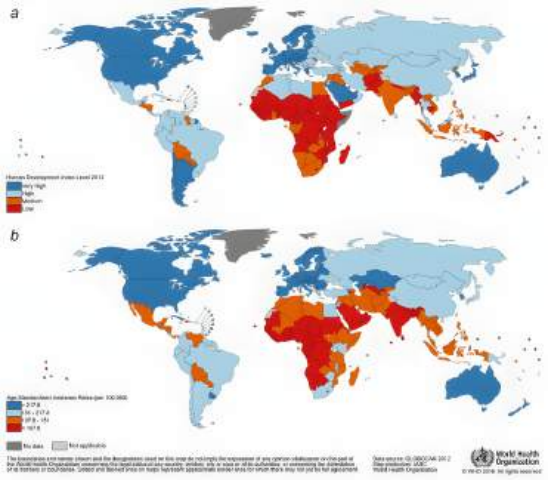
Implications

The results of this study can help create a hierarchy of selective sensitivity. As previous literature identified, DRE was selectively toxic to malignant* cells and was harmless to normal cells. By categorizing which types of cancer were more sensitive, this study suggests that DRE's efficacy is not uniform across every cancer type. This suggests that research institutes and agencies should focus therapeutic resources on Leukemias and Lymphomas (and other blood-based cancers) in their clinical trials, to eventually utilize natural anti-oxidants as therapies over synthetic therapies (that come with harmful side effects).

Additionally, synthetic chemotherapies prove to be unaffordable and expensive in developing countries. As demonstrated by the American Cancer Fund, synthetic chemotherapies

can range anywhere from $12,000 to $200,000 and higher (ACF, 2026). Most developing countries are able to afford such high-cost therapies. Due to higher population counts and other factors (i.e. hygiene), developing countries also demonstrate higher prevalence in cancer rate & require more readily accessible treatments. As demonstrated by the figure below, bright red countries including those in Africa and parts of Asia contain high incidences of cancers. Developing countries are unable to afford costly treatment but require it more than developed countries. Therefore, the results of this study further contribute to the quest of creating a natural therapeutic treatment that efficiently attacks malignant* (human) cancers. Since DRE is so widely grown & prevalent to the extent at which humans call it a pest, DRE is easily accessible and may provide crucial to creating these therapies.

Figure 2: HDI Global Cancer Incidence



Limitations

One limitation of this study is the lack of standardization of the Dandelion plant itself. Variances in the extract may occur due to differences in the sub-genere of the plant type or difference in plant origin. Various environmental factors that affect the growth of the plant may have induced certain compounds to appear more than others, thus influencing the composition of the extract (further affecting cytotoxicity* outcome). For example, dandelion plants harvested from Ghana may contain more flavonoid composition than plants harvested from Ukraine. This study will not be able to confirm whether the result of hematological cancers having lower IC50 averages will hold true to every Dandelion extract simply because the extracts in the studies are from various settings and environments.

Additionally, the findings of this study are not generalizable to human individuals, since data was collected upon in-vitro studies only. Complete systems may provide an outcome different than that of a laboratory study due to bacterial interactions (trillions of which are prevalent within humans), metabolism, and absorption differences. The findings of this study should only be considered from a “perfect-world perspective”, in which only the provided

variables are studied. Additionally, in-vivo models are constantly dynamic with varying levels of oxygen deposited, blood, and flow rates. Various cell lines interacting with each other in the organism may also affect how DRE gets absorbed or how it affects cancerous cells. Concentrations and dosages may also vary in-vivo to in-vitro, and human to human. Therefore, the results of this study should only serve as a general guide for researchers determining the type of cancer to focus on for DRE research.

Further Research

A smaller yet significant trend that was identified within the literature review is the effect of solvent type on cytotoxic activity. The type of solvent utilized to make the extract plays a key role in pulling out certain compounds of the plant that can affect how sensitive malignant* cells are to the extract. As some studies suggest, ethanolic extracts proved to be more cytotoxic compared to aqueous extracts (Kumnerdkhonkaen, 2018). Future research that focuses on cancer cell type and the solvent types effect on cytotoxicity* would indicate combinations that increase DRE's effectiveness. Additionally, since this study only focused on in-vitro studies and experiments, future research should collect experimental data on in-vivo models to get the benefit of a whole system. The complete system in in-vivo experiments can produce different effects that were unforeseen in the lab (McKim Jr., 2020; Jansson-Lofmark et al 2020). Therefore, further research is necessary to confirm the results of this study to be viable and successful as a pharmaceutical drug post lab (with human participants). Lastly, referring back to the third trend of "DRE combinations", further research could focus on combinations of DRE (accounting for solvent type/cancer type) and modern therapies (i.e. Metformin) to determine whether cytotoxicity* effects are higher or lower (Rezaie, 2023; Chatterjee, 2011). A generalizable finding further research could discover is that DRE is ineffective or loses its selective toxicity when combined with specific independent variables (solvent type, cancer cell line, etc).

Conclusion

This study addressed the gap of a lack of standardization of DRE's effects on various cell lines in terms of cytotoxicity*, and it utilized a meta-analysis approach to analyze studies across medical databases. The goal was to allow for a holistic comparison of DRE's effectiveness across certain cancers. There was a demonstrated selective effectiveness, in which DRE was far more effective in hematological tumors than solid tumors. Results of this study may influence further research into solvent type, in-vivo models rather than in-vitro and will ultimately guide DRE research as a potential natural therapy (a better suited alternative to synthetic drugs/chemotherapies).

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Appendix A- Key Definitions

Antiangiogenic	Prevents formation of new blood vessels
Anticarcinogenic	Cancer fighting
Antinociceptive	Substance that blocks pain
Anti-Oxidant	Natural substances that prevent cellular damage through the neutralization of free radicals. They donate e- to reduce oxidative stress.
Benign	Harmless cells that do not impair cellular/organ function
Bioavailability	Rate/extent a substance gets absorbed and useable in an organism
Cell viability	Number of health cells in a sample
Cytotoxicity	Ability to be toxic to cell(s)
Hematological	Branch within medicine studying blood disease
Innocuous	Harmless, innocent
Inhibition	Prohibiting of a process/behavior
In-Vivo	In body/organism
In-Vitro	In laboratory or clinical lab
Malignant	Harmful/cancerous cells
Melanoma	Most harmful type of skin cancer
Phenolic	Acts as an antioxidant, comprised of various other compounds such as flavonoids, lignans, tannins, etc

Appendix B- Full Table

Table 4: Summary results of 18 included studies

Ovadje2012	https://doi.org/10.1371/journal.pone.0030604	Root	Aqueous	0.2- 1.0 mg/mL	MV-4-11, HL-60, U-937, (all human CMML leukemia cell lines); non-cancerous PBMCs used as a control	Chronic Myelomonocytic **Leukemia** (CMML)	Moderate
Ovadje2011	https://pubmed.ncbi.nlm.nih.gov/20849941/	Root	Aqueous	~ 1.0 mg/mL	Dominant- negative FADD; PBMCs	Human Leukemia	Moderate
Appiah-Opong, 2016	Link	Root	Aqueous	540 µg/mL	Human promyelocytic	Leukemia	Moderate

					leukemia (HL-60) cells		
Chatterjee2011	https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0030604	Root	Aqueous	2.5 mg/mL	A375, G361	Melanoma	Low
Ovadje2016	https://www.oncotarget.com/article/11485/text/	Root	Aqueous	2.0 - 3.5 mg/mL	HCT116, HT-29	Colorectal	Low
Ren2020	https://www.frontiersin.org/journals/pharmacology/articles/10.3389/fphar.2020.00460/full	Root	Crude DRE powder	Dose dependent	SW480	Colorectal	Moderate
Nguyen2019	https://onlinelibrary.wiley.com/doi/10.1155/2019/2951428	Root	Aqueous	~2 mg/mL	BxPC-3, PANC-1	Pancreatic	Low
Zhu2017	https://www.iscientific.org/wp-content/uploads/157-ijcbs-23-24-10-157e.pdf	Root	Aqueous	3 - 4 mg/mL	SGC7901	Gastric	Low
Zhang2021	https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/epdf/10.1002/pca.3033	Root	Crude DRE powder	Dose-dependent suppression	BGC-823	Gastric	Low
Li2017	https://www.mdpi.com/1420-3049/22/9/1409	Root	Aqueous	Induced ER stress apoptosis	MDA-MB-231	Breast (TNBC)	Moderate
Qu2022	https://pubmed.ncbi.nlm.nih.gov/36413334/	Root	Aqueous	Metabolic interference identified	MDA-MB-231	Breast (TNBC)	Moderate
Deng2021	https://www.sciencedirect.com/science/article/abs/pii/S0378874121002051?via%3Dihub	Root	Crude DRE powder	Inhibited malignant phenotype	MDA-MB-231	Breast (TNBC)	Moderate
Trinh2016	https://www.scirp.org/reference/referencespapers?referenceid=3194867	Root	Methanol	low µg/mL	MCF-7 Stem	Breast CSCs	High potency
Sigstedt2008	https://doi.org/10.3892/ijo.32.5.1085	Root	Aqueous	Selective growth inhibition	MCF-7, LNCaP	Breast/Prostrate	High potency
Murtaza2023	https://krishikosh.egranth.ac.in/server/api/core/bitstreams/749a5f23-d52b-429a-983d-cf5c602582d1/content	Root	Ethanol	50 µg/mL	C4-2	Prostrate	High potency
Kim2011	https://www.sciencedirect.com/science/article/pii/S0278691513002561	Root	Crude DRE powder	Higher cytotoxicity	HepG2	Liver	Low
Lee2019	https://www.kjpr.kr/articles/article/ABbm/	Root	Crude DRE powder	Lower cell viability	HepG2	Liver	Moderate
Duan2021	https://pubs.rsc.org/en/content/articlelanding/2021/fo/d1fo01093j	Root	Crude DRE powder	2.22 mg/mL	KYSE450	Esophageal	Low

The Biology of Fear: Genetic, Neurobiological, and Epigenetic Mechanisms Underlying the Heritability of Fear Responses and Anxiety Vulnerability By Sanjana Shetty

Abstract

Fear is an adaptive survival mechanism that enables organisms to detect and respond to threats. However, substantial individual differences exist in fear sensitivity, fear learning, and susceptibility to anxiety disorders, raising questions about the biological origins of these variations. This paper investigates how fear responses can be transmitted across generations through biological mechanisms independent of direct learning and examines the extent to which genetic differences contribute to individual variability in fear and anxiety. Drawing upon contemporary research in genetics, neuroscience, and psychology, the paper evaluates genetic polymorphisms including BDNF, SLC6A4 (5-HTTLPR), FKBP5, and CRHR1, alongside their effects on neural circuitry, stress regulation, and fear extinction. Evidence from twin studies, genome-wide association studies, and epigenetic research is synthesised to assess the heritability of fear-related traits and anxiety disorders. The paper further integrates cognitive and evolutionary theories of fear to demonstrate that inherited biological predispositions interact continuously with environmental experiences throughout development. It concludes that fear responses exhibit moderate heritability through identifiable neurobiological pathways, but that inherited genes confer vulnerability rather than predetermined outcomes, with gene-environment interactions ultimately shaping the expression, intensity, and persistence of fear and anxiety.

Keywords

Fear heritability, gene-environment interaction, anxiety disorders, neurobiology of fear, epigenetics.

Introduction

A child who fears snakes without ever seeing one might be expressing more than imagination alone. Is it possible to fear something not because you learned to, but because your brain is preconfigured to?

Fear is an adaptive emotional state triggered by perceived threats or danger (Beckers et al., 2023; Grogans et al., 2023). Physiological and behavioural changes are activated that help us detect and respond to threats (Beckers et al., 2023; Grogans et al., 2023). Fear helps organisms detect dangers and produce defensive responses, supporting survival (Beckers et al., 2023; Grogans et al., 2023). Sometimes, the magnitude or duration of this crucial response becomes disproportionate to the real-life threat, making it maladaptive (Beckers et al., 2023). Disruptions in the fear-related neural systems can contribute to the development of anxiety disorders (Nuss, 2015). Anxiety disorders affected 359 million people globally in 2021, making them the world's most common mental disorders (World Health Organisation, 2025).

Fear responses are shaped by the interactions of multiple biological and psychological factors that contribute to varied fear responses between individuals, characterised by differences

in threat sensitivity, fear-learning, and fear-extinction (Steimer, 2022; Beckers et al., 2023; Grogans et al., 2023). Historically, these differences were explained with a focus on learned associations and conditioning (Steimer, 2022; Beckers et al., 2023). Modern neuroscience is beginning to identify evidence of genetic factors influencing pathways involved in fear responses (Arnsten, 2009; Steimer, 2022; Grogans et al., 2023).

Although genetic influences are evident, the precise pathways by which specific fear-related genetic factors give rise to alterations in neural circuitry remain incompletely understood (Arnsten, 2009; Steimer, 2022; Grogans et al., 2023). Investigating the specific processes linking genes, the environment, and fear-related neural systems can provide a deeper understanding of fear responses (Steimer, 2022); this could facilitate the development of targeted therapies for anxiety-related disorders (Arnsten, 2009; Beckers et al., 2023; Grogans et al., 2023). In response to this gap, the central research question of this study is: **How can fear responses be transmitted across generations through biological mechanisms independent of direct learning, and how do genetic differences contribute to individual variability in fear and anxiety?**

This research paper argues that fear responses exhibit moderate heritability through identifiable genetic and neurobiological mechanisms, yet their transgenerational transmission and individual variability arise not from genetic determinism alone but from interactions between biological predispositions and environmental-epigenetic factors.

Conceptual and Theoretical Background: Understanding Fear

From a psychological perspective, fear is an emotion that is evoked by specific stimuli perceived as threatening or dangerous (Steimer, 2022; Silva et al., 2016). In this emotional state, attention is directed towards the threat at hand, which is prioritised over other less relevant stimuli, and focus is directed toward assessing threat cues (Mobbs et al., 2015; Hofmann et al., 2012). Physiological and behavioural changes are initiated according to the type and context of the threat (Steimer, 2022; Silva et al., 2016). Neurobiological accounts describe fear-related responses as mediated by neural systems that detect and integrate threatening and contextual information (Mobbs et al., 2015; Silva et al., 2016). They then elicit defensive responses that are suited to the situation (Silva et al., 2016; Asok et al., 2019).

Sensory information relevant to threat can reach the amygdala through cortical and subcortical routes (Garrido et al., 2012; Johnson et al., 2011). In the quicker subcortical pathway, the sensory information can reach the amygdala directly through the thalamic route (Garrido et al., 2012; Johnson et al., 2011). As this route involves faster processing, it may help produce defensive responses more quickly and may promote survival (Garrido et al., 2012; Johnson et al., 2011). In the cortical ‘high road’, sensory information is processed in the cortex, after which it is relayed to the amygdala, allowing more detailed processing of the stimulus (Garrido et al., 2012; Johnson et al., 2011). The Basolateral Amygdala (BLA) evaluates stimulus valence as positive/reward-related or negative/aversive (Antonoudiou et al., 2025), and can then activate the

central amygdala (CeA), which then projects to the hypothalamus and brainstem, contributing to physical manifestations of fear or anxiety (Nuss, 2015).

When danger is detected, the sympathetic nervous system is rapidly activated (Harvard Health Publishing, 2024). Norepinephrine and adrenaline are released, resulting in increased heart rate and alertness, increased blood flow to muscles, and pupil dilation in preparation for fight-or-flight responses (Psychotraumatology, 2025; Harvard Health Publishing, 2024). In the next step of this response, the hypothalamus activates the hypothalamic-pituitary-adrenal (HPA) axis, stimulating cortisol release, which maintains this state of alertness (Harvard Health Publishing, 2024).

Fear is an emotional response that occurs when an individual perceives an immediate, identifiable threat (Gramlich, 2024; Schmidt et al., 2018; Nuss, 2015). Unlike immediate fear, anxiety disorders involve excessive and persistent anticipation of uncertain or future threats (Schmidt et al., 2018; Grupe & Nitschke, 2013). Many anxiety disorders involve the hyperactivation of the amygdala, which is linked to hypervigilance and inappropriate threat perception (Schmidt et al., 2018; Grupe & Nitschke, 2013). The activation of the output pathways of the CeA contributes to the physical manifestations of anxiety (Nuss, 2015), such as racing heart, nausea, and breathing changes (Mind Matters Mental Health, 2026; Gramlich, 2024). This expression of anxiety is regulated in part by mechanisms in which prefrontal cortical inputs interact with GABAergic intercalated neurons, thereby regulating BLA-to-CeA activation and inhibiting amygdala output (Nuss, 2015; Asede et al., 2022). GABA is a major inhibitory neurotransmitter widely used in the CNS, which helps maintain the balance between neuronal excitation and inhibition. GABAergic intercalated neurons surround the BLA and provide feedforward inhibition to amygdala nuclei, including the CeA and BLA (Jewett & Sharma, 2023; Nuss, 2015; Asede et al., 2022). Disruption of these inhibitory systems may impair fear extinction and fear generalisation, which are processes involved in anxiety disorders (Asede et al., 2022; Grupe & Nitschke, 2013).

Chronic stress is when a stimulus that triggers stress responses remains present for a prolonged period (Viradia et al., 2022). This is linked to hyperexcitability of the BLA and reduced GABA receptor response, which result in hypervigilance and responses similar to those observed with anxiety (Viradia et al., 2022). Chronic stress is also associated with structural changes in the prefrontal cortex and impaired function (Viradia et al., 2022; Arnsten, 2009). Specific phobias are a type of anxiety disorder; they are disproportionate fears associated with stimuli that can be certain objects or situations (Senftleben et al., 2025; Garcia, 2017). Individuals with phobias display increased amygdala activity when exposed to the feared stimuli (Moawad, 2016; Garcia, 2017). These stimuli are not necessarily commonly considered unpleasant (Moawad, 2016), and the specific phobia can either arise from a traumatic experience associated with those stimuli, or it could be a nonassociative specific phobia, wherein there has not been previous associative fear learning (Garcia, 2017). The development of this type of phobia is likely influenced by a combination of genetic and environmental factors (Garcia, 2017;

Senftleben et al., 2025). This brings us to the exploration of two major explanations for the development of fears: cognitive models and genetic explanations.

Learning theories of fear explain how fears can be acquired through experience (Field, 2006; Shechner et al., 2014). Classical conditioning is an associative learning model that explains fear acquisition through repeated pairing of a neutral conditioned stimulus (CS) with an aversive stimulus (UCS), leading to the formation of a CS-UCS association such that when the CS is presented alone, a conditioned response (CR) is produced, which can be fear responses such as changes in heart rate or fear potentiated startle (Shechner et al., 2014; Field, 2006). This explains fear learning through direct conditioning and is one of Rachman's pathways to the acquisition of fears and phobias (OpenEd CUNY, 2026; Field, 2006). Vicarious learning involves an individual producing a fear response to a stimulus after observing someone else display a fear response to that same stimulus; this explains fear acquisition without direct exposure, but through modelling others instead (OpenEd CUNY, 2026; Field, 2006). The third pathway is verbal transmission or information, in which individuals acquire fear after being told that a stimulus is dangerous (OpenEd CUNY, 2026; Field, 2006).

The expectancy model of fear suggests that there are two components that contribute to fear behaviour: the expectation of danger, and the expectation of anxiety, along with the anxiety sensitivity (Valentiner et al., 1993). Anxiety sensitivity is how much salience the individual assigns to the sensations of anxiety (Valentiner et al., 1993). The expectancy model may help explain how fear-related avoidance or escape behaviour is maintained after learning: the fear behaviour occurs not simply due to the presence of the stimulus, but because of the expected danger of exposure to it and/or anxiety (Valentiner et al., 1993). Cognitive bias theories suggest that phobias and anxiety disorders are associated with biases in information processing (Remmerswaal et al., 2014). Fearful individuals may selectively search for threat-confirming information (Remmerswaal et al., 2014).

Öhman et al. (2001) found that evolutionarily relevant threats (spiders and snakes) were detected more quickly than fear-irrelevant stimuli, and even faster by people who were fearful of those stimuli. This suggests that evolutionarily relevant threatening stimuli may have captured attention more effectively (Öhman et al., 2001), which may have accounted for the relatively lesser time taken to detect it. Seligman (1971, as cited in Armfield, 2006) suggested that fears were not uniformly distributed across potentially fear-relevant stimuli, and this was one of the ways he suggested phobias differed from conditioned fear. He proposed that phobias also differ from conditioned fears in that they are learned more easily, show greater resistance to extinction, and can appear irrational (Seligman, 1971, as cited in Armfield, 2006). Preparedness theory proposes that some stimuli are biologically prepared for fear conditioning because of their evolutionary significance, which might be linked to the easier acquisition of fear for ancestrally relevant threats (Seligman, 1971, as cited in Armfield, 2006; Öhman & Mineka, 2001). Öhman and Mineka (2001) proposed that, in aversive contexts, evolutionarily fear-relevant stimuli such as snakes are especially likely to activate the fear module, which may make them more readily associated with fear through learning. Together, these accounts support the idea that evolutionary

relevance may influence both attentional bias toward threat and the ease with which certain stimuli become associated with fear.

Genetic and Neurobiological Mechanisms of Fear Inheritance

Anxiety disorders and anxiety-related traits are polygenic (Purves et al., 2020). Rather than being governed by a single gene, they are influenced by many genetic variants, each generally contributing small effects (Purves et al., 2020). This contributes to variation in anxiety-related traits across the population (Purves et al., 2020). Genetic information is transmitted through the inheritance of DNA from each parent (Genetic Alliance & District of Columbia Department of Health, 2010). Single-nucleotide polymorphisms (SNPs) occur when a single nucleotide is changed in the DNA sequence; they are common across the human genome (Genetic Alliance & District of Columbia Department of Health, 2010; Antontseva et al., 2023). Some SNPs can affect gene function or regulation (Antontseva et al., 2023). Certain gene variants may affect the transcription rate of the gene, which is one of the factors that affect protein abundance (Antontseva et al., 2023; Hausser et al., 2019). In some cases, mutations in a gene can produce an altered protein product that has reduced, lost, or changed function (Genetic Alliance & District of Columbia Department of Health, 2010).

Brain-derived neurotrophic factor (BDNF) is a protein that is involved in neuronal development, differentiation, and plasticity (Notaras & van den Buuse, 2020). BDNF binds with high affinity to TrkB receptors and forms BDNF-TrkB signalling pathways (Notaras & van den Buuse, 2020). In fear-related neural circuits, this signalling contributes to contextual fear-memory consolidation and fear extinction (Notaras & van den Buuse, 2020). The BDNF Val66Met variant reduces activity-dependent BDNF release, lowering the efficiency of BDNF-TrkB signalling; this can impair extinction learning (Notaras & van den Buuse, 2020). It may also alter stress-related consolidation of emotionally salient fear memories (Notaras & van den Buuse, 2020).

Promoters are DNA sequences that allow RNA polymerase to bind and start transcription (Alberts et al., 2002; Clark et al., 2018). Promoter regions can include or be associated with nearby regulatory sequences where transcription factors bind; transcription factors are proteins that bind DNA. Many transcription factors bind specific DNA sequences, and they can enhance or repress transcription by influencing RNA polymerase activity or access at the promoter (Alberts et al., 2002; Clark et al., 2018). and either promote or inhibit RNA polymerase recruitment. Regulatory elements are DNA sequences that are bound by transcription factors and help increase or decrease transcription (Alberts et al., 2002; Clark et al., 2018).

Serotonin influences mood (Hariri et al., 2005), and a lack of serotonin may be a factor that is linked to depression (The Open University, 2015). SLC6A4 is the gene that encodes the serotonin transporter protein, also called 5-HTT (Hariri et al., 2005; The Open University, 2015). 5-HTTLPR is a common polymorphism in the promoter/regulatory region of SLC6A4 (Hariri et al., 2005; Lesch et al., 1996). The short (S) and long (L) versions are two forms of the 5-HTTLPR regulatory region (Hariri et al., 2005). Lesch et al. (1996) found that the long variant

showed greater transcriptional efficiency than the short variant in reporter-gene assays, and cells with the long form had higher 5-HTT mRNA and higher serotonin uptake. An approximately 50% reduction in 5-HTT availability was found to be associated with the short allele (Hariri et al., 2005), which is presumed to lead to reduced serotonin reuptake capacity and relatively more serotonin remaining in the synaptic cleft (Hariri et al., 2005; The Open University, 2015; Lesch et al., 1996). Individuals carrying the short 5-HTTLPR allele showed heightened right amygdala reactivity to fearful and threatening facial expressions (Hariri et al., 2005; Hariri et al., 2002). This suggests that the variant may influence the reactivity of the amygdala in the face of a threat (Hariri et al., 2005); this may increase vulnerability to affective disorders, including anxiety and depression, particularly in interaction with stressful life experiences or reduced cortical regulation (Hariri et al., 2005)

The hypothalamic-pituitary-adrenal (HPA) axis is activated after a trigger of stress is perceived. During stress, the hypothalamus releases corticotropin-releasing hormone (CRH), stimulating adrenocorticotropic hormone (ACTH) release from the pituitary gland, which triggers cortisol secretion from the adrenal glands (Mahon et al., 2013). Cortisol regulation involves negative feedback loops involving glucocorticoid receptors (GRs) and FKBP5 (Mahon et al., 2013; Klengel et al., 2013). The GRs in the cytosol are a part of GR heterocomplexes, which contain the co-chaperone protein FKBP5 (Mahon et al., 2013; Binder et al., 2008). FKBP5 decreases GR ligand-binding affinity and can reduce nuclear translocation of the GR complex (Klengel et al., 2013; Binder et al., 2008). Therefore, when cortisol binds to GRs, FKBP5 is replaced by FKBP4, which helps the cortisol-bound GR complex enter the nucleus (Mahon et al., 2013; Binder et al., 2008). When the GR moves into the nucleus, it interacts with the regulatory regions of the FKBP5 gene and increases its transcription rate (Klengel et al., 2013; Mahon et al., 2013). Increased FKBP5 expression can reduce subsequent GR nuclear translocation by retaining more GR complexes in the cytosol (Mahon et al., 2013). This restrains the GR signalling (Klengel et al., 2013; Mahon et al., 2013). Therefore, negative feedback mediated by GRs helps terminate the stress response after the threat ends, and FKBP5 modulates this feedback by restraining GR activity (Klengel et al., 2013; Mahon et al., 2013). Variants of FKBP5 have been found to be associated with differences in cortisol responses (Mahon et al., 2013), and changes in the expression of the FKBP5 gene or function of FKBP5 could affect the regulation of the stress response, which could cause an increase in cortisol burden (Mahon et al., 2013). One variant of FKBP5, rs1360780, can influence FKBP5 transcription by altering chromatin organisation (Klengel et al., 2013).

Epigenetic modification can affect gene transcription without changing the DNA sequence itself (CD Genomics, 2025). One type of epigenetic modification is DNA methylation (CD Genomics, 2025; biomodal, 2025), which involves the addition of methyl groups to DNA (biomodal, 2025; CD Genomics, 2025). In carriers of the version of the FKBP5 SNP rs1360780 that was associated with greater PTSD risk after childhood trauma, the combination of the presence of the risk allele and childhood trauma can lead to demethylation in the regulatory regions of FKBP5 (Klengel et al., 2013), making FKBP5 transcription more responsive to

glucocorticoid receptor activation; this may increase FKBP5 induction following glucocorticoid receptor activation (Klengel et al., 2013). This mechanism may contribute to increased vulnerability to stress-related psychiatric disorders, including PTSD, after childhood trauma (Klengel et al., 2013).

CRHR1 is the gene that codes for type 1 corticotropin-releasing hormone receptor (CRHR1), which is involved in HPA-axis regulation and stress reactivity, as it is a receptor involved in CRH signalling (GeneCards, 2026; Bradley et al., 2008). Certain polymorphisms of CRHR1 have been linked to adult depressive symptoms through interaction with childhood abuse (Bradley et al., 2008). CRHR1 polymorphisms rs7209436, rs110402, and rs242924 showed nominal associations with the highest cortisol response following psychosocial stress (Mahon et al., 2013). This suggests a link between genetic variants in the CRHR1 gene and the stress response (Mahon et al., 2013).

Genetic and neurobiological mechanisms provide a framework for understanding the inheritance of fear. However, fear responses reflect the combined effects of inherited variation and environmental exposure. A comprehensive understanding, therefore, requires gene-environment interaction frameworks.

Evidence for the Heritability of Fear: Twin and Family Studies

Monozygotic twins arise when a single fertilised ovum (zygote) splits into two embryos, resulting in individuals with nearly identical genetic material (National Human Genome Research Institute, 2024; Sahu & Prasuna, 2016). In contrast, dizygotic twins develop from the fertilisation of two separate ova by two different sperm, producing genetically distinct individuals who share approximately 50% of their DNA, similar to ordinary siblings (National Human Genome Research Institute, 2024; Sahu & Prasuna, 2016). Concordance rates refer to the proportion of twin pairs in which both twins display a given trait, given that one already displays this trait (Sahu & Prasuna, 2016). These concordance rates can contribute to estimating heritability (h^2) (Sahu & Prasuna, 2016; Wray & Visscher, 2008), the proportion of variance in a trait within a population attributable to genetics (Wray & Visscher, 2008; Sahu & Prasuna, 2016). It is estimated by comparing the similarity between monozygotic and dizygotic twins (Wray & Visscher, 2008; Sahu & Prasuna, 2016). It does not specify the extent to which an individual's trait is genetically determined; rather, it reflects differences across a population (Wray & Visscher, 2008). Twin studies can be used to examine genetic contributions to psychological traits, including fear-related traits, when measured in twin samples (Sahu & Prasuna, 2016; Wray & Visscher, 2008). Twin studies rely on the Equal Environments Assumption (EEA), which assumes that the shared environmental contribution to resemblance is similar for MZ and DZ twin pairs (Wray & Visscher, 2008; Sahu & Prasuna, 2016). Therefore, higher concordance rates in monozygotic than dizygotic twins may suggest a genetic influence (Sahu & Prasuna, 2016). This supports the use of twin studies to estimate, rather than perfectly isolate, genetic and environmental contributions to fear-related traits (Wray & Visscher, 2008; Sahu & Prasuna, 2016).

Twin studies and meta-analyses suggest moderate genetic influence across several anxiety-related disorders and traits (Hettema et al., 2001; Tambs et al., 2009; Van Houtem et al., 2013). Anxiety disorders such as generalised anxiety disorder (GAD) show moderate heritability, estimated at 0.32 (Hettema et al., 2001). A common liability across anxiety disorders was estimated at about 54% heritability (Tambs et al., 2009). Panic disorder also shows moderate heritability of 0.43 (Hettema et al., 2001), supporting a genetic contribution, with shared environmental influences appearing minimal (Hettema et al., 2001). Reviews of twin studies suggest PTSD heritability is around 30-40% (Almli et al., 2014), and twin-study estimates of PTSD heritability following trauma range from 24% to 72% (Duncan et al., 2018). A genome-wide association study (GWAS) estimated the SNP-based heritability of PTSD at 5-20% (Nievergelt et al., 2019). Specific phobias show moderate heritability (Van Houtem et al., 2013), and a previous meta-analysis estimated heritability at 0.20-0.37 for various types of phobias (Tambs et al., 2009). In a meta-analysis, animal fear exhibited the highest heritability, at approximately 45%, among fear subtypes (Van Houtem et al., 2013). Neuroticism, a personality trait strongly associated with anxiety (Hettema et al., 2004; Hettema et al., 2006), shows a broad genetic influence of 41% (Jang et al., 1996).

Although genetic influence is not exclusive and much of the remaining variation is often accounted for by nonshared environmental factors (Hettema et al., 2001; Van Houtem et al., 2013; Jang et al., 1996), genetics appear to play a moderate and meaningful role in vulnerability across several anxiety-related disorders and traits (Hettema et al., 2001; Tambs et al., 2009; Van Houtem et al., 2013).

A twin study that measured GAD diagnosis and neuroticism reported an estimated genetic correlation (r_g) of 0.80 (Hettema et al., 2004). This indicates a high genetic correlation and substantial overlap in genetic influences (Hettema et al., 2004). Another twin study used multivariate genetic modelling across neuroticism and several internalising disorders, including GAD, panic disorder, major depression, agoraphobia, social phobia, animal phobia, and situational phobia (Hettema et al., 2006). Across the internalising disorders, genetic factors shared with neuroticism were reported to account for between one-third and one-half of genetic risk. These findings suggest that GAD and panic disorder share substantial genetic liability with neuroticism (Hettema et al., 2004; Hettema et al., 2006).

Twin-study heritability estimates have methodological limitations. They rely on assumptions such as the EEA (Mitchell et al., 2007; Sahu & Prasuna, 2016). Monozygotic twins may experience more similar environments or treatment than dizygotic twins (Morris-Yates et al., 1990; Mitchell et al., 2007); if this occurs, it could increase heritability estimates (Mitchell et al., 2007). Anxiety-related traits influenced by genetic factors may affect the environments individuals experience, for example, by eliciting more controlling parental responses (Eley et al., 2010; Mitchell et al., 2007). This reflects gene-environment correlation (Mitchell et al., 2007). Genetic influences on fear- and anxiety-related outcomes may depend in part on environmental context, rather than operating deterministically or in isolation (Eley et al., 2010; Hutter, 2026; Van Houtem et al., 2013). Twin studies generally show moderate heritability across

anxiety-related disorders (Hettema et al., 2001; Tambs et al., 2009; Van Houtem et al., 2013), and while these findings do not imply genetic determinism, they support a genetic contribution to fear- and anxiety-related traits (Van Houtem et al., 2013; Hettema et al., 2001; Tambs et al., 2009). Therefore, genetic factors may contribute to fear- and anxiety-related vulnerability through both interaction with environmental context and gene-environment correlation (Hutter, 2026; Mitchell et al., 2007; Eley et al., 2010).

Conclusion

Fear triggers physiological and cognitive changes that enable rapid detection of both physical and psychological threats, making it essential for survival. However, when dysregulated, these processes can become maladaptive and underlie fear- and anxiety-related disorders, including generalised anxiety disorder (GAD), phobias, PTSD, and panic disorder.

Cognitive theories explain many acquired fears through conditioning, vicarious learning, information, expectancy, and cognitive bias. But learning alone does not fully explain why some fears are easier to acquire or explain individual differences in fear responses. Evolutionary theories, such as preparedness theory, suggest that biological predispositions may affect vulnerability to fear acquisition and fear conditioning apart from direct learning.

Increasing evidence indicates a genetic contribution to the biological mechanisms underlying fear responses. Twin studies consistently report moderate heritability for anxiety-related traits, with higher concordance in monozygotic compared to dizygotic twins, which can indicate a genetic contribution. A substantial amount of variance remains environmental, especially the nonshared environment. Genetic factors can affect the neurobiological mechanisms that regulate fear responses. BDNF variants can affect synaptic plasticity, contextual fear memory, and fear extinction. SLC6A4/5-HTTLPR variants can affect serotonin transporter expression and amygdala reactivity to threat. FKBP5 variation and expression can affect the sensitivity of glucocorticoid receptors, cortisol feedback, and recovery of the stress response. Polymorphisms within the CRHR1 gene can affect CRH signalling and HPA-axis stress response. Epigenetics can lead to altered gene expression without a change to the DNA sequence. These genetic factors are expressed through interactions with environmental factors.

Therefore, genetic factors can lead to changes in the biological mechanisms that influence fear responses, such as detection of threat, stress regulation, fear learning, and fear extinction. However, this does not mean that fear is genetically determined. These genetic factors therefore affect vulnerability, and the extent to which these vulnerabilities are expressed is heavily affected by the gene-environment interaction. Specific fears themselves are not inherited; rather, what may be transmitted from one generation to another is vulnerability within fear-related traits.

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Engineering Precision Bacteriophage Therapy for the Targeted Control of *Escherichia Coli* O157 Infections While Preserving the Gut Microbiome in the Age of Antibiotic Resistance By Leran (Laurel) Xu

Abstract

Foodborne diseases caused by Shiga toxin producing E. coli O157 is a major global health issue. Globally, unsafe food causes about 600 million cases of foodborne diseases and roughly 420,000 deaths annually. The inability to treat E. coli O157 infections can be catastrophic: leading to hemorrhagic colitis, hemolytic uremic syndrome, or even death and rising multidrug resistance is increasingly undermining the available antibiotic treatment options. Due to the possibility of resistance emerging rapidly and spread across strains, developing new antibiotics alone is unlikely to provide a durable silution. Bacteriophages have been used as an antibiotic alternative since the 1910s, in fact, researchers have engineered speciality bacteriophages with modified receptor binding proteins, CRISPR-Cas systems, and regulatory circuits that can narrow host range and control replication. Bacteriophages specificity for particular hosts, may also reduce some of the negative side-effects of antibiotics, by preserving the native gut microbiome. With these systems in hand, this paper aims to understand how engineered bacteriophages may be designed to selectively eliminate E. coli O157 while preserving commensal gut microbiota, and limiting the spread of antibiotic resistance. Drawing on recent studies, this paper finds that engineered bacteriophages and targeted cocktails can achieve rapid, multi-log reductions of E. coli O157 on food and in simulated gut environments, while maintaining specificity for pathogenetic strains. Overall, these findings suggest that precisely engineered phage therapies represent a promising alternative to antibiotics for managing E. coli O157 foodborne infections.

Introduction

Foodborne diseases are a pressing global health issue, causing millions of infections each year. Globally, unsafe food causes about 600 million cases of foodborne diseases and roughly 420,000 deaths annually, with about 30% of foodborne deaths occurring among children under 5 years of age (WHO). In the United States, domestically acquired foodborne illnesses contribute to tens of thousands of hospitalizations and deaths each year; a CDC estimate places numerous hospitalizations and hundreds to thousands of deaths across seven major pathogens in the U.S. annually (CDC). One particularly concerning foodborne pathogen is Escherichia coli O157, which is associated with severe illnesses, including hemorrhagic colitis and hemolytic uremic syndrome, which can present with symptoms ranging from severe abdominal cramps and bloody diarrhea to kidney failure in the most serious cases (Bush et al.). E. coli O157 grows best in environments with limited oxygen and can be acquired from contaminated foods such as undercooked ground beef, raw milk, and produce contaminated with animal waste (Bush et al.). E. coli O157 is a particularly threatening pathogen, responsible for illnesses such as hemorrhagic colitis and hemolytic uremic syndrome (Doyle). Hemorrhagic colitis presents with intense

abdominal cramps and bloody diarrhea, while hemolytic uremic syndrome can lead to kidney failure and life-threatening complications. The global burden of E. coli O157 contributes to substantial healthcare demands worldwide, with documented impacts on healthcare systems in both developing and developed countries.

Alarmingly, the treatment of E. coli O157 infections is becoming increasingly challenging due to the emergence of multidrug-resistant strains (Martínez-Vázquez et al.). The standard approach for treatment remains largely supportive, and as antibiotics can have limited efficacy and can worsen outcomes by increasing toxin release; care focuses on symptom management during recovery (Keddy et al.). This escalating multidrug resistance in E. coli O157 is significant because it demonstrates how antibiotic practices in livestock production can deplete the effectiveness of existing treatments, transforming common foodborne exposure into a much more dangerous health threat to the public.

Given these limitations, bacteriophage therapy is emerging as a promising approach. Bacteriophages are viruses that specifically infect bacterial cells and have been studied for decades for their potential to target bacterial pathogens. Historically, phage therapy has shown therapeutic relevance in various contexts, underscoring its potential as an alternative to broad-spectrum antibiotics. There are two types of bacteriophages: lysogenic and lytic phages. Extensive research has clarified the mechanisms controlling the switch between lysogenic and lytic cycle (Oppenheim et al.). Between the two, lytic phages are often preferred for therapeutic purposes because they lyse (to lyse means to break down, rupture, or destroy a cell by damaging its outer membrane or wall, causing the contents to spill out) bacterial cells rapidly without integrating into the host genome, thereby reducing the risk of horizontal gene transfer (Principi et al.). Horizontal gene transfer is able to move multidrug resistance genes between bacteria, so by limiting this process, it helps to prevent the further spread of antibiotic resistance in these bacterial populations. Engineering phages to enhance specificity and efficacy may yield additional therapeutic benefits, such as expanded host range or increased bacteriolysis, without collateral damage to the microbiome.

While bacteriophage therapy seems extremely promising, a major challenge in targeting E. coli O157 is maintaining the specificity of the phages. Although the E. coli O157 pathogen is extremely dangerous, the gastrointestinal tract hosts numerous beneficial and commensal E. coli species that support immunity, digestion, and overall health (Ogunrinola et al.). Disrupting these important microbes can result in severe health issues, emphasizing the necessity for engineered phages that selectively target pathogenic strains while preserving the integrity of the microbiome (Ma et al.). So, the disruption of these beneficial microbes can lead to severe and significant health issues, underscoring the need for engineered phages that selectively target pathogenic strains without harming the microbiome (Ma et al.). Given these considerations, this research paper will explore strategies that are being employed to engineer phages that effectively eliminate E. coli O157 without adversely impacting beneficial microbiota in the gastrointestinal system through maintaining the host specificity of the phages.

Molecular Engineering Strategies for Effectively Targeting E. coli O157

Bacteriophage engineering offers a promising path for treating antibiotic-resistant bacterial infections such as E. coli O157. One approach to maintain host specificity of engineered bacteriophages is to target the receptor-binding protein (RBP) of the phages (Gencay et al.). RBPs are proteins on the phage tail that recognize specific surface receptors on their bacterial hosts, such as outer-membrane proteins or o-antigen structures (Jia et al.). By modifying the RBPs of phages, scientists can more accurately engineer phages that target specific pathogenic bacterial strains, while leaving the commensal strains unharmed. A few different techniques are available to re-shape the binding interface so that it preferentially binds to, and lyses E. coli O157 (Dams et al.; Jia et al.; Klumpp et al.). One technique is called domain swapping, which involves replacing the native receptor-binding domain of a tail fiber with one from a different phage. Another is directed evolution where iterative rounds of mutagenesis and selection are performed for improved binding to a chosen host. A third technique is computational design, which involves structure-guided modeling to stabilize desired contacts and destabilize off-target ones. Overall, these RBP engineering strategies show that bacteriophages can be precisely retargeted to selectively destroy E. coli O157, offering an alternative to antibiotics to control this dangerous foodborne pathogen.

In practice, scientists build chimeric RBPs by swapping the tip of the phage's tail fiber - the part that touches the bacterium - with the tip of another phage that is closely related but has a different host range; this process creates a "mix and match”phage tail that is able to effectively recognize a new type of bacterial cell. This method is appealing because it reuses the binding parts of nature that already work well in nature, instead of forcing scientists to invent a whole new binding protein from zero. By swapping these already existing pieces between related phages, scientists are able to alter what bacteria a phage can infect with minimal gene editing. The success of RBP-based strategies depends on making the phage and host RBPs fit together in a way that lets the tail work like a mechanical syringe.

In a normal infection, the phage would use its tail to inject the genetic material into the bacterial cell, which then allows the phage to replicate. When scientists go through the process of altering RBPs, they try to change only the part that touches the bacterial receptor while keeping the rest intact, so that replication can still go through smoothly and the injection can still happen. By doing this, scientists focus phage activity on a very specific pathogen and minimize damage to the rest of the microbiome, in which a disruption can have serious consequences on health. However, this strategy is challenging because the engineered RBPs must fit into the phage tail exactly and be able to function correctly on the new host. When these constraints are met, RBP engineering can tightly focus phage activity on pathogenic E.coli lineages and thus offers a key route for developing therapies that could eliminate E.coli O157 pathogenic species from a host, while sparing the broader microbiome, and thereby preventing negative health outcomes from dysbiosis, and the potential for MDR E. coli O157 pathogens.

Another approach for engineering bacteriophages with increased host specificity for pathogens involves the use of CRISPR-Cas9. CRISPR-Cas9 is a gene-editing system that uses

guiding proteins to cut DNA at specific sequences, and allows scientists to precisely insert, delete, and modify genetic material (Chaudhary et al.; Jia et al.; Chitboonthavisuk et al.). CRISPR-Cas9 modified phages are engineered to carry CRISPR components (gRNA and Cas9) that target sequences unique to E. coli O157. These phages are engineered to package a programmable guide RNA (gRNA) which locates and binds to certain target DNA sequences in the pathogen, and a Cas-9 nuclease which acts like scissors to cut DNA at specific targeted spots (Klumpp et al.; Gencay et al.). When the CRISPR-engineered phages infect their target bacterial cell, the CRISPR machinery is expressed inside the bacterium, where the gRNA directs Cas9 (or a related nuclease) to a target sequence (Stirling et al.). Often, researchers will target genes within the bacterial cells that are responsible for their pathogenicity, rendering them either incapable of inducing infection or inducing cell death (Klumpp et al.; Gencay et al.; Stirling et al.). This strategy is powerful because the gRNA can be chosen to recognize genes that distinguish pathogenic E.coli species from commensal E. coli species.

Additionally, the gRNAs can be designed to target antibiotic-resistance genes. This method is valuable because it can prevent MDR pathogenic species from spreading antibiotic-resistance genes to other bacteria within their local environment (Chitboonthavisuk et al.). CRISPR-engineered phage designs are especially useful because they can disable and cut specific antibiotic resistant genes in the pathogenic bacteria, which reduces the chance that multidrug resistance will be transferred before the phage lyses them. Additionally, CRISPR gRNAs rely on sequence specificity to induce editing. This means that these engineered phages are less likely to infect and spread in the bacteria that do not have those targeted genes unless a usually harmless strain picks up the same resistance or virulence gene (Dams et al.; Klumpp et al.). This is why the guides need to target genetic features that clearly distinguish dangerous E. coli O157 from the commensal E. coli in the gut.

Compared with older genome editing methods - where scientists had to wait then isolate the phages that happened to have the desired genetic change - CRISPR phage editing is more straightforward because the phage is able to be retargeted easily by changing the gRNA sequence (Klumpp et al.; Chaudhary et al.). This is important because it substantially shortens the labor necessary to design and adapt to evolving pathogenic E. coli linkages over time (Brockhurst et al.). Since pathogenic bacteria like E.coli are constantly evolving and changing their genomes to respond to antibiotics, immune pressures, and other environmental stresses, this rapid evolution makes it really hard to design a specific phage therapy that would be able to stay effective over time (Klumpp et al.; Stirling et al.). This is because the targets on the bacterial surface in their genomes can change quickly. Nevertheless, advances in whole-genome sequencing now allows scientists to target new mutations much more quickly. Because CRISPR guided phages can be retargeted by just updating their gRNA sequences, they fit well with this method and can offer a flexible way to keep therapy methods matched to the latest E. coli variants (Klumpp et al.). This evolution follows a theory called the Red Queen Hypothesis where bacteria and their predators (in this case, phages) need to keep on adapting to survive. CRISPR editing helps phages win this race by allowing scientists to quickly edit gRNA sequences to target new bacterial targets, while

the whole genome sequencing are able to spot mutations immediately, and these keep the therapies matched to the evolving E. coli O157 without harming commensal gut microbiota (Brockhurst et al.). Together, these advances show that CRISPR engineered phages designed with rapid genome sequencing can keep up with the rapid evolution of E. coli O157 and maintain effective, targeted therapy without damaging beneficial gut bacteria.

Beyond CRISPR and RBP engineering - which focuses on targeting and recognition - regulatory gene control is able to add precision by using genetic "switches" that only turn on phage replication in the presence of certain environmental signals (Rottinghaus et al.). For example, synthetic promoters are artificial DNA sequences that act like on/off switches for genes. They can be designed to activate key lytic genes only when they detect transcription factors (proteins that turn genes on or off) or metabolites (small molecules produced by the bacteria). These synthetic promoters are produced in much higher amounts during E. coli growth in the colon, while engineered resposers are able to keep those genes turned off everywhere else (Rottinghaus et al.; Jia et al.).

Another useful technique is quorum sensing, which is a process by which bacteria communicate using chemical signals to detect when their population is getting dense (Dams et al.; Rottinghaus et al.; Klumpp et al.). Quorum sensing modules that are engineered into phages can respond to acyl-homoserine lactones or other related signals that E. coli produces, which further restricts phage replication in crowded bacterial populations typical of an active infection site (Dams et al.; Rottinghaus et al.; Klumpp et al.). These modules act like gates that block phage replication and cell lysing until the right signals emerge. Once they detect the molecular signs of E. coli colonization, they switch to complete destruction mode. Destruction mode lowers the risk of harming harmless gut bacteria and lets the phage numbers naturally drop (Dams et al.; Rottinghaus et al.; Klumpp et al.). These regulatory circuits reduce the risk of unintended lysis of non-target microbiota and ensure that replication ceases once the infection is cleared, thereby enhancing ecological safety and therapeutic efficiency. In practice, phages may be programmed to respond to molecular signatures such as specific gene-expression profiles or quorum signals that accompany E. coli O157 colonization, providing an additional safeguard against collateral damage to the gut ecosystem (Chitboonthavisuk et al.; H. Zhang et al.).

Such regulatory designs support the overarching aim of this work: constructing phage therapies that are not only strain‑specific but also context‑dependent, acting aggressively only when and where pathogenic E.coli have colonized the gastrointestinal system (Chitboonthavisuk et al.; H. Zhang et al.). Taken together, these regulatory control strategies show that phage therapies can be engineered to act only on specific settings where pathogenic E. coli O157 are present.

Limitations of Bacteriophages

One major concern with phage therapy is safety because it involves infecting people with a virus in order to kill pathogenic bacteria within their body. As a result, researchers have engineered safeguards (Rottinghaus et al.; Stirling et al.) such as genetic kill switches and

containment strategies. Genetic kill "switches" trigger phage self-destruction or replication inhibition, prevent the uncontrolled spread of bacteriophages within the body, and can prevent horizontal gene transfer (Rottinghaus et al.; Stirling et al.). If replication is not tightly controlled, phages that acquire bacterial genetic information during their life cycle, could potentially spread pathogenic, antibiotic resistant, or toxin-inducing genes to other bacteria present in their environment (Rottinghaus et al.; Stirling et al.).

One example of a kill switch within a phage system is an inducible toxin–antitoxin module (Rottinghaus et al.; Stirling et al.). In this system, a toxin gene is placed under a promoter that becomes active within specific conditions (Rottinghaus et al.; Stirling et al.). These conditions can include incidences such as a phage infecting a bacterium outside its specified host-range, or after a defined number of replication cycles (Rottinghaus et al.; Stirling et al.). In these contexts, the antitoxins (a neutralizing protein) is either absent or quickly broken down. This allows the toxin to build up unchecked, which stops the phage from replicating and spreading (Rottinghaus et al.; Stirling et al.).

Other containment systems work even better at eliminating leftover phages after a treatment, and this acts as a key safety feature (Rottinghaus et al.; Stirling et al.). For example, some phages are engineered so their capsomer or tail genes can only turn on when a specific synthetic chemical or host protein is present (Rottinghaus et al.; Stirling et al.). This means that therapeutic phages stop replicating efficiently once they leave the infected site or host (Rottinghaus et al.; Stirling et al.). Overall, these kill switches and containment designs show that phage therapies can be controlled and safely cleared after use, making them a realistic option for managing antibiotic resistant E. coli O157 infections.

Bacteriophage Delivery Mechanisms

Overall, the described engineering strategies of RBPs and CRISPR-Cas9 strategies, paired with kill-switches help ensure that engineered phages act precisely to kill their pathogenic target, and do not replicate in commensal species, or become uncontrolled in their replication during treatment (Wang et al.; Cass et al.). When delivering engineered phages into the human body, an important aspect is the precise engineering of the phages and site specific release, where the phages only activate on arrival (Wang et al.; Cass et al.). These steps together maximize the efficacy and specificity of the phages. When transferring bacteriophages via oral route, stomach acid can destroy them before they reach the infection site in the intestines, therefore rendering the treatment useless (Wang et al.; Cass et al.). As a result, encapsulation methods like packaging phages in protective nanoparticles or alginate beads are able to shield them from stomach acid and help the phages to be released where they are needed safely (Wang et al.; Cass et al.). More advanced methods use phage-binding peptides or nanoparticles that are specially coated that are able to stick to bacteria-covered gut surfaces to assemble phages at infection sites (Wang et al.; Cass et al.). Some even use tiny magnetic beads that doctors are able to move to the right spot using an external magnet to concentrate the phages where they were needed (Wang et al.; Cass et

al.). This prevents phages from spreading to other areas in the body and harming commensal gut bacteria.

Integrating molecular, genetic, and delivery innovations yields a comprehensive framework that not only selects for particular pathogens within the body, but also ensures that they arrive at the site of infection to eliminate the pathogens, while preventing harm to the broader gut ecosystem (Chitboonthavisuk et al.; Wang et al.; X. Zhang et al.). For example, Zhang et al. redesigned the phage's RBP by switching its host recognition tip with one from a phage to better target E. coli like strains (X. Zhang et al.). They combined this by CRISPR to cut a specific gene locus that was unique to the pathogen, which creates a “double hit” that has a higher efficacy in killing target cells (X. Zhang et al.). In their experiments, this combination reduced viable E. coli O157 cells by ~4.5 logs (over 99.997%) after 24 hours, compared to ~1-2 logs for controls (X. Zhang et al.).

Additionally, Chitboonthavisuk et al. took a different approach by engineering synthetic regulatory circuits into phages. For instance, promoters responsive to host gene-expression or quorum-sensing signals can restrict phage replication to conditions that mimic an active infection. In their work, phages carrying these circuits showed strong lytic activity, reducing target bacterial counts by up to 99% when exposed to signals that mimicked a real infection. However, the phages remained nearly inactive with over 90% less replication under off-target conditions without these signals (Chitboonthavisuk et al.). This shows how a gene-circuit design could cut unwanted killing of non-target bacteria with maximal precision.

Safety-focused studies by Rottinghaus et al.demonstrated that inducible kill switches, such as toxin-antitoxin systems that would trigger phage self destruction when certain chemicals are absent and other containment circuits like dependency on specific host factors for replication can drastically limit residual phage (leftover phages that are still active after treatment) after treatment (Chitboonthavisuk et al.). For example, phages equipped with genetic kill switches showed much lower persisting phage titers in animal models and in lab grown bacterial communities compared with non-contained phages even though phage titers decreased naturally as the target bacteria were cleared (Wang et al.).

Without such safeguards, rare host-range-expanding mutants or phages that mediate horizontal transfer of virulence or resistance genes could persist in the gut, posing long-term biosafety risks even after the initial infection is resolved. From a biosafety perspective, these data support the view that inducible kill switches and containment strategies are not optional, but instead necessary safeguards that protect against the unintended spread of genes through horizontal gene transfer to other bacteria (Rottinghaus et al.; Stirling et al.). Together, these elements provide an adaptable blueprint for advancing phage therapies that selectively eradicate E. coli O157 while preserving the beneficial microbiota of the gastrointestinal tract. They also offer a path forward for combating antibiotic resistant pathogens that are harmful to human health (Carter et al.; Cieplak et al.; Rivas et al.). Ultimately, these integrated engineering strategies show that phage based therapies can be designed to clear E. coli O157 reliably while

minimizing disruption of the microbiome, offering a good alternative to antibiotics for managing resistant foodborne infections.

The Development, Design, and Real-World Challenges of Advancing Effective Phage Therapies for E. coli O157

Engineered phage strategies involve the design or selection of the core components of existing bacteriophages to maximise their efficacy in a clinical setting (Carter et al.; Cieplak et al.; Rivas et al.). For example, a bacteriophage cocktail composed of three phages - ECML-363, ECML-122, and ECML-359 - was shown to have effective lytic activity against E. coli DSM 10058, showing their specificity (Carter et al.; Cieplak et al.; Rivas et al.). Different phage RBPs recognize different parts of these bacterial antigens, so to maximize the chances of lysing cells of interest, a powerful strategy has evolved: phage cocktails (Carter et al.; Cieplak et al.; Rivas et al.). Phage cocktails are made by combining phages that have RBPs against the same (or sometimes different hosts). This combination of highly specific lytic phages greatly reduces the probability that a bacterial cell is able to escape infection via mutation (Carter et al.; Cieplak et al.; Rivas et al.).

In some cases, phages may be bioengineered before combining them into a single cocktail. For instance, work by Cieplak et al., Rivas et al., and Carter et al. demonstrated that phage cocktails were efficacious in killing E. coli O157 without harming beneficial microbiota (Carter et al.; Cieplak et al.; Rivas et al.). In addition, Carter et al.'s in vitro Ecoshield three phage cocktail achieved ~1-2 log reductions of E. coli O157 on beef and lettuce in minutes and was able to maintain this effect during a week of refrigeration, while Cieplak et al.'s in vitro Ec17B153DK1 cocktail rescued a Crohn's disease associated E. coli strain by ~2.5 logs in a simulated small intestine but left a seven species commensal consortium unaffected, unlike ciprofloxacin. Finally, Rivas et al. engineered a cocktail of the following phages:e11/2/e4/1c which was able to drive E. coli O157 below detection in a rumen model within 1-2 hours using an experimental rumen model (Carter et al.; Cieplak et al.; Rivas et al.). Together, these examples illustrate how selected phage cocktails can be more potent and specific in vitro.

Even though phage therapy has shown success in vitro, there are many more challenges when transitioning this work to in vivo systems. For instance, bodies have mechanisms in place (e.g., physical barriers)that could prevent the phage from successfully reaching their targets. These physical barriers include mucus, mucosal tissue (membranes), skin, and other physical and mechanical features like tears and urine, which can help to eliminate pathogens (Carter et al.; Cieplak et al.; Rivas et al.). Additionally, in bodies, implanted phages must also compete against the existing phage and microbes present within the body, which can lead to competition with other microbes, and may hinder their success at lysing pathogens of interest. Overall, the variety of barriers and challenges makes it harder for the phages to be able to reach and target E. coli O157 (Carter et al.; Cieplak et al.; Rivas et al.).

Rationally selected phage cocktails - cocktails assembled by intentionally choosing phages with the most complementary host range and lytic activity - are able to demonstrate

impressive in vitro potency and specificity against E. coli O157 without harming commensal bacteria. However, overcoming in vivo barriers like mucus and microbial competition is extremely important for effective clinical therapies targeting foodborne and gut pathogens. In E. coli O157:H7 infections, phage cocktails reduce the probability of these pathogens mutating to gain resistance against a single phage. Phage cocktails are able to strengthen the treatment efficacy against E. coli O157 strains by using multiple phages, making them a targeted solution for combating antibiotic resistant foodborne pathogens central to this research.

A recent product, EcoShield™, emerged in 2011 as a phage cocktail composed of three Myoviridae family phages aimed at targeting and killing E. coli O157. EcoShield™ is a phage cocktail composed of three Myoviridae family phages aimed to target and kill E. coli O157. These three distinct phages have long contractile tails that apply mechanical force to inject DNA after binding to the receptor located on the bacterial cell. Importantly, each phage targets a different receptor on the surface of E. coli O157, which reduces the probability of treatment escape - meaning the bacteria are less likely to mutate or change in a way that allows them to survive the phage cocktail - as previously mentioned. In result, this treatment only targeted the intended E. coli strain and did not affect other beneficial bacteria, which proves that it is highly specific (Carter et al.).

EcoShield™ was tested in vitro on both beef and lettuce surfaces. On beef, it reduced E. coli O157 by 94-98% within 5 minutes using ~3–5 × 10^6 PFU/g, and this decrease was held at 94-98% over seven days days of storage at 4°C (Carter et al.). On lettuce, it achieved an 87% reduction at 10^6 PFU/g or 60% at the lower dose of 10^5 PFU/g, also within just 5 minutes (Carter et al.). This data shows that a one time spray of EcoShield™ rapidly kills most target bacteria and maintains lower contamination levels during fridge storage, and is able to do all of this without harming beneficial bacteria on the food. This is important because it shows that phage cocktails like EcoShield™ offer a safe, targeted way to exterminate E. coli O157 risks right at food processing.

Carter et al. designed EcoShield™, a three phage Myoviridae cocktail to target E. coli O157 on beef and lettuce – foods particularly prone to E. coli outbreaks – by spraying ~3–5 × 10^6 CFU (colony forming units)/g onto artificially contaminated surfaces and analyzing reductions over a time period at 4°C. To confirm their results, they also performed whole genome sequencing to verify the absence of toxin genes, antibiotic resistance genes, and 16S rRNA genes, to ensure specificity and no transduction risks (Carter et al.). On beef, Carter et al. saw reductions in CFUs of E. coli O157 of 94-98% within 5 minutes when compared to the control without EcoShield™. Further, the researchers saw these reductions persist up to 24 hours after using EcoShield™ on the beef.On lettuce, 87% at the 10^6 CFU/g level and by 60% at the 10^5 CFU/g level within just 5 minutes (Carter et al.). However, recontamination of treated beef with fresh E. coli O157 showed no protection despite previous successes, and that revealed that there were physical access limitations that favored surface exposed targets, the solution was suggesting repeated dosing or improved delivery systems (Carter et al.).

Researchers have developed model systems that more closely mimic the conditions of the human GI tract, allowing investigation of how bacteriophages interact with the GI tract in vivo. Additionally, other researchers such as Rivas et al. constructed O157-specific phages and tested them in vivo in both mice and calves, as well as simulated gut models ex vivo. The importance of these model systems is that they allow researchers to evaluate both the efficacy of O157 specific phages and their impact on the natural microbiota in the controlled, GI relevant environments (Rivas et al.). And, Rivas et al. were able to evaluate both the efficacy and the impact of the bacteriophage treatment on the natural microbiota both in vitro and in vivo (Rivas et al.). Overall, their experimental design involved introducing O157 into a simulated or animal GI tract, adding either a mono-phage or cocktail, and tracking the changes in pathogen load while assessing the community structure changes using 16S rRNA sequencing–which is a technique where the bacterial ribosomal gene is used as a clear taxonomic marker (Rivas et al.). The results of their study conclusively demonstrated that while O157 specific phages like e11/2 and e4/1c excel ex vivo in rumen models, with e11/2 at multiplicities of infection (MOI) 0f 1-100 driving viable E. coli O157 below direct detection within one hour and e4/1c achieving similarly strong reductions within two hours, corresponding to greater than 90% decreases in pathogen load relative to the initial inoculum (Rivas et al.).

In contrast, in the live calves, the in vivo efficacy of these phages were much more limited: phage treatment produced only modest reductions in E. coli O157 compared with controls because the phages were difficult to deliver and maintain at colonization sites, and the existing microbiota interfered with establishing a O157 infection (Rivas et al.). There were limitations within this study, however, as no antibiotics were tested in this study for direct comparison of the impact of microbiome pre- and post- treatment. In addition, although the phage-treated rumen fluid maintained a stable pH around 6.8 with no reported microbiota disruption, there was no 16S rRNA sequencing to fully support the claim that there was no microbiota disruption upon phage treatment (Rivas et al.). Taken together, these findings highlight both the promise of O157-specific phages in GI relevant models and the significant practical challenges of translating this approach into reliable, microbiome safe therapies in vivo.

To overcome the possibility of phage resistance, phage cocktails (a mixture of different phages targeting the same or different hosts) have emerged as a promising solution. To develop phage cocktails, researchers are beginning to use bioreactors and microcosms that imitate human gut conditions under controlled laboratory settings (Cieplak et al.). Unlike animals models, which involve complex host physiology and immune responses, these in vitro gut systems use defined microbiome consortia - known groups of microorganisms assembled specifically for experiments - and allow scientists to inoculate the community with E. coli O157, odd phage cocktails, and quantify reductions in E. coli by counting colonies (Cieplak et al.). Because these models can show whether a phage treatment would disrupt the microbiota without major ethical issues arising, they provide an important way to test both the efficacy and the safety of treatments (Cieplak et al.). Overall, these gut models help show whether fate cocktails can effectively reduce E. coli O157 while protecting the native microbiome, although they cannot

fully capture the complexity of in vivo conditions or allow direct one to one comparisons with animal studies.

Studies investigating the targeted phage treatment in controlled in vitro gut models generally showed a reduction, and in some cases, complete elimination of E. coli O157, with only little temporary shifts in the native microbiota (Cieplak et al.). This suggests that phage cocktails can suppress or remove the pathogen while largely preserving the surrounding microbial community, a key goal for gut directed therapies (Cieplak et al.). Although phage therapy offers a promising alternative to antibiotics, phage-resistance can still emerge, which is why researchers re-test resistant E. coli O157 mutants to determine whether changes in their surface receptors alter the susceptibility to the phages (Cieplak et al.). In most cases, the resistance was related to receptor changes that stopped phage binding, but these mutations did not appear to further infect the commensal bacteria, which shows both the specificity and the remaining evolutionary challenge of phage therapy (Cieplak et al.). This also emphasizes why rationally designed phage cocktails, which target multiple receptors or strains, may be necessary to more fully eliminate fast replicating pathogens that can rapidly evolve phage resistance.

Across these case studies, there are several common testing and engineering strategies necessary to treat E. coli O157 infections. For example, some strategies include using obligately lytic phages, host-range screening before deployment, and evaluating the safety of the phage in realistic models (Carter et al.; Cieplak et al.; Rivas et al.). At the same time, limitations include spatial barriers that limit infection, recontamination issues, and resistance development via mutations(Carter et al.; Cieplak et al.; Rivas et al.). These findings highlight the potential of rationally designed phage therapies to selectively target E. coli O157 while preserving the native microbiome. Nevertheless, factors such as gut spatial heterogeneity, environmental re-exposure, and rapid bacterial evolution remain significant barriers to replacing antibiotics with these precision therapies in clinical practice.

Discussion & Future Directions

Engineered phages offer a precision medicine alternative to antibiotics in a world where foodborne diseases already cause hundreds of thousands of deaths globally each year.E. coli O157 is becoming increasingly multi drug resistant, turning infections that were once manageable into potentially life-threatening diseases. By modifying receptor binding proteins, adding CRISPR-Cas systems, and using environment dependent regulatory circuits, researchers have created phages that infect particular strains carrying the specific markers or pathogenic genes that turn on their light of programs only in the environments where the pathogen is present (i.e. the colon) (Klumpp et al.; Stirling et al.; Chitboonthavisuk et al.). These designs directly address one of the most central challenges of gut targeted therapy: how to eliminate a highly pathogenetic E. coli lineage and ecosystem or many related, commensal E. coli strains are important for digestion and immune function (Klumpp et al.; Stirling et al.; Chitboonthavisuk et al.).Taken together, these advantages show that engineered phage therapy is rapidly expanding

and offers a promising, targeted solution to the growing problem of antibiotic resistant E. coli O157.

To address the development of phage resistance, work on phage cocktails that are bordered, including commercial preparations such as EcoShield, are able to reduce E. coli O157 contamination without disrupting microbiota, which can lead to long-term negative health outcomes (Carter et al.; Cieplak et al.; Rivas et al.). Together these molecular and ecological strategies provide a blueprint for a microbiome safe phage therapy that is both highly specific and effective, while remaining adaptable to different clinical and environmental settings (Carter et al.; Cieplak et al.; Rivas et al.). Overall, these findings suggest that phage-based approaches have strong potential as a targeted and microbiome-preserving strategy for addressing antibiotic resistant E. coli O157.

A major research priority moving forward, is to understand how resistance to engineered phages and phage cocktails arise in E. coli O157 infections, and what this means for both the evolution and pathogens and patient safety (X. Zhang et al.). Long term evolution experiments and genomic surveillance in animal or human-like in vivo models could track how often E. coli O157 gains resistance, and whether these changes make strains more or less virulent to a patient over time. Coupling this monitoring with rapid genome sequencing, and modular engineering platforms, would make it possible to update RBP designs or CRISPR guide RNAs so that phage therapies can keep up with pathogen evolution (X. Zhang et al.). In line with the Red Queen hypothesis, which describes how hosts and parasites must continuously adapt simply to maintain their relative fitness, phages and E. coli are expected to coevolve - offering the possibility that engineered phage therapy could function as living treatment that adapts alongside its bacterial targets rather than remaining static like conventional antibiotics (Anzia and Rabajante; Brockhurst et al.). More broadly, this perspective suggests that engineered phage therapy must be intentionally flexible, leveraging modular design, rapid sequencing, and combination approaches so that they just can continually run in this evolutionary race and remain affected and safe as pathogens keep evolving.

On the clinical side, future work is necessary to establish mechanisms that deliver these phage therapies safely and reliably to real patients (Ding et al.; Wang and Yu; Fukaya-Shiba et al.). This includes optimizing formulations - for example, enteric coated capsules or encapsulated phages - so that phages are able to survive stomach acid, bile, and digestive enzymes and reach the colon at high enough doses, while also limiting strong immune reactions to repeated phage exposure (Ding et al.; Wang and Yu; Fukaya-Shiba et al.). Further, this emphasizes one of the central challenges of phage therapy delivery to the GI tract: phages must be capable of surviving the conditions of the GI tract, which includes, most notably, surviving the high pH of the stomach before arriving to the site of infection within the colon for E. coli O157 infections. Additionally, research on phage pharmacokinetics (how the body absorbs, distributes, and clears phages) and dosing schedules, while keeping in mind the Red Queen hypothesis (add citation) in humans would also be needed to understand how often phages must be given, how long they persist and how they interact with the standard of care for foodborne infections.

Finally, there are many important regulatory and implementation questions that need to be addressed before microbiome safe age therapies for E. coli O157 can move from experimental tools to actual treatments. Regulators are starting to set up approval pathways for phage based products, but they still need to gather more evidence on quality control, how to standardize changing these phage cocktails, and how to prove safety in patients. Experience from cancer-focused phage work suggests that well-designed phage Therapies could become personalized tools that work alongside antibiotics rather than replacing them completely. for E. coli 0157 combining molecular engineering with the clear view of the ecosystem and regulation would be key to turning targeted pages into real world treatments that can protect both patients and their gut microbiomes. In the context of hundreds and thousands of global foodborne deaths and rising multi drug resistance, this additional work is very urgent. While more research and regulatory clarity are still needed, phage therapy offers an exceptionally promising path to prevent otherwise treatable E. coli 0157 infections from becoming life-threatening.

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Comparative Analysis of Immunotherapy Strategies for Triple-Negative Breast Cancer: Benefits, Side Effects, and Future Research Priorities By Olivia Cheon

Abstract

Breast cancer is the leading cause of death among female patients, estimated to be 670,000 deaths globally in 2022. This review paper compared four different types of immunotherapy as an emerging treatment for triple-negative breast cancer, inspecting the mechanisms, modes of delivery, clinical trial, benefits, side effects, and future directions for each of them. It was observed that all four modalities demonstrated a trade off between efficacy and toxicity, and the combination immunotherapy emerged as the most promising therapy if its toxicity and cost limitations could be solved in the future.

Introduction

Currently, breast cancer is the most commonly diagnosed cancer among women and the leading cause of death among female patients. It is a worldwide concern, accounting for an estimated 670,000 deaths globally in 2022. (*Breast cancer*) Among its molecular subtypes, triple-negative breast cancer (TNBC), which is defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2), is one of the most aggressive forms of breast cancer and has a high early recurrence rate. TNBC typically exhibits poor cellular differentiation and prognosis, high invasiveness and recurrence rates, and a strong tendency for both local and distant metastatic spread. (Liu et al.) As such, TNBC has historically demonstrated poor response to conventional treatments due to its limited therapeutic targets and aggressive biology.

Recent advances in immunotherapy have provided new treatment opportunities for TNBC. Under normal conditions, the immune system identifies and eliminates abnormal cells through immune surveillance. However, cancer cells can evade immune detection by suppressing immune activation or exploiting inhibitory signaling pathways. (Guerrouahen et al.) Immunotherapy seeks to overcome these immune escape mechanisms by helping the immune system better recognize and destroy cancer cells. It uses substances produced by the body or synthesized in a laboratory to strengthen immune activity or to provide artificial components that mimic the actions of natural immune system components. (*What is immunotherapy?*) Multiple immunotherapeutic strategies are currently under investigation for TNBC.

Four major immunotherapeutic modalities are compared here: immune checkpoint inhibitors (ICIs), cancer vaccines, adoptive cell therapy (ACT), and combination immunotherapy. Comparing these approaches is important because each differs in mechanisms, clinical stage, and toxicity profile, all of which influence treatment selection and future research priorities for TNBC.

Methods

This review paper collected and analyzed relevant literature from the PubMed and Google Scholar databases. Articles were chosen using keywords including “triple-negative breast cancer,” “immunotherapy,” “immune checkpoint inhibitors,” “cancer vaccines,” “adoptive cell therapy,” and “combination immunotherapy”. The literature search included studies published between 2020 and 2026, and articles published in English were only included. The priority was given to peer-reviewed review articles, clinical trials, and publications from recognized scientific or medical organizations to ensure reliability. The selected studies were evaluated and synthesized to compare the mechanisms, clinical evidence, therapeutic benefits, side effects, limitations, and future directions of different immunotherapy strategies for TNBC.

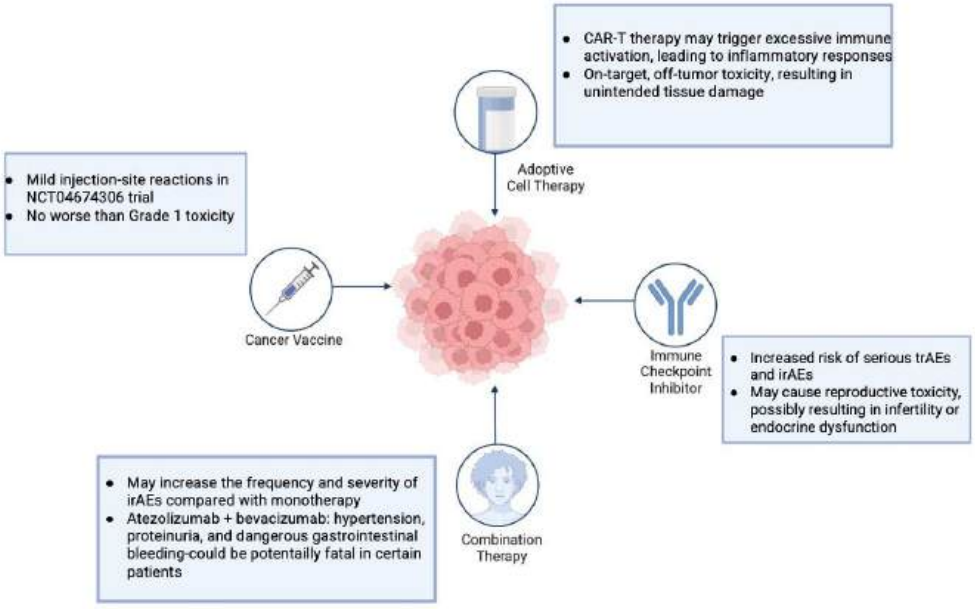

Figure 1. Schematic overview of four immunotherapeutic modalities for TNBC and their side effects. Immune checkpoint inhibitor (ICI) has an increased risk of serious trAEs and irAEs, and may cause reproductive toxicity that could potentially result in infertility or endocrine dysfunction. Adoptive cell therapy (ACT) has on-target, off-tumor toxicity, which results in unintended tissue damage. Also, CAR-T therapy may trigger excessive immune activation, leading to inflammatory responses. The cancer vaccine caused a mild injection-site reaction in the NCT04674306 trial, and the toxicity was no worse than Grade 1. Lastly, combination therapy may increase the frequency and severity of irAEs compared with monotherapy, and the combination of atezolizumab and bevacizumab resulted in hypertension, proteinuria, and dangerous gastrointestinal bleeding that could be potentially fatal in patients with advanced liver cancer and high blood pressure in the portal vein. Created in https://BioRender.com.

Literature Review

Immune Checkpoint Inhibitors(ICIs)

Mechanism

Immune checkpoint inhibitors (ICIs) restore anti-tumor immunity by blocking inhibitory signaling pathways that suppress T-cell activity. These agents target immune checkpoint molecules such as CTLA-4, PD-1, and PD-L1, which normally regulate T-cell activity. By blocking these pathways, ICIs restore T-cell activation and enhance the immune system's ability to recognize and attack tumor cells. (Liu et al.)

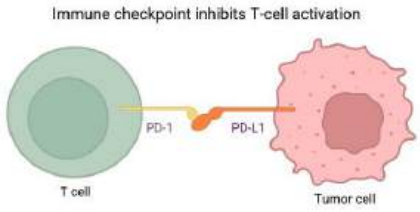

Figure 2. Mechanism of ICI. Schematic depicting the mechanism of ICI between T cell and tumor cell through PD-1 and PD-L1. Created in https://BioRender.com.

Clinical evidence

Clinical trials have demonstrated that ICIs produce durable anti-tumor activity in a subset of patients with TNBC, particularly those with PD-L1-positive tumors. (Farshbafnadi et al.) In the phase I KEYNOTE-012 trial, pembrolizumab demonstrated an objective response rate (ORR) of 18.5% in metastatic TNBC, with a median progression-free survival (PFS) of 1.9 months and a median overall survival (OS) of 11.2 months, while maintaining a favorable safety profile. Subsequent studies additionally confirmed durable anti-tumor activity in PD-L1-positive TNBC. Similarly, PD-L1 inhibitors atezolizumab and avelumab have demonstrated clinical benefits in metastatic TNBC, with atezolizumab demonstrating an ORR of 10.0% overall and 12.0% in PD-L1-positive patients, whereas the phase 1 JAVELIN study reported an ORR of 44.4% in patients with PD-L1≥10% but only 2.6% in those with PD-L1<10% for avelumab, showing the influence of PD-L1 expression on treatment response. (Liu et al.)

Benefits

ICIs provide clinically considerable improvements in pathological complete response (pCR) rates and survival outcomes, particularly in patients with PD-L1-positive tumors. (Yan et al.) ICIs enhance anti-tumor immune responses by restoring T-cell activity and overcoming immune suppression within the tumor microenvironment. Clinical benefit appears greatest in tumors with high PD-L1 expression or substantial immune cell infiltration. Furthermore, combination strategies involving ICIs have demonstrated higher response rates than monotherapy. (Liu et al.)

Side effects

Although ICIs improve clinical outcomes, they are associated with immune-related toxicities. ICIs substantially increase the risk of serious treatment-related adverse events (trAEs) and immune-related adverse events (irAEs), such as hypothyroidism, pneumonitis, adrenal insufficiency, and infusion-related reactions. (Yan et al.)
Furthermore, ICIs may cause reproductive toxicity through immune-mediated damage to gonadal tissues or disruption of the hypothalamic-pituitary-gonadal axis, possibly resulting in infertility or endocrine dysfunction. (Tanase-Damian et al.)

Limitations

Although their treatment potential seems encouraging, the clinical benefit of ICIs remains limited to a subset of patients because both primary and acquired resistance frequently develop. Primary resistance has been associated with factors such as insufficient antigen expression, loss of HLA expression, and PTEN deficiency, whereas acquired resistance can emerge after an initial response. For example, some melanoma patients initially respond to ICIs but subsequently experience disease progression. Moreover, while ICIs can improve antitumor immunity, excessive immune activation may result in irAEs including hematologic, endocrine, pulmonary, and gastrointestinal toxicities. (Dobosz et al.)

Future directions

Future research should focus on expanding the clinical benefit of ICIs. One encouraging strategy is combination therapy, as effective cancer treatment often requires the integration of multiple therapeutic modalities. This includes ICIs combined with chemotherapy, radiotherapy or targeted therapy. Another important approach is identifying more reliable biomarkers to accurately predict patient response and resistance to immunotherapy, thereby enabling better patient selection and tailored therapy. Finally, a better understanding of both primary and acquired resistance mechanisms is key for overcoming treatment failure and maximizing the lasting effectiveness of ICIs. (Dobosz et al.)

<u>Cancer Vaccines</u>

In addition to immune checkpoint inhibitors, cancer vaccines represent another major immunotherapeutic strategy for TNBC. Unlike ICIs which restore existing anti-tumor immune

responses, cancer vaccines aim to actively induce tumor-specific immunity by presenting tumor-associated antigens.

Mechanism

Cancer vaccines are designed to stimulate an anti-tumor immune response by presenting cancer-associated antigens or cellular components to the host immune system. In TNBC, vaccine strategies under investigation include adagloxad simolenin, which is an active anti-cancer immunogen that targets the Globo-H antigen and could be used as a target to identify cancer cells and eliminate malignant tumors; α-lactalbumin engineered vaccine, which is a redundant protein that may provide proactive immune protection prior to the expression of α-lactalbumin protein; DR5 DNA vaccine, which is capable of attaching to TNF-related apoptosis-inducing ligands and trigger cellular apoptosis; DC fusion vaccine, which demonstrated substantial stimulation of T cell expansion; and personalized peptide vaccine, which is composed of synthetic long peptides derived from the unique mutations (neoantigens) found in a patient's tumor-it appeared in the phase II study not to lead any serious adverse effects in the patients with metastatic and recurrent TNBC, showing a potential effect. By enhancing immune recognition of tumor antigens, these approaches aim to generate targeted anti-tumor immunity. (Liu et al.)

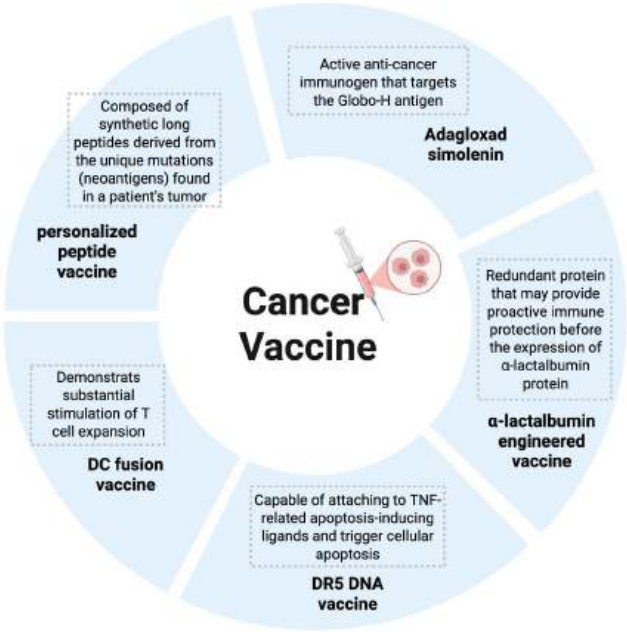

Figure 3. Different types of cancer vaccines. Figure demonstrating the different types of cancer vaccine and their characteristics. Created in https://BioRender.com.

Clinical evidence

Clinical evidence for cancer vaccines in TNBC remains limited and is largely preclinical or early-phase. Among the approaches described, the study by Tuohy et al. has consistently demonstrated clinical proof. The study focused specifically on the α-lactalbumin engineered

vaccine in murine breast cancer. The α-lactalbumin engineered vaccine is highly expressed in many TNBC cells but not in normal non-lactating breast cells, making it an encouraging target antigen for vaccine development. The completed Phase 1 clinical trial (NCT04674306) evaluated the α-lactalbumin vaccine as a preventive breast cancer vaccine. Among the 35 enrolled participants, 74% developed an immune response, supporting the vaccine's immunogenic potential. Adagloxad simolenin progressed to a Phase III clinical trial (GLORIA), although the study did not meet its primary endpoint and was terminated. This shows the challenges of translating promising vaccine candidates into clinical benefit. (Rugo et al.)

Benefits

Cancer vaccines offer the potential advantage of high antigen specificity and a relatively low incidence of adverse effects compared with some other treatment modalities. By directing the immune response toward tumor-associated antigens, these vaccines may provide a targeted immunotherapeutic strategy for TNBC. However, broader claims regarding cancer prevention or treatment replacement should be interpreted with caution, as current evidence remains preliminary. (Liu et al.)

Side effects

In the Cleveland Clinic Phase I trial of the α-lactalbumin preventive breast cancer vaccine (NCT04674306), adverse events consisted primarily of mild injection-site reactions. At the maximum tolerated dose, 14 of 15 participants experienced no worse than Grade 1 toxicity, showing a favorable early safety profile. (Johnson et al.)

Limitations

Despite their therapeutic promise, responses to tumor vaccines remain unsatisfactory with current technology. Vaccine effectiveness may vary among patients because of genetic differences in tumors, and multiple mechanisms of immune escape or evasion can limit efficacy. (Fines et al.) In addition, cancer cells may lose MHC molecules and antigen-presenting machinery, along with reduced expression of target antigens. Another limitation is that many cancer vaccines may not yet be time- or cost-effective, underscoring the need to develop shared tumor antigens that could expand the population likely to benefit. Potential adverse reactions, including toxicity, also remain a concern.

Future directions

Subsequent research should focus on carrying out additional clinical trials to determine the preventive and therapeutic effects of cancer vaccines in TNBC. Major challenges include improving efficacy and durability, identifying optimal patient populations, and addressing cost-effectiveness along with accessibility. (Imtiaz et al.) Addressing these concerns will be important for determining whether cancer vaccines can progress from early clinical investigation to a more established role in breast cancer control.

Adoptive Cell Therapy (ACT)

While cancer vaccines aim to stimulate endogenous anti-tumor immune responses by presenting tumor antigens, adoptive cell therapy takes a different approach by directly administering tumor-reactive immune cells.

Mechanism

Adoptive cell therapy (ACT) is an immunotherapeutic strategy that improves the body's ability to recognize and eliminate cancer cells by using ex vivo expanded or genetically engineered immune cells. In this approach, immune cells such as tumor-infiltrating lymphocytes (TILs), T cells engineered with chimeric antigen receptors (CAR-T), or natural killer (NK) cells are collected from the patient or donor, expanded or genetically modified in the laboratory, and reinfused into the patient. These modified immune cells can then recognize tumor-associated antigens and directly target cancer cells. (Fuentes-Antrás et al.)

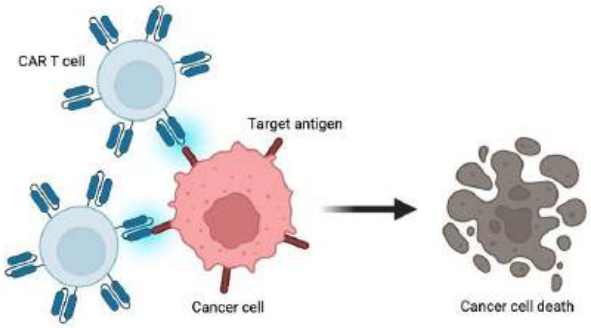

Figure 4. Mechanism of ACT. Schematic showing the mechanism of ACT, the modified CAR-T cells recognizing tumor-associated antigens and targeting cancer cells, resulting in cancer cell death. Created in https://BioRender.com.

Clinical evidence

Clinical evidence for ACT in breast cancer remains limited but promising. A case study reported complete durable tumor regression in a patient with metastatic breast cancer treated with tumor-antigen-specific TIL therapy combined with IL-2 and anti-PD-1 therapy. Genomic analysis identified 62 nonsynonymous somatic mutations, with four mutant proteins eliciting strong TIL reactivity. In addition, several clinical trials are currently investigating ACT in TNBC, including TIL therapy (NCT04111510) and CAR-T cell therapies targeting NKG2D

(NCT04107142), MUC1 (NCT04025216), and c-Met (NCT01837602). However, most studies remain in the early clinical stages, and further research is required to establish the therapeutic efficacy of ACT in breast cancer. (Fuentes-Antrás et al.)

Benefits

ACT offers a highly personalized immunotherapeutic approach by targeting tumor-specific antigens in individual patients. It can also generate strong and targeted anti-tumor immune responses by expanding tumor-reactive immune cells. CAR-T therapy may overcome certain immune escape mechanisms because CAR-T cells recognize tumor antigens in an MHC-independent manner. In addition, NK cell-based therapies can eliminate tumor cells without requiring antigen presentation, potentially enhancing the targeting of tumors that evade T-cell recognition. (Fuentes-Antrás et al.)

Side effects

ACT may cause serious treatment-related adverse effects. CAR-T therapy can trigger excessive immune activation, leading to inflammatory responses such as cytokine release syndrome and multi-organ toxicity. Another important limitation is on-target, off-tumor toxicity, which occurs when engineered immune cells recognize antigens expressed on both tumor and normal tissues, resulting in unintended tissue damage. (Fuentes-Antrás et al.)

Limitations

Despite its treatment potential, several challenges continue to limit the clinical application of ACT in breast cancer. Tumor heterogeneity and immune escape mechanisms may reduce the effectiveness of immune cell targeting. In addition, isolating and expanding tumor-reactive immune cells such as TILs remains technically challenging. Limited persistence of transferred cells, insufficient trafficking of immune cells into tumor tissues, and the immunosuppressive tumor microenvironment further reduce the therapeutic effectiveness of ACT. (Fuentes-Antrás et al.)

Future directions

Further research should focus on improving the efficacy of ACT by identifying more effective tumor-associated antigens through genomic analysis and developing more advanced gene-engineering strategies for immune cells. Improvements in areas such as cell proliferation techniques, immune cell trafficking to tumors, and persistence of transferred cells are also currently being investigated. Moreover, combining ACT with other immunotherapies such as ICIs may enhance anti-tumor responses and overcome immunosuppressive mechanisms within the tumor microenvironment. (Fuentes-Antrás et al.)

<u>Combination Immunotherapy</u>

While ICIs, cancer vaccines, and ACT each target distinct aspects of anti-tumor immunity, combining these approaches has emerged as a promising strategy to enhance therapeutic efficacy and overcome the limitations of individual modalities.

Mechanism

Combination immunotherapy aims to improve treatment efficacy by simultaneously targeting multiple pathways involved in cancer progression. Effective combination strategies can be developed by identifying interconnected targets within signaling networks that drive tumor growth and metastasis. Systems biology approaches have been used to identify key molecular targets and to design network-based therapies, thereby inhibiting multiple mechanisms associated with TNBC progression at the same time. (Pandey et al.)

Clinical evidence

Clinical evidence supporting combination immunotherapy in TNBC has expanded considerably. The phase III KEYNOTE-522 trial (NCT03036488) evaluated pembrolizumab in combination with neoadjuvant chemotherapy followed by adjuvant pembrolizumab in patients with previously untreated stage II or III TNBC. The co-primary endpoints were pCR and event-free survival (EFS). Pembrolizumab plus chemotherapy significantly improved the pCR rate compared with chemotherapy alone (64.8% to 51.2%), with an early improvement in event-free survival also observed. This evidence supports the therapeutic potential of combination immunotherapy in TNBC. (Schmid et al.)

Benefits

Combination drug therapy has presented promising therapeutic potential in TNBC and other cancers. The combined treatment strategies have been associated with improvements in pCR, PFS, and OS. Furthermore, combining PI3K/AKT/mTOR inhibition with chemotherapy demonstrated considerable improvements in PFS among TNBC patients, suggesting that combination approaches may improve treatment outcomes compared with single-agent therapies. (Schmid et al.)

Side effects

Combination immunotherapy may increase the frequency and severity of immune-related adverse events compared with monotherapy, creating additional challenges for medical management. For instance, the combination of atezolizumab and bevacizumab has been associated with high blood pressure, protein in the urine, and dangerous gastrointestinal bleeding that can be fatal for patients with advanced liver cancer and high blood pressure in the portal vein. Therefore, fixed-dose administration may require optimization to balance the tumor microenvironment, maximizing treatment success while minimizing treatment-related toxicity. (Lu et al.)

Limitations

Combination immunotherapies face several important limitations. Standard inclusion criteria remain insufficient for optimal patient selection. In addition, administering these combinations at fixed doses is still a major challenge, as inappropriate dosing can trigger excessive vessel pruning and severe intratumoral hypoxia rather than vascular normalization. Furthermore, clinical management is hindered by the lack of reliable predictive immune and vascular biomarkers to guide treatment. Finally, from an economic standpoint, their regimens are not cost-effective, with financial costs remaining well above the standard economic acceptability thresholds. (Lu et al.)

Future directions

Future research should focus on designing more effective combination immunotherapy strategies that go beyond immune checkpoint inhibitor monotherapy. One proposed approach suggests that successful tumor elimination may require integrating at least four components: a tumor-targeting antibody, an ICI, a potent T-cell vaccine, and a T-cell-stimulating cytokine. Recent preclinical and clinical studies have shown the potential of these combination strategies. In particular, incorporating neoantigen- or tumor-associated antigen-based vaccines may enhance the induction of targeted anti-tumor immune responses in a larger population of patients. (Lu et al.)

Results

1. Historical Perspective of TNBC and the Emergence of Immunotherapy

Historically, TNBC had been associated with poor clinical outcomes because of its aggressive biology, high recurrence rates, and lack of ER, PR, and HER2 expression, which limits available treatment targets. To address these challenges, immunotherapy has emerged as a promising treatment strategy that enhances the immune system's ability to recognize and eliminate cancer cells. Current research has focused on several immunotherapeutic approaches, including immune checkpoint inhibitors (ICIs), cancer vaccines, adoptive cell therapy (ACT), and combination immunotherapy. The major characteristics, clinical evidence, safety profiles, and limitations of these modalities are summarized in Table 1.

2. Comparison of Immunotherapy modalities

2.1 Clinical Evidence and Efficacy

Clinical evidence for immunotherapeutic strategies in TNBC varies considerably across modalities, with ICIs and combination immunotherapy demonstrating the most extensive clinical evidence. ICIs have shown clinically meaningful improvements in pCR rates and survival outcomes. For example, in the Phase I KEYNOTE-012 trial, pembrolizumab demonstrated an ORR of 18.5%, with a median PFS of 1.9 months and a median OS of 11.2 months. Similarly,

atezolizumab demonstrated an overall ORR of 10.0% and 12.0% in PD-L1-positive patients, while avelumab achieved an ORR of 44.4% in patients with PD-L1≥10% compared with 2.6% in those with PD-L1<10%, highlighting the influence of PD-L1 expression on treatment response. Combination immunotherapy has further improved clinical outcomes. In the Phase III KEYNOTE-522 trial, pembrolizumab combined with chemotherapy increased the pCR rate from 51.2% to 64.8%, supporting the therapeutic potential of combination strategies.

In contrast, clinical evidence for cancer vaccines and ACT remains limited, largely confined to early-phase clinical studies. The α-lactalbumin vaccine is currently undergoing Phase I clinical evaluation, while adagloxad simolenin advanced to a Phase III clinical trial before failing to meet its primary endpoint. Likewise, most ACT approaches remain in the early stages of clinical development. However, a case study reported complete durable tumor regression in a patient with metastatic breast cancer treated with tumor-antigen-specific TIL therapy combined with IL-2 and anti-PD-1 therapy. Genomic analysis identified 62 nonsynonymous somatic mutations, with four mutant proteins eliciting the strongest TIL reactivity. In addition, early-phase clinical trials investigating TIL therapy (NCT04111510) and CAR-T cell therapies targeting NKG2D (NCT04107142), MUC1 (NCT04025216), and c-Met (NCT01837602) are currently underway.

2.2 Side Effects and Safety Profiles

The safety profiles of the four immunotherapeutic modalities differ considerably, ranging from localized reactions to severe systemic toxicities. Cancer vaccines exhibit the most favorable safety profile, with adverse events consisting primarily of mild skin inflammation at the injection site. In the Phase I α-lactalbumin vaccine trial, 14 of 15 participants experienced no worse than Grade 1 toxicity, indicating favorable early tolerability.

In contrast, ICIs substantially increase the risk of treatment-related and immune-related adverse events (irAEs), including systemic complications such as hypothyroidism, pneumonitis, adrenal insufficiency, and infusion-related reactions, as well as potential reproductive toxicity caused by immune-mediated damage to gonadal tissues or disruptions of the hypothalamic-pituitary-gonadal axis.

ACT introduces distinct inflammatory toxicities associated with excessive immune activation. CAR-T therapy may induce cytokine release syndrome (CRS) and multiorgan toxicity, while on-target, off-tumor toxicity remains a major concern when engineered immune cells recognize antigens expressed on both malignant and healthy tissues.
Combination immunotherapy presents the greatest challenge for toxicity management because combining multiple agents frequently increases both the incidence and severity of immune-related adverse events compared with monotherapy. For example, atezolizumab combined with bevacizumab has been associated with hypertension, proteinuria, and potentially fatal gastrointestinal bleeding in patients with advanced hepatocellular carcinoma (HCC) and portal hypertension.

2.3 Technical and Economic Limitations

Despite their therapeutic promise, all four immunotherapeutic modalities face important biological, technical, and economic limitations. ICIs remain limited by both primary and acquired resistance, which have been associated with insufficient antigen expression, loss of HLA expression, PTEN deficiency, and other mechanisms of immune escape.

Cancer vaccines and ACT are similarly challenged by tumor heterogeneity and immune evasion mechanisms. Cancer cells may lose MHC molecules, antigen-presenting machinery, or targeted antigen expression, consequently reducing therapeutic effectiveness. Furthermore, many cancer vaccines remain neither time-effective nor cost-effective, while ACT meets considerable technical barriers related to isolating, expanding, trafficking, and maintaining the persistence of transferred immune cells.

Combination immunotherapy also faces considerable clinical and financial challenges. These regimens remain costly and exceed standard economic acceptability thresholds. Their clinical implementation is further limited by the absence of reliable predictive immune and vascular biomarkers, insufficiently precise patient-selection criteria, and the difficulty of optimizing dosing schedules, as inappropriate fixed-dose administration may induce vascular pruning and severe intratumoral hypoxia rather than vascular normalization.

Collectively, these findings show that each immunotherapeutic modality possesses distinct strengths and limitations with respect to efficacy, safety, and clinical feasibility. A comparative summary of these characteristics is provided in Table 1.

Table 1. Comparison of the four immunotherapeutic modalities for TNBC

Feature	ICIs	Cancer Vaccines	ACT	Combination Therapy
Mechanism	Blocks PD-1/PD-L1/CTLA-4 inhibitory signaling	Presents tumor antigens to prime new immunity	Infuses expanded or engineered immune cells	Targets multiple pathways simultaneously
Clinical Stage	Approved agents; trial data in TNBC	Phase I; one candidate reached terminated phase III	Early clinical/case studies	Phase III data available (KEYNOTE-522)
Key efficacy data	KEYNOTE-012 ORR 18.5%; JAVELIN ORR 44.4% vs 2.6% (PD-L1)	NCT04674306: 74% developed an immune response; GLORIA Phase III terminated	Case report: durable complete regression (TIL+IL-2+anti-PD-1) ; NCT04111510 and others	KEYNOTE-522 pCR 64.8% vs 51.2%
Main side effects	irAEs (hypothyroidism, pneumonitis, adrenal insufficiency)	Mild injection-site reactions; 14/15 ≤ Grade 1	CRS; on-target, off-tumor toxicity	Amplified toxicities; GI bleeding, hypertension, proteinuria
Main limitations	Primary/acquired resistance; immune escape	Tumor heterogeneity; immune escape; cost-effectiveness	Manufacturing; trafficking; cell persistence	Cost; fixed dosing; biomarker limitations

Discussion

The comparison of the four immunotherapeutic modalities shows a consistent balance between clinical efficacy and systemic toxicity. These findings suggest that a single treatment strategy may not be appropriate for patients with TNBC.

While ICIs and combination immunotherapy currently provide the strongest clinical evidence, including improvements in pCR, PFS, and OS, these benefits are accompanied by an increased risk of immune-related adverse events. (Liu et al.) In contrast, cancer vaccines have demonstrated favorable safety profiles, with adverse events largely limited to mild injection-site reactions in early clinical studies, but their therapeutic efficacy remains under clinical investigation. (NCT04674306) Likewise, ACT has shown encouraging results in individual case reports and early-phase clinical trials, yet its broader clinical application remains limited by technical challenges and safety concerns. (Fuentes-Antrás et al.) These results indicate that treatment decisions should balance expected clinical benefit against the potential risks of each modality.

Another important finding is that the future success of immunotherapy depends not only on improving efficacy but also on overcoming biological, technical, and economic barriers. Primary and acquired resistance continue to limit the effectiveness of ICIs, whereas tumor heterogeneity and immune escape reduce the efficacy of both cancer vaccines and ACT. (Dobosz et al.; Fuentes-Antrás et al.) Furthermore, ACT remains technically demanding because immune-cell isolation, expansion, trafficking, and persistence remain challenging, while cancer vaccines and combination therapies continue to face concerns regarding cost-effectiveness and accessibility. (Fuentes-Antrás et al.) These data underscore the need for reliable predictive biomarkers, improved patient selection, and more customized treatment strategies to maximize therapeutic benefit while limiting unnecessary toxicity.

At the same time, the comparison presented in this review should be interpreted with caution because the four modalities are at substantially different stages of clinical development. ICIs already have extensive clinical evidence from large randomized clinical trials, whereas many cancer vaccines and ACT approaches remain in Phase I studies or early clinical development. Consequently, direct comparisons of efficacy and safety should be viewed as comparisons of the current state of evidence rather than as definitive evidence of therapeutic superiority.

Overall, combination immunotherapy appears to represent the most promising future direction for TNBC treatment because it addresses multiple mechanisms of tumor progression simultaneously. Evidence from KEYNOTE-522 demonstrated that pembrolizumab combined with chemotherapy significantly improved the rate of pCR compared with chemotherapy alone, supporting the potential of combination strategies in TNBC. (Schmid et al.) However, combination therapy also produces more frequent immune-related toxicities than monotherapy and therefore requires further refinement. Future strategies will likely depend on personalized dosing, biomarker-guided patient selection, and more sophisticated multi-component regimens. A recent review has proposed that optimal tumor eradication may ultimately require integrating

multiple complementary therapeutic components, including a tumor-targeting antibody, an immune checkpoint inhibitor, a potent T-cell vaccine, and a T-cell-stimulating cytokine to generate coordinated anti-tumor immunity. (Pandey et al.) Continued research addressing toxicity, resistance, and accessibility will therefore be essential for translating these promising strategies into broader clinical practice.

Conclusion

In conclusion, this review synthesized the mechanisms, clinical evidence, safety profiles, limitations, and future directions of four major immunotherapy modalities for TNBC: ICIs, cancer vaccines, ACT, and combination immunotherapy. Collectively, these findings prove that each modality has distinct strengths and limitations, and no single approach currently offers both optimal efficacy and safety. Although cancer vaccines exhibit favorable safety profiles, their clinical evidence remains limited, whereas ICIs, ACT, and current combination therapies offer greater therapy efficacy but are associated with increased toxicity and treatment-related challenges.

Among the four modalities, combination immunotherapy appears to have the greatest potential to improve TNBC treatment because it targets multiple mechanisms of tumor progression simultaneously. However, its successful clinical implementation will depend on reducing treatment-related toxicities, identifying reliable predictive biomarkers for patient selection, optimizing personal treatment strategies, and developing sophisticated multi-component therapeutic approaches, including a tumor-targeting antibody, an immune checkpoint inhibitor, a potent T-cell vaccine, a T-cell-stimulating cytokine, and other complementary therapeutic components. (Pandey et al.) Continued progress in overcoming these biological and clinical challenges may ultimately transform TNBC from a historically poor-prognosis disease into a more manageable condition with improved long-term outcomes for patients.

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Microglia in the Central Nervous System: Development, Function, and Neurodegeneration
By Erica Ying

Summary

Microglia are key immune cells in the Central Nervous System (CNS) and play a vital role in multiple physiological mechanisms, such as immune defense, brain homeostasis, synaptic pruning, and neurogenesis. Microglia originate from yolk sac erythro-myeloid progenitors, with highly dynamic phenotypes. Recent research has shown that microglial dysfunction is closely linked to neurodegenerative diseases. In particular, in Alzheimer's disease, microglia undergo a complex transition from protective plaque phagocytosis to impaired, pro-inflammatory states, which accelerate neuroinflammation, cognitive decline, and the pathological spread of tau proteins. These findings show the potential of microglial signaling pathways as a therapeutic strategy to preserve microglial homeostasis and mitigate chronic neuroinflammation.

Introduction

Microglia are fundamental resident mononuclear macrophages resident in the Central Nervous System (CNS), despite being one of the smallest glial cell types (Colonna & Butovsky). During their development, microglia primarily originate from primitive erythromyeloid progenitors and migrate to the CNS, where they begin to adapt to the brain's microenvironment, self-maintaining locally into adulthood by self-renewal, rather than being replenished by circulating monocytes (Casali & Reed-Geaghan; Mehl et al.). Microglia respond to a diverse range of signals, neurotransmitters, danger molecules, and cytokines, which they produce to communicate an immune response (Colonna & Butovsky). Removing apoptosis debris, and inducing apoptosis in old, damaged, or waste cells, pruning weak or unnecessary synaptic connections, and supporting adult neurogenesis, are also a part of their unique role of maintaining homeostasis in the CNS (Arnoux & Audinat; Cornell et al.; Mehl et al).

Microglia have two states, activated and "resting". In their resting state, microglia are ramified and are constantly scouting the cerebral microenvironment, using their dendrites to extend and retract to patrol neuronal and non-neuronal activities (Cornell et al.). However, rather than microglia being inactive in their "resting" state, which is an outdated idea, they are highly dynamic, continuing to carry out functions essential for a healthy brain. With recent advances in imaging, genetics, and single-cell sequencing, microglia have been found to be much more diverse than scientists had once thought, as they adopt a wide spectrum of phenotypes that vary based on context and disease, rather than simply existing in two stages. They converge on microlesions, pathogens, and foreign substances, almost immediately after detection, at which point they become highly activated, transitioning to an amoeboid shape, and producing pro-inflammatory/neurotoxic mediators as an immune response, induced by ATP/ADP-P2Y12 signaling (Davalos et al.). Though the inflammatory molecules are produced with the intent to protect the brain from infection and debris, the prolonged effect of this response is detrimental, causing chronic inflammation, increased risk of heart issues, and neurodegenerative diseases

(Casali & Reed; Heppner et al.; Mehl et al.). This effect makes microglia major players in certain neurodegenerative and overall CNS diseases, such as Alzheimer's, Parkinson's, ALS, etc.

Alzheimer's especially seems to be related to microglia in certain aspects (Ulland & Colonna). This disease, which is the most common cause for dementia and is the leading cause of death in the United States (Heppner et al.; Ulland & Colonna), is characterized by amyloid beta plaque accumulation, tau and neurofibrillary tangles, chronic microglia activation, synaptic dysfunction and loss, among others ultimately leading to cognitive decline, memory loss, and behavioural/personality changes (Ulland & Colonna).

Microglia development

Microglia differentiate from the majority of the immune cells, which originate from the bone marrow. Rather, they begin their development early from erythromyeloid progenitors in the yolk sac. Studies conducted in mice have shown that in 7.5 days the primitive cells then migrate to the developing brain, where they settle and take root (Ginhoux et al.). Unlike peripheral monocytes which also arise from erythro-myeloid progenitors, microglial development relies on the transcription factors PU.1, IRF8, and Sall1 (Casali et al.). Spalt-like transcription factor 1, or Sall1, regulates organogenesis, the process by which embryonic germ layers differentiate into tissue, and microglia gene expression (Fixsen et al.; Donovan). Eventually, the progenitors specialize into microglia with the help of two transcription factors, SPI1 and IRF8 (Kierdorf et al.). The microglia precursors proliferate rapidly within the brain and spread across the neural tissue (Matcovitch-Natan et al.). The development of their function is primarily due to the help of the transforming growth factor-β (TGF-β), which activates numerous genes that are exclusively expressed in microglia, including P2RY12, which encodes a marker that maintains homeostasis within microglia, and TMEM119, which has similar functions. Together, they regulate microglia and ensure they are homeostatic within the CNS (van Wageningen et al.).

Other notable growth signals are CSF1 and IL-34, which both work through the CSF1 receptor to help the microglia grow and survive as the brain grows as well (Easley-Neal et al.). Microglia grow as the brain does, gradually shifting from an amoeboid resembling shape, which initially helps the microglia clean dead cells in the dynamic brain, to their adult ramified form. This reflects their specialized functions, including the ability to prune synapses and maintain brain circuits (Masuda et al.). Recent studies using single-cell RNA sequencing have shown that even in a mature brain, microglia are not all the same, as their gene expression and behaviour differ depending on their location and local environment, influenced by factors like neuronal activity and cytokine signals (Hammond et al.).

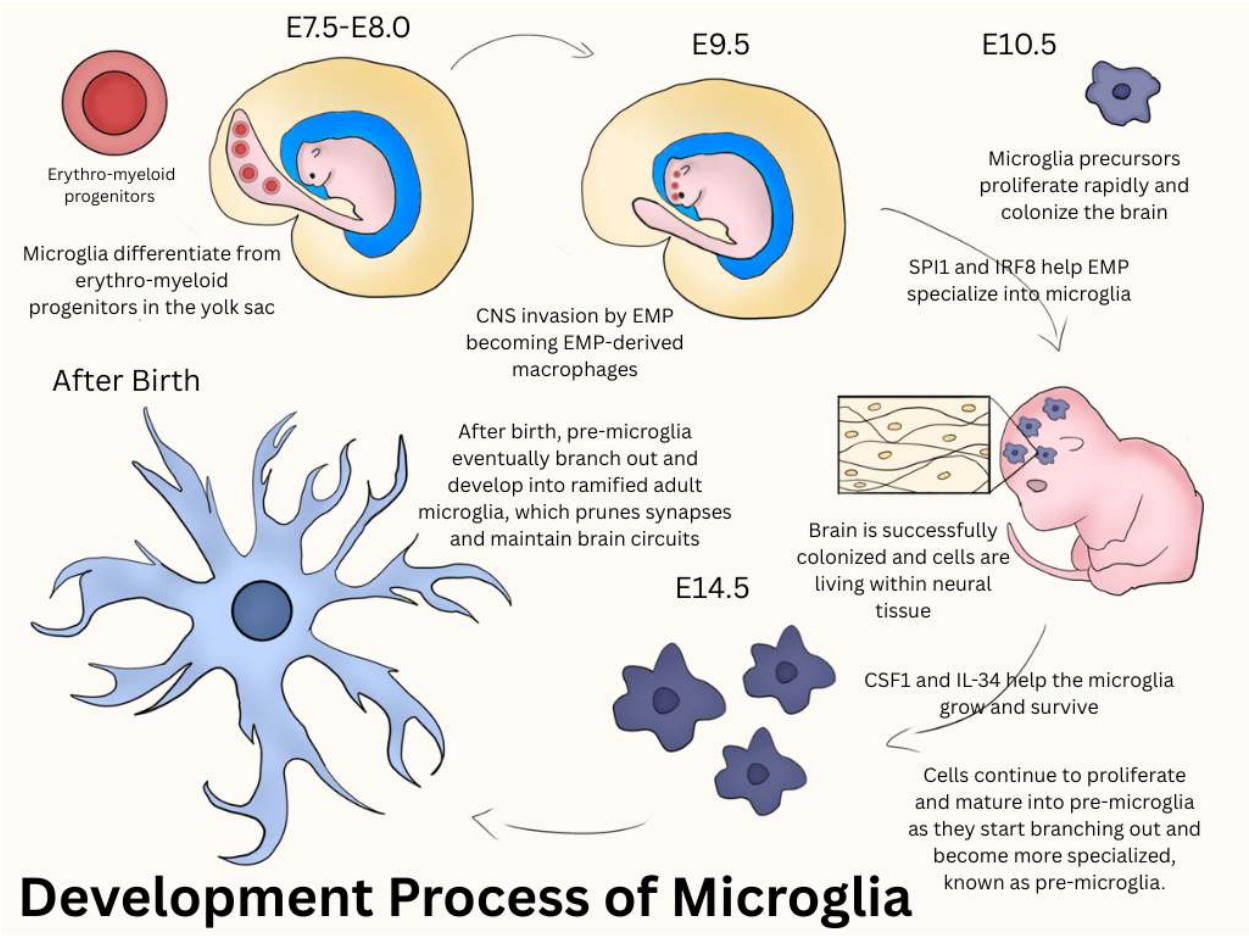

Figure 1: Development process of microglia

Immune and Defense

Microglia are the primary innate immune effector cells of the CNS, with their role being to constantly survey the parenchyma to maintain tissue integrity. Their surveying ability is a result of pattern recognition receptors, toll-like receptors, and NOD-like receptors. These receptors allow the microglia to detect pathogen-associated or damage-associated molecular patterns arising from infection or cell stress (Kigerl et al.). Upon activation, they undergo a morphological transformation by communicating with neurons and glia because they carry receptors for neurotransmitters, while also undergoing transcriptional reprogramming to support a coordinated inflammatory response. Microglia undergo phagocytosis to eliminate several factors that threaten the brain's homeostatic state, this means apoptotic cells, misfolded proteins, and pathogenic organisms. Receptors such as TREM2 and CR3 facilitate efficient debris recognition and clearance, thereby preventing secondary inflammation and promoting tissue repair (Ulland & Colonna). Microglia also use the receptors, Tyro3, Axl, and Mer, in order to opsonize apoptotic cells, which essentially makes the cell more vulnerable to phagocytosis. By producing cytokines and chemokines, they serve as regulators of the brain using the process of inflammation. Early response mediators, TNF-α, IL-1β, IL-6, and various other

pro-inflammatory cytokines, help the innate immune response, while anti-inflammatory cytokines, like TGF-β and IL-10, contribute to the resolution of inflammation and the restoration of homeostasis after an immune response (Colonna & Butovsky). During an adaptive immune response, the microglia stimulate MHC class II, CD40, and B7 molecules, which enable antigen processing and presentation to infiltrating T-Cells (Srinivasan et al.).

Synaptic Pruning and Adult Neurogenesis

During the development of neural tissue in the brain, microglia prune synapses, allowing the nervous system to be refined by eliminating useless synapses and allowing meaningful and stronger pathways to remain. Microglia achieve this by relying on the signals from the complement system, where proteins such as C1q, caused by astrocytes releasing TGF-beta, allowing the synapses to express this protein, and C3 "tag" weaker synapses for clearance (Stevens et al.). Microglia recognize these tags with CR3 receptors and phagocyte the tagged synapses. Live imaging has shown that even in healthy adult brains, microglia still constantly make contact and adjust their structure depending on how active they are, as they continue to support lifelong synaptic plasticity, which allows the brain to adapt, learn, and store memories (Wake et al.). Microglia extend their support to the hippocampus dentate gyrus and the subventricular zone as well, as it aids the process of neurogenesis, where new neurons are formed. They support this process by releasing growth factors such as IGF-1 and BDNF that promote progenitor proliferation, increasing neuronal survival rates. They also clear away any apoptotic cells that failed to integrate into a neuron, which prevents secondary inflammation and maintains a healthy microenvironment for surviving neurons (Sierra et al.). They aid in neurogenesis by also communicating bidirectionally with stem cells through fractalkine signaling pathway via the microglia-specific receptor CX3CR1, thereby regulating microglial states that contribute to the neurogenic niche, as well as aiding in the proliferation and survival of cells (Arnoux & Audinat). But as the brain ages or undergoes neurodegenerative pathology, microglia can adopt a pro-inflammatory phenotype, which inhibits progenitor proliferation through cytokines TNF-α and IL-1β, as they begin to experience dysfunctions and decline in adult neurogenesis with time (Pérez-Rodríguez et al.).

Microglia in Alzheimer's

As mentioned before, microglia are central factors that play into the spread and function of Alzheimer's disease. As neurotoxic amyloid-β plaques begin to build up, which is the hallmark of Alzheimer's, they recognize and engulf them to prevent the build up of extracellular plaques and to limit neuronal stress through the receptor TREM2, which allows microglia to enter a specialized state that recognizes tissue damage (Krasemann et al.). This transcriptional state is known as disease-associated microglia (DAM), which is characterized by the down regulation of homeostatic genes, such as the P2RY12 and TMEM119. Though ultimately the DAM response is activated towards clearing debris, its activation causes neuronal inflammation (T Casali et al.). However, microglia protective functions waver as plaques accumulate to a point

where homeostasis through phagocytosis becomes challenging. Microglia begin to shift into a chronically activated and pro-inflammatory state, releasing an excessive amount of cytokines such as IL-1β and TNF-α which accelerate the process of cognitive decline, synaptic loss, and necrosis across neuronal tissue (Heppner et al.). In normal function, microglia can degrade tau, a neuronal protein that stabilizes microtubules, but in neurodegenerative diseases, such as Alzheimer's, they become hyperphosphorylated, clumping inside of neurofibrillary tangles within neurons, leading to cell death (Avila et al., 2004). Inadvertently, microglia cause the spread of more misfolded tau proteins, by attempting to break them down, and spreading the seed-competent tau through exosome-mediated mechanisms (Asai et al.). Their synaptic pruning function is also disturbed in Alzheimer's, as C1q and C3 inappropriately tag synapses for elimination, pushing the process of memory loss even further, even before wide-spread neuronal loss (Hong et al.).

Conclusion

Microglia play a key role in keeping the central nervous system healthy throughout life. They are involved in much more than just the brain's immune defense, contributing to processes such as clearing debris, regulating inflammation, pruning synapses, and supporting neurogenesis. As research has advanced, scientists have discovered that microglia are far more dynamic and adaptable than believed, as they change their behavior depending on the situation of the brain, such as during disease.

However, these functions, meant to protect the brain, can become harmful when they remain activated for long periods. Alzheimer's chronic activation disrupts their function, which contributes to neuroinflammation, the accumulation of amyloid-β and tau, synaptic loss, and cognitive decline. Understanding these functions helps researchers develop treatments that slow the progression of Alzheimer's. As scientists continue to learn more about microglia, they will remain key to understanding treatments for Alzheimer's and other neurodegenerative diseases.

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Understanding Recent Trends in Adolescent Mental Illness By Yiyi Yang

Abstract

The significant rise in adolescent anxiety diagnosis in recent years has sparked widespread concern about overdiagnosis. The paper argues that this rise in diagnosis reflects a greater awareness of mental health disorders, rather than clinical over-diagnosis. Three mechanisms explain this trend: advances in developmental neuroscience have improved our ability to distinguish normal adolescent development from clinical anxiety; greater recognition of stressors such as parental expectations, peer pressure, and social media has reduced stigma and encouraged individuals to seek help; and validated screening tools, such as Mini-SPIN and SPAI-B, have enabled schools and communities to identify previously undetected cases. The conclusion suggests that greater mental health awareness, scientific understanding, and access to resources may increase reported overdiagnosis rates. Nonetheless, identifying more young people is a step forward in youth mental health rather than an increase in the misdiagnosis of normal adolescent development as mental illness.

Keywords adolescent, mental health, anxiety, diagnosis, stress

Introduction

In recent years, the number of young people diagnosed with social anxiety disorder has increased significantly (Salari et al. 795). To most of the people in the society, this trend appears to be clear evidence of over-diagnosed, a tendency to pathologize normal teenage discomfort. However, “over-diagnosed “is never simply defined and can be understood in at least three distinct ways.

At the clinical level, it refers to states that should not be considered as disordered— normal fluctuations, developmental differences, or personality traits— being classified as mental illness (Tikkinen et al.). At the threshold level, it means the diagnostic bar has been lowered. Previously, only severe, persistent, and functionally impairing cases, like those who refuse school attendance entirely and experience recurrent panic attacks that stop them from engaging in daily social interactions, were classified in this category (National Institute of Mental Health). Nowadays, even milder, earlier, or more ambiguous presentations, which would be discussed later in the paper, can be included. Furthermore, at the social or systemic level, diagnosis becomes a gateway to resources, support, and legitimate identity. As a result, there is a tendency to bring more problems into the diagnostic framework.

Each of these concerns deserves serious consideration. However, this essay argues that the rising diagnosis rates of adolescent mental illness, especially social anxiety, are not primarily explained by these forms of over-diagnosed. Instead, they reflect the fact that these mental diseases are now better recognized and diagnosed, which can be shown through three basic reasons. First, detection methods and healthcare equipment and services, such as screening tools, have improved significantly. Second, the stigma surrounding mental illness has decreased. More

teenagers now realize that some emotional vulnerabilities are caused by natural adolescent brain development and are therefore more willing to ask for help. Finally, with the widespread use of social media, peer pressure and parental expectations are becoming more visible and widely discussed. For these reasons, more genuine cases are being better identified. However, it is importantly to distinguish between different categories of mental illness rather than treating them as a single block. For example, adolescent social anxiety, unlike depression or attention-deficit/hyperactivity disorder (ADHD), has a unique developmental trajectory that makes rising rates largely expected and reasonable.

I. Why Is Adolescent Social Anxiety Better Recognized, Not Over-diagnosed

First, at the clinical level, adolescent social anxiety is sometimes mistakenly labeled as a "disorder". Adolescence does involve heightened social sensitivity, largely because of normal brain development. However, a clinical diagnosis of social anxiety disorder requires persistence and significant functional impairment. Although adolescents with social anxiety tend to feel anxious and nervous before events, many are still able to function normally and adjust quickly soon after. This suggests that temporary social unease is a normal developmental reaction for adolescents, instead of a pathological condition. Therefore, those experiences alone are insufficient for a clinical diagnosis of disorder. Therefore, the concern about over-diagnosis at clinical level does not hold.

Second, at the threshold level, whether diagnostic criteria have been lowered has long been debated among mental health studies. However, lowering the thresholds for diagnosing mental illness is not necessarily equivalent to over-diagnosis. On the contrary, if identification and intervention can take place at an early stage, preventing symptoms appearing on patients from progressing into severe impairment, then lowering diagnostic thresholds may represent responsible preventive medicine. Research from the past few years have shown that adolescence is the peak period of social anxiety. If an early intervention like CBT or group therapy for social anxiety works better during adolescence than in adulthood, lowering the threshold may reflect a better understanding of mental health than an over-diagnosis.

Third, at the social or systemic level, some people believe diagnosis of mental illness functions as a gateway to resources, therefore causing an increasing number of diagnosis cases (Jutel; Bruchmüller et al. 128; Frances). However, this concern applies differently depending on the type of mental illness. For example, diseases like ADHD and autism diagnoses have indeed influenced resource allocation. But for social anxiety, the situation is the opposite. In fact, social anxiety remains substantially under-recognized globally - the vast majority of adolescents suffering from social anxiety never recede diagnosis or help. Therefore, this specific type of mental illness is more likely to be neglected rather than being over-diagnosed. Overall, none of the above three levels of over-diagnosis sufficiently explain the rising diagnosis rates of adolescent social anxiety.

II. Brain Development

The rise of social anxiety during adolescence is, to some extent, a natural developmental phenomenon. The first mechanism of better recognition comes from deeper scientific understanding of adolescent brain development. According to Tillfors and Van Zalk, social anxiety naturally rises in adolescence because the time for the brain's emotion system to mature is usually earlier than the brain's control system (Tillfors and Van Zalk 71). To be specific, the limbic system including the amygdala, which is responsible for emotional reactions, fear and vigilance, becomes highly active in early adolescence in the development of the brain. However, the prefrontal cortex, which controls emotion regulation, impulse control, and strategic thinking, does not normally become fully mature in adolescence. This developmental asynchrony means that teenagers often experience stronger emotional reactions to social evaluation, peer rejection, or public performances before their cognitive control systems are fully developed (Casey et al. 225).

This phenomenon is completely normal for adolescents and is something people experience, not pathological. However, previous generations in the past have possessed the same developmental track in their brains when they were young but lacked the recognition and support from society. Instead, their social anxiety was very likely to be categorized as "introverted personality", "shyness" or "normal teenage behavior" (Lane). Their mental health has been neglected and belittled and therefore resulting in a small number of diagnosis cases.

Nowadays, we can diagnose social anxiety more efficiently. This improvement is not caused by turning the "normal status" into diseases, it is because through all those years of studying mental health deeply, clinicians are now more able to distinguish between ordinary adolescent nervousness and clinically anxiety that requires attention from people around. This belongs to the "detection improvement" mechanism of better recognition.

III. Stress Acknowledged

The more accurate acknowledgement of stress level and social anxiety stemmed from the source that causes pressure and anxiety which had been underestimated or ignored for a long time and now finally being considered seriously.

Being pressured by peers seems to be the most important stressor of social anxiety. Research conducted by Wen & Hu indicated that peer relationship is the leading cause connecting academic stress with anxiety as stated above (Wen and Hu 48).

Academic pressure is rarely independent of peer pressure and can manifest itself through various means including peer comparison and/or social standing among peers and/or the fear of being left out. Historically, this was often considered to be the adolescent go-through associated with teenage years and seen as a normal condition for teenagers. Now we have begun to recognize peer pressure as a genuine and serious cause of stress. Where we have improved is that by recognizing this pressure as a source of stress we are now recognizing more and more cases of anxiety.

Parental expectation is the other type of stressor that must be mentioned. The research by La Greca and Ranta indicated that parental expectation is the fourth most important reason for adolescent anxiety (La Greca and Ranta).

Overprotecting parenting styles and emotionally cold parents are two main risk factors of parental expectation. Adolescents who come from over-protected families or emotionally cold parents will have higher rates of anxiety as compared to other adolescents because they lack all sense of identity. The reason why this was not stated as a mental health problem is that it has always been attributed to the way parents raise their children or family relationships as opposed to being a mental health issue.

Including social anxiety in our developmental model reflects two important improvements in this field: we have become more aware of the stigma associated with mental health, and our scientific knowledge has grown substantially. This is extremely significant as previous models often treated social anxiety as purely a personality trait or a moral failing, rather than a neurodevelopmental phenomenon.

The third type of stressor is social media. According to the research, while social media could intensify adolescents' social anxiety, it eliminated the stigma attached to mental health (Masri-Zada et al. 111). Now, teenagers have access to public posts or discussions of anxiety through social media, learn to recognize symptoms themselves, and become more willing to admit their need in receiving help from professionals. This means that the rise in diagnosis rates associated with social media does not reflect an increase in underlying morbidity, instead, it reflects a significant increase in visibility. Therefore, we can know that reduced stigma leads to greater willingness to acknowledge psychological difficulties, and this is the key mechanism of better recognition.

IV. Better Screening Tools

The third mechanism of better recognition is methodological. Garcia-Lopez, Salvador, and De Los Reyes's Chapter 6 in Ranta et al., Social Anxiety and Phobia in Adolescents, Springer.

Systematically examined brief examining tools for adolescent social anxiety including Mini-SPIN (Mini- Social Phobia Inventory) and the SPAI-B (Social Phobia and Anxiety Linventory-Brief form). These tools have been rigorously validated and proved can effectively distinguish adolescents with clinical- level social anxiety (Garcia-Lopez et al. 121). More importantly, they are suitable and can be used in school settings for daily examination.

The significance of the development of screening tools shows clearly that better tools can locate more real cases instead of false alarms. In the past, even if an adolescent completely matched with symptoms showing that he or she met the diagnostic criteria for social anxiety disorder, they would not be identified or examined unless they happen to enter a clinical setting, which most of the teenagers at that time did not. Nowadays, school counselors can use brief, validated tools to identify potential students with social anxiety and provide them with initial support as well as offer them professional assessment when appropriate. The rise in diagnosis

rates is, to a considerable extent, a direct consequence of improved measurement. It is not that we have created more cases of social anxiety, rather, more patients who have never been seemed now being discovered. And this is better screening tools leading to better recognition.

Conclusion

To sum up, rising rates of adolescent social anxiety should not be simply interpreted as over-diagnosis. Instead, we need to distinguish among the three levels of over diagnosis—clinical level, threshold level, and systemic level— and examine their applicability to social anxiety carefully. Through detailed analysis, we can find out that a more reasonable explanation of rising rates of adolescent social anxiety reflects the improvement in recognition nowadays. Scientific understanding of brain development has shown that the phenomenon has always existed, no matter forms the past or in modern society.

Environmental factors like peer pressure, parental expectations, and social media are finally taken into consideration carefully when looking at the stressor, bringing more cases to be exposed to public. Validated screening tools and improved methods have enabled schools and communities to identify genuine cases. At the same time, without treating the mental diseases as a single category makes better recognition a far more accurate description than over-diagnosed due to the unique developmental trajectory of adolescent social anxiety. Having more adolescents being seen, understood, and supported over time is the real progress behind rising diagnosis rates.

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Lost in Translation?
The Secrets Behind the U.S.'s Most Trusted Clinical Practice
By Diya Chandarana

Introduction

There is a translatability crisis in clinical trials in the United States. Animal testing is the most widely used clinical testing method in the U.S. Medications that are animal-tested are heavily relied on, and many people need them to stay healthy. As various medical companies are searching for solutions to cure different diseases, they are being held back, with only 5% of animal-tested drugs receiving Food and Drug Administration (FDA) regulatory approval for human use. (Ineichen et al. e3002667).

Translation Gaps

A lack of an animal model that translates directly to humans is holding back scientists from finding a powerful drug for ischemic strokes. When a blood vessel is blocked, it cuts off oxygen, causing a stroke. This leaves patients with severe neurological damage and no cure (Narayan et al. 2). Numerous preclinical studies report promising drugs for managing neuropathic pain—pain due to a damaged nervous system. However, replication in human trials has so far failed. Countless drugs for strokes have been found effective for neuroprotection in animals, but few have worked in human trials. Advancements in translatability are overdue and will continue to plague medical research.

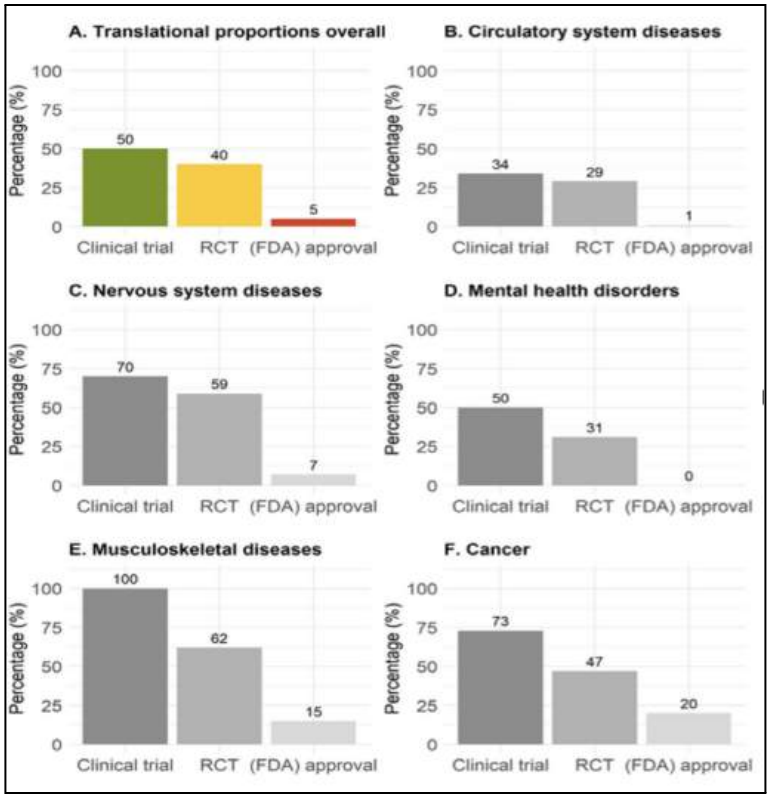

Fig. 1. Parkinson's Data: This table shows three environmental methods used to model Parkinson's disease in animals — how the disease-like state is induced, the resulting symptoms in the animal, and where each model falls short of representing real human Parkinson's (Marshall et al. 107).

Parkinson's

Parkinson's (PD) is a neurodegenerative disease, meaning it is an incurable brain disease; it results in slight tremors, balance issues, stiffness and movement issues. My grandmother, Indira Chandarana, has been living with Parkinson's disease since 2019. When her symptoms get worse, she has trouble with daily tasks like walking and holding a simple conversation (Chandarana). PD causes neurons to weaken and then ultimately die. There are approximately 1 million people in the U.S. currently living with Parkinson's, and that population is only expected to grow, with sources saying there will be 1.2 million people by 2030. To add on, worldwide, the total costs related to Parkinson's are estimated to be around $52 billion, including the costs of medications, care, and loss of income (Marshall et al. 106).

There is currently no cure for Parkinson's, only medicines that are proven to be effective in reducing symptoms, but these come with side effects such as depression. According to a study done by the National Library of Medicine, "some patients tended to delay the initiation of medications due to concerns about the early onset of side effects and a shortened response to medications as PD progresses. Nevertheless, subjective experiences after patients have been taking medications for longer periods remain to be characterized" (Chen et al. 2018769).

Even though scientists can replicate brain diseases like Parkinson's in animals, the animal models fail to reproduce the same pattern of neuronal cell death in humans, which is essential in recreating the diseases in animals. There was medical improvement shown in 90% of mice studies and 67-80% of primate studies, but only 32% of human studies showed improvement (Marshall et al. 106). As a result of this, healthcare companies have invested heavily in attempting to find a remedy for Parkinson's, but are hindered by the low transferability of animal models.

Alzheimer's

Every year, 10 million new cases of dementia occur worldwide. Alzheimer's disease (AD) is the most common, with 60-70% being dementia cases. In addition, the success rate in Alzheimer's drugs is very low, with less than 1% of drug trials showing promise. No animal models translate perfectly to the symptoms shown by humans in AD (Marshall et al, 103). For instance, transgenic mice can develop amyloid plaques and show signs of cognitive decline, but they don't display substantial neuron loss. A medicine called Aduhelm was approved by the FDA after demonstrating success in mice models. In human trials, however, there was little evidence of cognitive improvement (Marshall et al. 103).

13-year-old Dylan Amin's grandfather suffers from Alzheimer's. For 8 years, her grandfather has been unaware of her name, how old she is, and even her relation to him. "Every

time I see him, my grandfather's Alzheimer's is just a little bit worse than last time. We make sure we can go [visit him] as much as we can because we don't know how bad it could possibly get," says Dylan (Amin). Many medicines have been developed for AD, yet only 6 drugs have been approved by the FDA since 1995. These drugs can help improve cognitive symptoms, but have not been effective for all patients. Relying on animal models that fail to translate to humans leads to a high failure rate in clinical trials (Marshall et al. 103).

Traumatic Brain Injury

Traumatic brain injury (TBI) is a widespread problem, with 18.2% of Americans suffering a TBI in their lifetime (Karamian et al. 558). Additionally, TBI care is estimated to cost the global economy 400 billion USD annually (Radabaugh et al. 1433). When Nadia Turay was 3 years old, she suffered a TBI—left-side nerve damage and partial paralysis; there was no cure. 20 years later, her seizures came back again. Her medications helped but came with side effects such as depression and fatigue. Now, she doesn't remember parts of her early life and how it felt to move freely without pain (Turay). Eric Miller suffered a TBI with no cure, and 30 years later, he still has memory problems and blind spots in each eye (Miller).

Numerous medical trials have attempted to find remedies for types of TBI's, nevertheless, many have failed during animal lab testing. During a trial for TBI, a medicine called Progesterone, which had been tested on animals like rats, showed that it could help with neuroprotection (Narayan et al. 2). In human trials, however, the medication did not show such effects on humans. Additionally, it failed in numerous tests, portraying the large gap in translation (Carroll et al. 451). During another trial for Ischemic strokes, a medicine named citicoline was tested on rats and mice. It was revealed to help memory and cognitive function after neurological damage. However, according to the MDPI in the National Library of Medicine, in humans, it provided little help in repairing the neurological connections that support cognitive function.

Model generation	Means of induction	Disease features	Shortcomings
6-hydroxydopamine	Injection into the brain.	L-Dopa responsive motor deficits.	Acute toxicity.
			Lacks non-dopaminergic pathologies.
			No Lewy bodies.
MPTP and MPP+	Usually intravenous/intraperitoneal injection.	Neuroinflammation, reduced striatal dopamine, degeneration of nigrostriatal dopaminergic neurons, motor deficits.	Induces acute pathology.
	Different dosing routes/regimens are used to create different mouse models.		No extra-dopaminergic symptoms.
			Lack of proteinaceous inclusions.
			No degeneration of the locus coeruleus.
Paraquat	Usually intraperitoneal injection.	Neuroinflammation, nigrostriatal degeneration, degeneration of the locus coeruleus, accumulation of α-synuclein, reduced striatal tyrosine hydroxylase.	Accumulation in areas of the brain without a blood–brain barrier.
			Reduced motor and behavioural symptoms.

Fig. 2. Percentage of Drug Approvals
This graph displays the percentage of diseases that have passed clinical approval, regular clinical trial (RCT) approval, and FDA approval (Ineichen et al. e3002667).

Culmination

Lindsay Marshall, a researcher for the Humane Society of the United States, states, "the absence of concordance between animal and human responses to treatment suggests that scarce research funds would be better dedicated to identifying more human-relevant methods to address the issues at stake"(Marshall et al. 106). The overall estimate of drugs progressing from animal clinical trials was 50% to human studies, 40% to regular clinical trials, and only 5% received FDA approval (Marshall et al. 109). There is an overuse of animal models in medical testing and low translatability to humans, severely affecting people living with incurable diseases. By continuing to use animal models, we risk slowing down advancements in the medical field and putting all of our medical resources and time into something with a high attrition rate.

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Rewiring Parkinson's Disease: Optogenetics as a Window into Neural Circuit Dysfunction and Therapy By Rishika Vennapusa

Abstract

Optogenetics, a technique that uses light to control cell activity, has been advancing rapidly across medical fields since its recent discovery. This paper aims to reveal the potential of optogenetics, and explore how it can be used to investigate the behaviors and symptoms of Parkinson's Disease (PD). This in-depth review of the extensive research on both optogenetics and PD provides four main themes: (1) optogenetic techniques, (2) the effects of PD, (3) the application of optogenetics in PD animal models, (4) the future implications for optogenetics. Our study reveals numerous instances when optogenetics successfully mitigated PD-related symptoms on animals. Furthermore, we explore multiple optogenetic methods and outline their advantages and disadvantages. Beyond discussing the science behind optogenetics and PD, we elaborate on its potential as a crucial tool in PD research. We specifically point to the lack of research on optogenetics and the importance of future research. It is established that optogenetics can enable new discoveries in PD and, with proper attention, possibly aid in accurate treatment of the disease. The implications of optogenetics for patients with PD are discussed, along with its limitations to provide a clear picture of current-day optogenetics and present potential next steps for advancement. Optogenetics can greatly impact PD and the process of attempting to mitigate the condition in patients, from reversing symptoms to targeting the root cause.

Introduction

PD is the second most prevalent neurodegenerative disease, classified as the progressive degeneration of dopaminergic neurons in the substantia nigra (Zafar et al.). Although the disease is not fatal, its motor and non-motor symptoms–such as bradykinesis, cognitive impairment, and sleep dysfunction–can contribute to death (Connolly and Lang; Zafar et al.). PD has effective medications and therapies to alleviate symptoms, but there has been no cure to stop or reverse the disorder (Gouda et al.). PD's complex nature involves a variety of genetics, environment, and other risk factors that have made the discovery of disease-altering cures difficult.

In recent years, optogenetics has emerged as a powerful engineering technology that manipulates the activity of neurons and other cells using light and has the potential to help address numerous issues in the human body, including the neurological effects of PD (El Hajj et al.). Developed in the past 20 years, optogenetics has changed the field of neuroscience by allowing researchers to manipulate neural circuits. Furthermore, it has also begun to provide insights into numerous aspects of the brain. With the current limitations of PD therapies and medications, optogenetics proves to be a capable tool to explore the neural circuits and dysfunctions of PD with high spatial and temporal precision (Parker et al.). Here, we review how precise manipulation of brain activity via optogenetics can help prevent neuronal cell death in PD, highlighting potential benefits for patients. Finally, we conclude by outlining current

challenges and limitations that hinder the use of optogenetics and potential solutions to address these hindrances.

Optogenetic Techniques

Optogenetics, a genetic engineering method in which cells, such as neurons, are controlled by light, uses optogenetic actuators (Lehtinen et al., 2021). Actuators are light-reactive proteins that are used to regulate and/or modify the functions of a cell. The most common actuator used for this procedure is an opsin. However, opsins are ineffective without retinal-based cofactors, as they require retinal (a Vitamin A derivative) to be light-sensitive (Choi et al.). Since mammals contain these retinal-based cofactors, optogenetics is effective on humans and other animals without extra steps.

There are many opsins used in optogenetics; the most common light-sensitive protein in the process being channelrhodopsin-2 (ChR2). This protein is not found in animal bodies but rather present in the green alga, *Chlamydomonas reinhardtii*. These ChR2 proteins attach to the outside of the cell membrane, acting as channels for each cell. When these proteins absorb light, typically at 473 nm blue light, a change in their 3D shape allows cations (positively charged ions) to pass (Britt et al.). This opsin aids in depolarizing neurons with light, thereby increasing membrane potential and enhancing the chances of neuronal firing (Gradinaru et al.).

Another important opsin is halorhodopsin (NpHR). It is produced by the bacterium *Natronomonas pharaonis* (Sato et al.). This opsin functions as a chloride pump that adds chloride ions (Cl-) to hyperpolarize the membrane, inhibiting neuronal activity. However, this can only develop with continuous absorption of yellow light around the 589–593 nm range (Gradinaru et al.). Another opsin used for optogenetics through hyperpolarization is archaerhodopsin-3 (Arch-3). This protein comes from the archaebacterium, *Halorubrum sodomense* (Chow et al.). These light-driven pumps respond primarily to 570 nm yellow light (Herasymenko et al.) and 550 nm green light (Williams et al.). They are useful for optogenetics as they can pump protons, specifically hydrogen ions (H+), when exposed to the correct wavelengths to aid in inhibiting activity in the cell. However, this protein has only been highlighted in recent years, leaving little known about Arch-3 and its photocycle (Clair et al.).

Progress in light-delivery systems used for activating opsins have come a long way. There are many different types of devices used for optogenetics. One device is an external LED array, a light-emitting diode arranged in patterns to create a strong energy source (Bernstein et al.). They are placed outside the body, unlike most optogenetics procedures. However, this method is only effective for areas near the skin surface. Another technique is to use fiber-optic cables, which are a large number of optical fibers–thin strands of glass or plastic used to transmit light–bundled together to transmit light (Gorris et al.). This can be done by attaching lasers directly to the cable (tethered devices) or by using external sources that aren't directly attached to the fiber (light-coupled cables). These fiber optics are attached near the problematic surface and sometimes even target nerve axons, using cuffs that wrap around the axons. Therefore, the properties of the method and the materials can potentially damage the delicate tissues or prevent

the organism from moving freely (Chowdhury and Yamanaka). These limitations make fiber-optic cables a risky proposition, even though they are widely used in optogenetics. Recently, the idea of using fully wireless devices has been experimented with. This has been done through inductive coupling, which transfers energy between circuits and creates a change in the shared magnetic field (Mickle and Gereau). They also rely on LEDs and antennas, which can cause temporary power issues; however, rechargeable batteries or supercapacitors can help prevent power loss. These devices improve reliability and longevity due to the lack of large batteries and wired devices that other optogenetic methods used (W. Khan et al.). Overall, advancements in light delivery systems through technology have continued to enhance the safety and effectiveness of optogenetics

There are two primary genetic delivery methods to insert opsins into the body, including viral vectors and transgenic animal models. Viral vectors, such as adeno-associated virus (AAVs) and lentiviruses, are injected into an organism to deliver the opsins required for optogenetics (Emiliani et al.). Viral vectors are genetically modified entities that spread pathogens and are used to deliver genetic material to cells in optogenetics (Wang et al.). Viral vectors usually contain different types of opsin genes, photoreceptor-encoding DNA extracted from an organism, and are injected with promoters as well as targeting sequences to control gene expression (Fig. 1) (Emiliani et al.). Viral vectors can produce proteins rapidly in cells, conferring strong immunity and long-lasting effects (Holman et al.).

Another delivery method is the use of transgenic animal models, which is more permanent than using viral vectors. The foreign DNA, called a transgene, is bred into the germline, resulting in the opsin gene being present in the reproductive cells of the animal (Fig. 1) (Ting and Feng). This allows opsin genes to be passed down to future generations through biological processes (Zeng and Madisen). In conclusion, the various delivery methods are used based on the required duration of optogenetics on an organism.

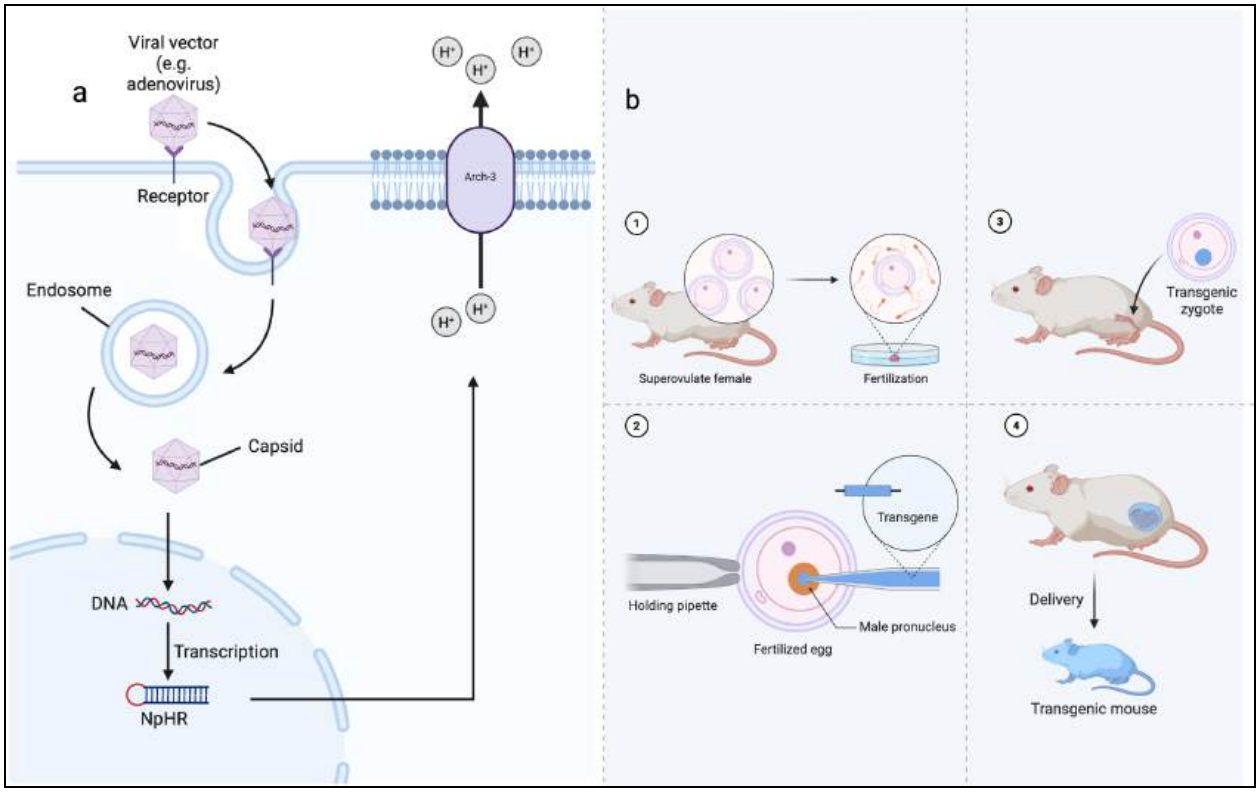

Fig 1. The mechanisms for optogenetic delivery systems
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The cell (a) engulfs the genetic material to form an endosome for transportation through endocytosis. The viral vector then escapes the endosome, moving closer to the nucleus before the capsid undergoes uncoating to release the genetic material (Riyad and Weber). The material then travels into the nucleus to add the genes to the DNA of the cell. The foreign material is then a part of the mRNA as it goes through transcription and translation to initiate protein synthesis. The genetic material then produces the opsin protein, such as Arch-3 to continue the optogenetic process. Transgenic animal models (b) begin by separating the egg away from the body of the female species. The male sperm is then fused with the egg for fertilization to occur. The transgene is then injected into the male pronucleus before the two pronuclei combine (M. A. Larson). The transgenic zygote is then reimplanted into the female rat to become a transgenic embryo; future generations can inherit the new gene through breeding if the transgene is successfully embedded into the germline.

The Overview of Parkinson's Disease

PD has a long history of discoveries and mistakes to reach what we know about the disease today. PD was first recognized as a neurological condition in 1817 by James Parkinson (Goetz). However, conditions of PD in civilization have been described in earlier texts. Additional discoveries were made later in the century, such as studies by Charcot and his students over 50 years after the first discovery of PD, which distinguished the symptoms of PD

from those of numerous other neurological disorders (Goedert and Compston; Goetz). After differentiating PD from other tremor disorders, William Gowers' accurate analysis of the disease's prevalence in males compared to females continued to redefine the scientific understanding of PD and its underlying causes (Klein and König). After a century, in 1919, Frederick Lewy discovered that PD patients contained Lewy bodies in their brain tissue, suggesting that genetics could play a role in the disease (Li and Le). This can be proven with numbers: 5-10% of current PD patients have some form of genetic variation, most likely inherited. However, further research also suggested that environmental factors may be the cause of PD. For instance, the influenza outbreaks in the early 20th century were believed to be connected to PD because of the increase in PD patients during that time, leading to a strong belief that toxins or disease could cause PD (Logroscino). Unfortunately, at present time, the exact etiology of PD remains unknown.

Among the various symptoms in PD, one of the more identifiable ones are Motor Symptom Severity (MSS), including resting tremors, bradykinesia, balance impairment, and rigidity (Pacheco-Barrios et al.). Motor issues are initiated by the misfolding and clustering of α-Synuclein (α-Syn), an essential protein believed to regulate neurotransmitters such as dopamine. This creates Lewy bodies, which can harm the nervous system. Furthermore, before Lewy bodies form, oligomers are created and kill dopaminergic neurons in the substantia nigra, an important brain region responsible for motor skills (Pacheco-Barrios et al.). The substantia nigra is a part of the basal ganglia, a group of brain structures that focus on motor control. When there are fewer dopaminergic neurons, dopamine levels decrease, resulting in interruptions in information from the basal ganglia to the cortex, a condition known as Cortical deafferentation (Berardelli et al.). Although MMS is easily detectable, pain is the most common underlying symptom, with around 67.6% of patients experiencing the symptom (Pacheco-Barrios et al.). These painful sensations are suspected to arise from abnormal processing of pain input in the nervous system (Berardelli et al.).

Due to the complexities and uncertainties surrounding PD, both genetic and non-genetic models can be used to study its pathophysiology. Most genetic models aid in discoveries on the molecular side of heritable PD (E. Khan et al.). These genetic models can be developed by injecting viral vectors that encode overexpression or mutations of putative genes identified in PD patients (E. Khan et al.). Using genetic engineering techniques, researchers introduced knock-in (KI) mouse models by inserting the G2019S mutation into the genomes of mice. This altered the PD-associated LRRK2 gene into the G2019S-LRRK2 mutation (Garofalo et al.). This mutation causes a change in the kinase domain through amino acid substitution, altering the structure of the protein (Longo et al.). This model does not follow the process of neurodegeneration, a hallmark of PD, but reveals age-dependent changes in resting DA levels similar to the symptoms of early-stage PD (Longo et al.). The G2019S KI mice also contain an increased homovanillic acid-to-DA turnover ratio, which indicates that dopamine in the body is being broken down faster than it can be made, forming a deficiency. This mimics the increased DA turnover that occurs in the striatum in the early stages of PD, which produces harmful metabolic byproducts

(Sossi et al.). Genetic models involving the LRRK2 gene provide lots of insights; however, the implications of these models do not end there.

A-Syn has been associated with a vital role in the formation of Lewy Bodies. By mutating α-Syn, researchers can mimic aspects of PD pathophysiology, such as the formation of Lewy Bodies (Mizuno et al.). Another important gene, parkin (PRKN), and its mutations directly contribute to neurodegeneration (Imai and Takahashi). PRKN is an E3 ubiquitin ligase, which is an enzyme that aids in the degradation of proteins, often known as ubiquitination. This ubiquitination process continues by targeting damaged or misfolded proteins for degradation by the proteasome, a cellular machine that degrades unneeded proteins. Mutations in PRKN increase the rate of damaged proteins in neurons, leading to neurodegeneration (Mata et al.). However, the loss of dopaminergic neurons is not usually detected in genetic models (E. Khan et al.).

Due to certain limitations of genetic models, non-genetic animal models can alternatively be used to study PD. Non-genetic animal models can be generated by administering neurotoxins that directly target dopaminergic neurons. A major neurotoxin is 6-hydroxydopamine (6-OHDA) (Varešlija et al.). This 6-OHDA model is unique because it is used unilaterally: the neurotoxin is injected into only one hemisphere of the brain. The neurotoxins then only target specific dopamine neurons, creating an asymmetric motor deficit (Tieu). This means that only one side of the body, usually the opposite of the part of the brain affected, is affected by motor issues. This reduces mortality rates while creating a control group and an experimental group in the same body, stabilizing the control variables. However, this model does not contain Lewy bodies (Tieu). For both genetic and non-genetic PD models, results vary depending on the animal species used as the test subject, as the species chosen can determine what aspects–both behavioral and pathological–of the disease are recapitulated. Therefore, none of the PD animal models can replicate human disease properly (Le et al.).

However, the most accurate experiments are induced pluripotent stem cell (iPSC) models. The IPSC model can be generated by reprogramming fibroblasts–a type of somatic skin cells–into IPSCs, which can then be differentiated into dopaminergic neurons (Fig 2.) (Beevers et al.). This brings new insights into the disease, especially the influences in humans, as dopaminergic neurons are not easily accessible (Xiao et al.).

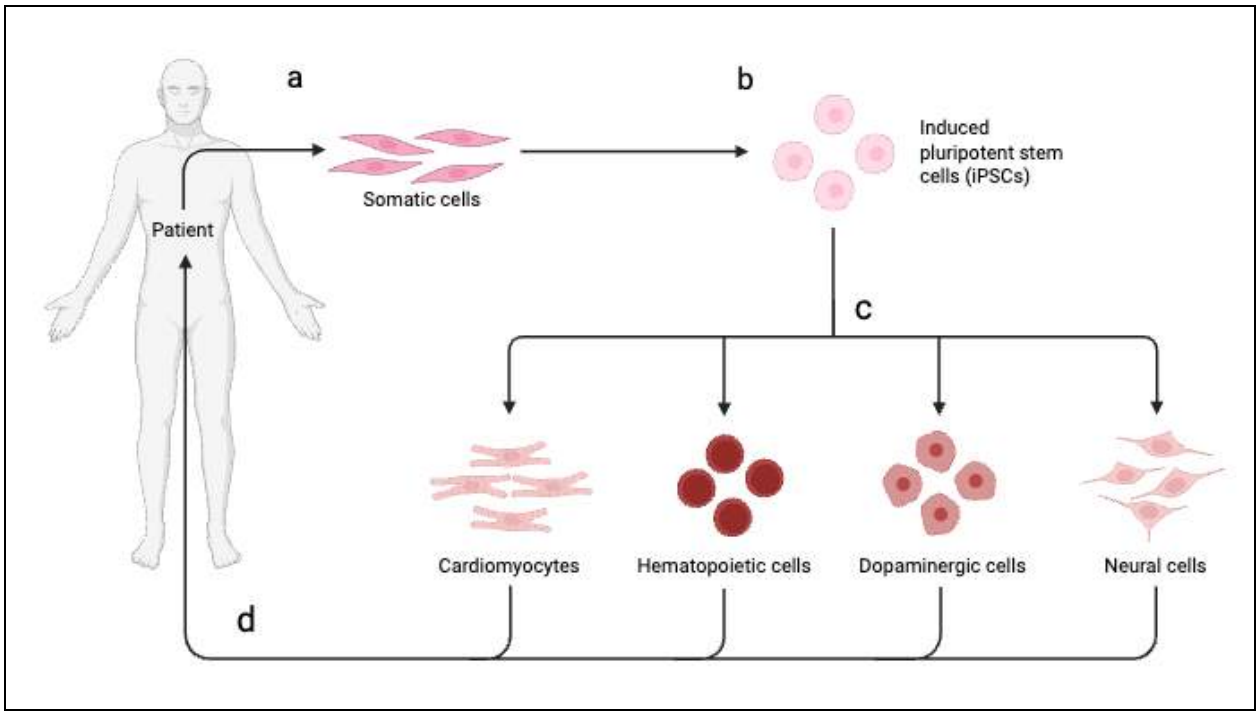

Fig 2. The pathway of a iPSC model
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The use of iPSCs is a way to convert somatic cells into cells that are destroyed by neurodegenerative diseases such as PD. First, (a) somatic cells–usually more accessible ones such as skin cells–are removed from the body. They are then (b) reprogrammed using transcription factors to set their gene expression back to the state of embryonic stem cells (Takahashi and Yamanaka). These iPSCs are then engineered to activate some genes while shutting down others, (c) allowing for a pluripotent state (Okita et al.). Based on the genes expressed and the functions they carry out, cells can be identified as specialized cells in the body such as dopaminergic cells to combat the dopaminergic neurodegeneration of PD. Finally, (d) these cells are reimplanted into the patient's body for them to duplicate.

Applications of Optogenetics in Parkinson's Disease

Optogenetics has been used in many genetic animal models of PD, such as ChR2, to explore the basal ganglia and its role in the production of PD-related symptoms. The basal ganglia contain the striatum, a crucial brain region for movement, and comprise two types of pathways: indirect and direct. The indirect pathway inhibits movement, while the direct pathway increases it. Since they are intertwined, it is difficult to study them separately (Calabresi et al.), hence the use of optogenetics. The hosts were mice that expressed a protein called Cre-recombinase: D1-Cre mice had the protein in direct-pathway MSNs, and D2-Cre mice had the protein in indirect-pathway MSNs. By using AAV1, the researchers delivered the Cre-dependent ChR2 that is fused with an enhanced yellow fluorescent protein (ChR2-YFP) into the MSNs of the pathways (Kravitz et al.). The specific MSNs that came in contact with the

Cre-recombinase protein would be light-sensitive to blue light (473 nm) and allowed for stimulation. The symptoms of PD often result from dopamine deficiency, as the lack of the neurotransmitter decreases the direct pathway and increases the indirect pathway (Kravitz and Kreitzer). The use of optogenetics helped activate the direct pathway without the need for dopamine, providing a potential solution to the dopamine damage caused by PD.

To explore another model, optogenetics can also be applied to the PINK1 fruit fly genetic model. The fruit flies tested are *Drosophila melanogaster*, a common fruit fly species, and have a genetic deletion in the PINK1 gene that makes them PINK1B9 flies. Due to the mutation in the PINK1 gene, which is located in the mitochondria, they lose PTEN-induced putative kinase 1 protein (PINK1), a type of enzyme that adds phosphate groups to other proteins (Ingles-Prieto et al.). The loss of PINK1 is associated with genetic PD, specifically mitochondrial dysfunction and dopamine neuron degeneration, creating a good environment for testing (Ge et al.). Scientists modified natural receptor proteins in the flies (dRET receptors) so they can be activated by light rather than natural biochemical signals. This is done by adding light-sensitive domains from the light-sensitive photoreceptor protein aureochrome1 (AUREO1), which is derived from the yellow-green algae *Vaucheria frigida*. AUREO1 contains a natural Light-Oxygen-Voltage (LOV) domain that creates changes in the protein when exposed to blue light at 470 nm. By combining the LOV domain with dRET receptors in flies, it can trigger RET signaling that activates pathways such as PI3K/Akt after exposure to light (Ingles-Prieto et al.). These pathways then encourage cell survival and mitochondrial function that rescues biochemical issues and morphological defects related to PD (Klein et al.).

However, exploring various PD models is crucial to understand how optogenetics interacts differently across models. An example of a non-genetic PD model is 6-OHDA, a neurotoxin that destroys dopamine-producing neurons, in Wistar rats. 6-OHDA is injected into these mice, leading to motor defects due to dopaminergic neuron degeneration (Campos et al.). Furthermore, respiratory dysfunctions are also caused by motor defects resulting from dopaminergic neuron degeneration (Aquino et al.). The research began by delivering the gene for ChR2 in the RTN through a viral vector. The opsins then functioned as a channel that depolarized the neurons upon the illumination of around 473 nm (Fig 3.). After implementing optogenetic stimulation of the RTN neurons, the activation encouraged breathing patterns and respiratory issues began to gradually improve (Hajj et al.). To further test the range of optogenetics, stimulation of the subthalamic nucleus (STN) was performed to successfully reverse pain hypersensitivity. Overall, the positive results indicate the potential of optogenetics for non-genetic PD.

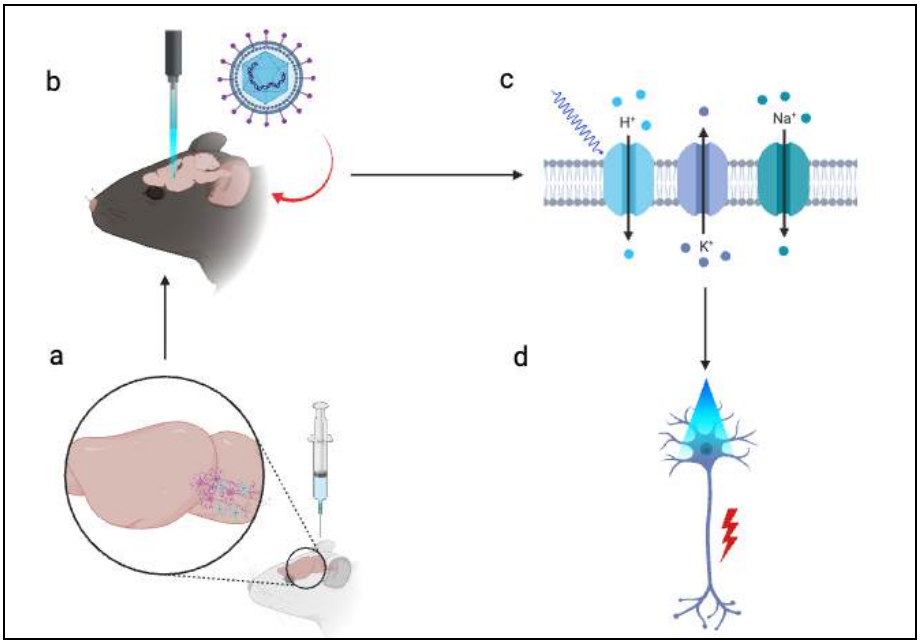

Fig 3. Application of ChR2 in a 6-ODHA PD animal model
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The 6-ODHA injection begins (a) dopaminergic neurodegeneration in the midbrain region. To combat the Pd symptoms, (b) a gene that encodes for ChR2 is introduced through viral vectors and blue light in the wavelength 470-473nm is delivered through an implanted fiber-optic cable (Hajj et al.). The light is introduced to the brain and (c) activates the ChR2, forming a passive channel that transports cations to depolarize the RTN neurons (Boyden et al.). The depolarization of the RTN region then (d) initiates the stimulation of the neurons to restore respiratory rhythm.

Another non-genetic PD model that is often used is the administration of a potent neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to primates such as monkeys. The implementation of MPTP was most successful in monkeys, whereas other animals, such as rats, were resistant to the toxin (Porras et al.). The most prominent effect of MPTP was mimicking PD's ability to lower dopamine (DA) levels in the striatum (Alam et al.). However, the strong connection between MPTP and PD has caused less research on MPTP's broader impacts beyond the nigrostriatal system and on other neurotransmitters (Porras et al.). Unfortunately, the scientific literature on MPTP is incomplete, and its link to optogenetics encourages further research on MPTP to support future optogenetic projects.

Using rodent and other animal PD models, researchers also transplanted dopaminergic neurons derived from human embryonic stem cells. They then initiated the optogenetic process through enhanced Natronomonas halorhodopsin 3.0, a modified version of halorhodopsin that displays stronger expression. The transplanted neurons then released dopamine to the striatum, a region of the brain focused on movement, during the optogenetic procedure. By releasing dopamine, it enhances excitatory post-synaptic potentials (EPSPs), which are rapid electrical

impulses used to transmit signals throughout the body. By enhancing the EPSPs, medium spiny neurons (MSNs) are ensured to have healthy firing patterns, protecting motor function (Kriks et al.). This method was later also applied to *Drosophila larvae*, demonstrating that using excitatory opsins in optogenetics to activate the relocated DA neurons can significantly benefit PD patients (Ordaz et al.).

Therapeutic Potential and Limitations

The immense potential of optogenetics in medicine makes it an important tool for studying various neurodegenerative disorders. A great example is the use of optogenetic tools in clinical applications for retinal disorders that can cause severe visual impairment and other symptoms (Kasem et al.). This reveals promise that these techniques can also be implemented to neurodegenerative diseases such as PD.

One medical therapy that supports the implementation of optogenetics is deep-brain stimulation (DBS). This gene therapy approach is currently used for mental health and movement disorders–including PD–where medication isn't effective or causes troublesome side effects (P. S. Larson; Zarzycki and Domitrz). A trait of electrical DBS is that the therapy often has better results the longer that it is implanted into the body. This is proven through the RNS Neuropace closed-loop trial, where newer DBS devices only stimulated the brain region when they detected abnormal neurological activity. When used on patients with epilepsy, people with the implant for 6 years had a 70% median reduction rate in seizure frequency, while those who only had it for 2 years had a 53% median reduction rate (Geller et al.). The use of DBS indicates the demand for new practices to overcome the nuances of neurological disorders such as optogenetics. The technique can even be a better tool than conventional electrical DBS, as optogenetics can target specific cell types precisely where electrical DBS activates all cells in one region (Gittis and Yttri). Since DBS is already widely practiced and beneficial within humans, it shows promise that optogenetics can be as well.

However, comparing electrical DBS and optogenetics comes with its limitations as well. DBS often becomes a long-term procedure as it can produce benefits up to 10 years (Limousin and Foltynie), similar to optogenetics, which relies on sustained gene expression to produce effective results (Chen et al.), making human trials difficult. Optogenetics often uses a viral vector to deliver light-sensitive proteins that stay in the body and an active implantable light source for triggering the proteins, such as fiber-optic cables and wireless devices involving LEDs. Optogenetics causes permanent modifications on the body, making the technique not ethically justified to test on healthy people. In fact, during standard drug tests, the policy is to administer the drug at only around 10% of the predicted dose at the starting stages for safety against unexpected body reactions (White et al.). This can pose a threat to evaluating optogenetics if a minimum amount of gene expression is required for the therapeutic benefits to become apparent. Nevertheless, there are ways to combat these ethical challenges. By focusing these trials on specific PD patients who are considering surgical interventions, these trials provide an opportunity for a reversible approch–as neural control can be altered based on light

expression–rather than permanent lesioning procedures (Vazey and Aston-Jones; Sharma et al.). If the trial doesn't provide the needed results, the patients can still opt in to the surgery based on the trial design.

There are scientific challenges to optogenetics as well. The behavior of brain tissue decreases light intensity, scattering the distribution of photons and leading to non-uniform absorption in the region (Gysbrechts et al.). Therefore, when using light to express opsins, it is difficult to determine the amount of light each opsin receives. This complicates choosing a specific level and amount of light for research to ensure the desired results are produced all across the region. Additionally, neurons do not express all opsins at the same level due to dissimilar cell behavior. Viral transduction, promoter activity, transportation of opsins, and other factors differ based on the cell (Mattis et al.), meaning one light-sensitive opsin can have more opsin channels—therefore larger photocurrent and can respond stronger to the stimuli–than another even under the same circumstances (Mattis et al.; Sims et al.). These limitations are much more difficult to overcome, as they require bending biological laws and the physics of the world.

Some other challenges worth mentioning include invasive technical limitations. The use of viral vectors to introduce opsins into the body proves to be effective as viral vectors are introduced directly into the nervous tissue. Furthermore, a recent trial on dogs has linked viral vectors to an increased risk of cancer, highlighting their harmful side effects (Sun et al.). This troublesome discovery could become an ethical dilemma that prevents human trials involving viral vectors from testing optogenetic therapies. Previously mentioned light delivery methods, such as power cables and fiber optics, are usually tethered to the animal in a PD model, thereby restricting movement and daily activities. The implementation of these light sources can also cause heat damage in areas such as the brain, as these lights need to be expressed for long periods of time to produce results (Ordaz et al., "Optogenetics and Its Application in Neural Degeneration and Regeneration"). It is crucial to address these issues before exploring optogenetics for PD, including finding alternatives to opsin delivery and light delivery methods. Translational limitations are also important to consider, such as the anatomical differences between primates and humans. Humans show specialized white matter patterns and unique human-specific connections in the cortical area not found in other primates (Rilling and van den Heuvel). Therefore, key mechanisms such as light delivery may become more difficult in human brains, as they are larger, have increased tissue depth, and have different connectivity patterns that promote greater light scattering and absorption, decreasing the light intensity that reaches the cells (Mohanty and Lakshminarayananan; Dufour and De Koninck). While non-human primates provide closer approximations for animal models than rodents, important differences in the brain structure limit the accuracy of translational models. Issues with the replication of PD also occur as human PD is complex and progressive, while animal models often reproduce selected features of the disease (Zhang et al.). For instance, neurotoxic animal PD models such as 6-OHDA and MPTP produce the progression of dopaminergic neurodegeneration more rapidly than observed in humans and lack the formation of Lewy bodies (Blesa and Przedborski), displaying that

animal models don't represent all aspects of human PD. Overall, there will be variables that remain unpredictable as optogenetic experimentation moves to human trials, posing major risks.

Conclusion

Optogenetics has been a growing area of interest in recent years. Its ability to target specific cells makes it a crucial tool for sensitive tissues, such as the brain. This review examined the potential for optogenetic therapies to alleviate PD-related symptoms and evaluate ongoing efforts through animal models. The analysis suggests that these tools can further research on understanding the underlying mechanisms of PD and aid in potentially alleviating symptoms in the future.

However, even with its countless benefits, optogenetics has limitations that prevent its use in clinical settings. Ethical concerns about human trials, viral vectors, and light delivery methods continue to hinder the implementation of optogenetics. Scientific and technological challenges that come with this process underscore that optogenetics has a long way to go before it can be implemented beyond models. Therefore, there should be an emphasis on the lack of research done on optogenetics and its impact on diseases such as PD, encouraging researchers to focus on this tool. Further research on identifying non-harmful viral vectors and effective wireless light sources can help overcome existing barriers, bringing it much closer to exploring the nuances of PD.

Nevertheless, optogenetics and its potential impacts extend beyond PD. By focusing on highly targeted therapies, researchers can gain crucial information to address treatment for other neurological disorders. Additionally, its cell-specific qualities allow for precision that can prevent treatments that end with unintended effects. Although barriers remain, continued research can transform optogenetics into a basis for PD and other brain conditions.

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A Review of Psychological Intervention in Sports Injury Rehabilitation By Morgan Greco

Abstract

Psychological factors play an important role in athletic injury rehabilitation by influencing motivation, treatment, and mental well-being. This literature review examines three psychological intervention methods used to aid athletes recovering from injury: motor imagery, mindfulness-acceptance commitment, and goal setting. Research on motor imagery has revealed that the practice of mentally rehearsing movements can increase activity in movement-related neural pathways and overall improve physical practice. Studies investigating mindfulness-acceptance commitment suggest enhancements in emotional regulation, athletic performance, and psychological flexibility. Research on goal setting demonstrates the benefits in self-efficacy and recovery outcomes. While each intervention targets different aspects in the rehabilitation process, the literature suggests that they can be paired with physical therapy by addressing the mental challenges associated with injury and recovery. Further research is still needed to discover how these methods of intervention can be individualized and combined in accordance with an athlete's psychological needs, injury, and stage of recovery.

Intro

Sports psychology is the scientific study of behavior in sports and has been a growing field since the 1980s. Over time, it has evolved from focusing solely on performance to acknowledging mental health as an equally important objective. The field examines how interactions among emotions, thoughts, and behaviors affect an athlete's well-being and performance. It aims to improve performance, increase enjoyment, enhance mental health, and reduce stress (1). Numerous unique methods of psychological intervention, split into three categories, Psychological Skills Training (PST), Third-Wave Mindfulness Intervention, and Positive Psychology Intervention, have been utilized to help athletes recover from mental issues as well as injuries.

Motor Imagery

Motor Imagery (MI) is a psychological intervention method in which athletes mentally rehearse a movement without physically performing it or activating the muscles involved (2). This consciously activates the brain regions responsible for planning and executing a movement while inhibiting the actual movement. The cognitive process is most often used to supplement physical rehabilitation when movement is limited in an athlete.

While recovering from an injury, pain and immobilization reduce sensory input to the brain. This can cause weakened or impaired motor representations, decreased body awareness, and cortical reorganization. MI aids in preserving neural representations even when physical movement is possible (after surgery, tendon repairs, etc).

During these mental movements, areas in the brain that play a role in movement, balance, and posture become activated. This includes the cerebellum, parietal cortex, supplementary

motor areas, and more (2). Movement-specific activations, when the brain activates the neural pathways for an exact movement, depend on the imagined movement. For example, imagining forearm flexion specifically increases excitability in the biceps muscle representation.

Athletes can undergo two types of MI. Kinesthetic (Internal) Imagery is the process in which individuals "feel" themselves performing the movement in a first-person perspective. This method is considered more effective for motor learning as it includes expected sensory experiences and produces stronger corticospinal activation (the brain activating the pathway that sends movement signals to muscles). Visual (External) Imagery is the process in which individuals watch themselves perform the movement in third-person perspective. The process activates somewhat different neural networks that are less directly connected to corticospinal activation, and overall may be less effective for improving motor performance.

Mental isochrony supports MI as an effective method for recovering athletes. The term indicates that imagined movements take approximately the same amount of time as actual movements, and suggests that the imagined movement follows normal biomechanical constraints rather than being simple visualization. There are also physiological responses that reveal MI as a successful method. During visualizations, the heart rate and respiration in athletes increase without movement.

Sports researchers investigating MI also deem that the method improves the performance of the athlete. Studies have found that combining physical practice with imagery is superior to physical practice alone. For example, athletes who mentally rehearse shooting a basketball free throw report greater performance improvement than athletes who rely only on physical practice(2). Research also indicates that imagery can increase muscle strength despite no muscle activation, suggesting that visualization can strengthen neural pathways involved in movement.

Mindfulness-Acceptance-Commitment (MAC)

Mindfulness-Acceptance-Commitment (MAC) is a method that targets athletes who are struggling mentally with negative thoughts. Instead of eliminating these thoughts, athletes learn to accept uncomfortable emotions, observe thoughts without judgment, commit to behaviors consistent with personal values, and to stay engaged despite discomfort (3). MAC is used for a multitude of reasons. Athletes frequently experience anxiety, stress, failure, and emotional distress. Psychological factors can interfere with performance and rehabilitation adherence. Traditional psychological skills training (PST) teaches athletes to control or replace negative thoughts, but trying to suppress thoughts can increase them in many cases. MAC instead teaches athletes to accept thoughts and emotions while remaining focused on the present task (4). The core values of the method include mindfulness, acceptance, cognitive defusion, present-moment awareness, values-based action, and commitment. These principles work together to help athletes come out of their negative state of mind.

To evaluate the effectiveness of MAC in sports, researchers have conducted several randomized controlled trials comparing the MAC approach with PST. These studies help identify

which psychological strategies most effectively impact and enhance athletes' well-being and performance.

In Study 1, 69 elite athletes from a range of sports (wrestling, soccer, golf, etc) were randomly assigned to either a seven-week MAC program or a traditional PST program. Similarly, in Study 2, female collegiate athletes were assigned to either a MAC or PST program randomly. Both interventions were delivered over several weeks, and participants completed assessments before the intervention, immediately afterward, and again one month later.

The research teams of both studies concluded that improved emotional regulation is one of MAC's primary mechanisms, and the athletes in the MAC groups had much lower DERS (Difficulties in Emotion Regulation Scale) scores (4). This is made evident in the athletes' reduced overall psychological distress, generalized anxiety, hostility, substance use, and eating concerns. (3). The studies additionally measured psychological flexibility in both groups, which is an athlete's ability to adapt to difficult situations, accept unwanted thoughts, and continue meaningful behaviors despite discomfort. Psychological flexibility is associated with lower risk of psychopathology and improved well-being. In the study findings, MAC increased psychological flexibility, which coincided with reductions in psychological distress (4).

Finally, the studies examined the effectiveness of the programs on athletes' performances. In study 1, athletes reported improved training performance after MAC, and improvements were explained by increased mindfulness and better emotional regulation. In study 2, Coach ratings showed significant improvements in performance in MAC groups.

Neither study involved injured participants, but the findings are applicable because injured athletes experience mental health issues such as anxiety, fear of reinjury, reduced confidence, and emotional distress. Mechanisms suggest mindfulness may have a role in rehabilitation by improving emotional regulation, increasing acceptance of pain or setbacks, reducing avoidance behaviors, maintaining focus on rehabilitation goals, and improving adherence to rehabilitation programs.

Goal Setting

The goal-setting (GS) method is a fundamental component of modern sports injury rehabilitation, providing a structured framework for athletes and rehabilitation professionals to work together toward recovery. Goals help improve both physical recovery and psychological well-being. It also promotes effort, persistence, motivation, and commitment (5). GS is a beneficial method for athletes facing mental hardships or working through recovery, as it establishes measurable standards for progress, gives injured athletes a sense of purpose, and builds confidence through small achievements.

Effective GS programs rely both on short-term and long-term goals, each serving a distinct purpose during rehabilitation. Short-term goals are more effective in improving adherence to rehabilitation exercises. They provide daily direction, improve concentration during rehabilitation, and increase effort during treatment sessions. They also help athletes recognize progress, which helps keep them motivated. Long-term goals are used to improve confidence in

athletes during long rehabilitation. They work on increasing hope for returning to a sport, providing the athlete with an overall purpose, and maintaining motivation throughout recovery.

GS interventions are also classified by their focus. Process goals focus on completing rehabilitation exercises correctly, following rehabilitation protocols, and improving technique. There is a strong emphasis on consistency and accuracy. Performance goals focus on returning an athlete and their body to their old physical abilities and functions. They include focusing on measurable outcomes, improving strength, and increasing range of motion (5).

There are many psychological benefits to GS, one of the most prominent being an increase in motivation. GS significantly increases an athlete's persistence and effort during physiotherapy. It also helps commit them to recovery. Greater self- efficacy is an additional psychological benefit of GS. Confidence in an athlete grows as well as their perceived readiness to return to their sport.

Professionals and researchers have reported many behavioral and physical benefits of GS. On the behavioral side, increased attendance at rehabilitation sessions has been shown among athletes practicing GS, along with completion of home exercises (5). Athletes are also more engaged during physiotherapy and remain focused with high effort. On the physical side, athletes in GS programs report fast recovery times and achieve high rehabilitation success. They also gain better functional outcomes, with 83% of athletes reporting that they achieved the functional goals they selected (5). This consequently increases the likelihood of achieving rehabilitation milestones during recovery.

Conclusion

Psychological factors can influence motivation, confidence, adherence, and other factors essential to injury recovery and overcoming mental barriers. The three discussed intervention methods approach these factors differently. Motor imagery focuses on maintaining and strengthening a movement to activate the neural pathways involved when the movement becomes physical. Mindfulness-Acceptance helps athletes manage emotions, accept difficult situations, and remain focused on rehabilitation. Goal setting provides structure and motivation with measurable progress. These approaches may be complementary rather than competing. For example, an injured athlete could set short-term goals, use mindfulness to manage anxiety, and use motor imagery to mentally rehearse movements before their body is physically ready. Psychological intervention should be used alongside physical rehabilitation, with future research examining how interventions could be combined and individualized according to an athlete's stage of recovery, psychological needs, and injury.

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Effects of Canine and Equine Therapies on Children's Physical and Mental Well-being
By Jixin Su

Abstract

Do you ever wonder why more individuals are turning to pet cafes when they want to destress? Living in a more competitive society like today's has resulted in an increasing level of stress and anxiety among people, including young children due to academic pressure or social expectations. As a result, there is also a growing interest in alternative therapeutic approaches that are more effective and less intimidating for them compared to traditional therapy sessions, in which patients interact directly with a therapist in a structured setting. Animal-Assisted Interactions, such as those found in structured therapies like canine therapy and equine therapy have become more popular as they provide a less formal therapeutic environment that may be more approachable for children. These therapies may also help to reduce cardiovascular arousal and cortisol levels, as well as anxiety and stress symptoms. These benefits may be especially helpful for those with social difficulties arising from trauma, as AAT can help boost their self-confidence and enhance self-esteem through interactions and bonding with therapy animals. This literature review explores the mechanisms behind these therapies and the effects of canine and equine therapies on the physical and mental well-being of children. Based on the existing literature, equine therapy and canine therapy may help children improve their physiological and psychological well-being, especially those who have difficulty engaging in social situations.

Introduction

It was first documented by child psychologist Boris Levinson that animals could be used in counselling. He observed that one of his young patients made significant progress in speech and movement after interacting with his dog, Jingles (Lange et al.). Although this was an unexpected observation, it does make people realise that animals can be used for animal-assisted therapies for their potential benefits for mental well-being. Some of the most common types of Animal-assisted therapies such as canine and equine therapies have been proposed to help children better adapt to these challenges and even improve their mental well-being. In fact, it was found that children may feel calmer and even more relaxed during therapy sessions involving interactions with animals which also increases their willingness in expressing themselves (Fine). This may help ensure a smoother and more effective therapy session, allowing therapists to make full use of the session to engage younger patients and maximise the benefits they receive. If children can perceive a trusting relationship between the therapist and the animal, they may also become more willing to interact with the therapist, particularly individuals with trauma-related issues (Germain et al.).

Animal-Assisted Therapies (AAT) are therapies that must be carried out by qualified professionals in a formally structured manner to support physical, psychological or therapeutic goals. AAT can also be combined with standard therapies such as Cognitive Behavioural Therapy, Occupational therapy and counselling to suit every patients' unique needs (Compitus).

As AAT is used increasingly along conventional therapies, many studies have started to examine the effectiveness of AAT on their patients (Marino; Fine; Brandão et al.). The growing popularity has also led to a widespread application in diverse contexts, including schools and hospitals (Marino). There are many facilities that offer AAT programmes, with dogs and horses being the most commonly used animals (Fine). However, most existing literature reviews tend to focus more on the physiological and psychological benefits of canine and equine therapies on children separately rather than synthesising both the psychological and physiological effects on them, particularly on those experiencing difficulties engaging in social situations due to trauma or stress-related issues. Focusing on both physiological and psychological effects is crucial for evaluating the effectiveness of AAT, as they provide insights into different perspectives of how AAT affects children's development, leading to a more balanced evaluation. This is important as childhood is a developmental period during which many children face psychological and interpersonal challenges. The increasing stress load on individuals also contributes to a higher risk on their cardiovascular health (Roemmich et al.). Thus, this literature review explores how equine therapy and canine therapy may help children improve their physical and mental well-being, especially among those with social, emotional, or trauma-related difficulties.

In an animal-assisted therapy programme called "A Magic Dream", Gragnon et al found that 16 participating parents and children showed reduced levels of psychological distress. Furthermore, animals have been found to provide companionship and meaningful social engagement for individuals with disabilities and mental health conditions, thereby promoting their psychological well-being (Hart and Yamamoto). This suggests that the emotional bonds formed between patients and animals play an important role in supporting their overall well-being. In addition, the soothing presence of animals may contribute to improvements in physical and emotional well-being by encouraging frequent social interaction and fostering a sense of stability (Melson; Hart and Yamamoto). However, its applicability may vary depending on individual circumstances due to challenges like cost and the need for trained professionals limit its widespread implementation. Therefore, besides examining the psychological and physiological benefits of AAT among children, this literature review explores its practical applications, and evaluates the ethical considerations associated with the use of animals in therapeutic settings.

Physiological benefits

The physiological effects of canine and equine therapies have been studied through biological markers of individuals such as blood pressure, heart rate and cortisol levels. In a study at the UNMC College of Nursing, young children were found to have significant declines in systolic blood pressure, mean arterial pressure, and heart rate after interacting with a dog, indicating that AAT was found to be useful in redirecting the children's attention away from their stress factors and could indeed be used to support them (Nagengast et al.). Research also suggests that animals can help to reduce human blood pressure, which translates into lower cardiovascular responses. This suggests that individuals are usually more relaxed and calm after

the session (Vormbrock and Grossberg). This is shown in Vormbrock and Grossberg's study, in which students who either had neutral feelings about dogs or were dog lovers showed lower heart rates after interacting with a dog. The researchers further confirmed that, through physical contact with the dog, participants experienced "contact comfort" with the animal. Through close physical interaction with therapy animals, participants' cortisol levels, a commonly used biological marker of stress, were found to decrease. This is particularly significant for younger patients who may feel anxious or intimidated when discussing experiences related to past trauma with an unfamiliar therapist in a traditional therapeutic setting, as such discussions may trigger distressing memories (Bachi and Parish-Plass). The reduction in cortisol levels suggests that AAT can help lower physiological stress, thereby enabling younger individuals to remain calm and engage more effectively in the psychotherapy process.

Besides decreasing cardiovascular arousal and cortisol levels, therapy dogs are also found to have a positive effect on children's motor coordination skills. When a dog is present, children tend to complete physical tasks such as long jump and high jump faster and with better accuracy (Gee et al.). Dogs by the side may serve as a motivating factor which stimulates children to perform better in their tasks when they see the dogs doing the same tasks as them. They may be less distracted and more engaged in sessions involving dogs. It was also found that one's motor skills are tightly linked to one's language skills; those with language impairments were found to benefit from AAT as well. The study by Gee et al. also suggested that improvements in motor skills are associated with increased self-confidence and self-esteem, which may encourage greater social engagement among preschool children with developmental difficulties, such as learning, behavioural, or social skills deficits, who were involved in the study. Although the quantitative data including the measure of physical functioning of a 10-week therapeutic horse-riding programme, showed no significant improvement in AAT participants' physical health such as body pain, qualitative data from participating families' feedback showed improvements in their children's behaviours and coordination skills (Davis et al.). The difference suggests that quantitative and qualitative methods may capture different aspects of the effects of AAT. What is not measured in numbers might still be reflected in how participants feel about the whole process, thus providing a more comprehensive evaluation of the effectiveness of AAT.

Overall, the findings of all these studies suggested different extents of physiological improvement shown by decreased heart rate, cortisol levels and improved physical functioning skills from interacting with the animals. These changes in biological markers indicate an improved physiological state across different age groups, especially for younger children, suggesting that AAT may serve as a useful tool in promoting physiological well-being among them. While these objective measures imply the benefits of AAT to a certain extent, it is still important to use subjective measures to understand the participants' experience and the psychological benefits behind canine and equine therapies.

Psychological benefits

Beyond their physiological benefits, canine and equine therapies have also been found to enhance the psychological well-being of children. One of the most common benefits of AAT, such as equine therapy, is the reduction of anxiety and depression symptoms (Punzo et al; Brandão et al.). A study involving participants aged between 7 and 18 with mental health disorders found that one of the most significant improvements was a reduction in stress and anxiety levels (Punzo et al.). The authors suggested that these improvements may be partly explained by the tranquility provided by the natural environment, which is full of greenery and animals. This study lasted for 12 to 14 months during which many of the participants gave positive feedback about equine-assisted therapy as they felt secure and calm in ways they could not experience elsewhere. Most participants also shared that they were not treated seriously by their previous therapists which had worsened their situation until then.

In addition, a study by Brandão et al. involved 21 children who participated in AAT sessions involving therapy dogs for 20 to 60 minutes showed improvements in their feelings. Before the AAT session, 40% of the participants reported feeling worried, while another 40% reported being very worried. After the experiment, 55% of the children reported feeling more relaxed and less worried. Similarly, another study conducted by Brandão et al. included 153 children aged between 7 and 17 years old who had experienced child abuse. They were divided into three groups — Group 1 with no dogs, Group 2 with dogs but no stories, and Group 3 with both dogs and stories. These stories consisted of therapeutic stories about dogs that were tailored to each session topic which included self-esteem, trust, boundaries, emotions, and how to identify unwanted touch. Questions related to the stories were also used to connect the dogs' visits with the discussion topics. The results showed that the most significant improvement was found in group 3, suggesting that the combination of interaction with dogs and therapeutic stories may have contributed to the psychological improvements observed. The results also indicated that children in the dogs-with-stories group reported lower levels of anxiety, depression, showing how animals can play an important role in creating a more comfortable environment for children to engage with difficult emotional topics, thus helping to support their psychological well-being.

One proposed mechanism underlying these psychological benefits is the human–animal bond (HAB), which refers to the positive relationship developed between patients and therapy animals. A strong HAB can develop even after a short AAT session (Rodrigo-Claverol et al.). As suggested by Rodrigo-Claverol et al., the psychological benefits of AAT may be influenced by the strength of this bond, as therapy animals provide emotional support and act as a social buffer, helping children feel less stressed and more willing to engage with therapists and the therapeutic process. This is reflected in the friendships formed between the young participants and the horses during equine-assisted therapy. When they encounter challenges and difficulties in their lives, these horses were referred to as their best friends and companions whom they could safely confide in (Punzo et al.). This friendship is significant and cannot be easily replaced.

In addition, children, in particular, tend to benefit greatly from AAT in terms of their self-esteem and self-confidence. Psychologist Gail Melson suggests the idea of the biocentric approach to children's development, which values meaningful relationships with non-human

species (Melson). According to Melson, children may choose to confide in a dog or cat rather than another human being. This suggests that animals act as a source of emotional support. Similarly, in a therapeutic setting, children can gain immense emotional support from a therapy animal. Feeling emotionally supported may give them stronger confidence to interact with the therapists as well. Consequently, learning how to care for animals helps children to develop nurturing skills, increasing their social competence and empathy (Melson). As children become more capable of caring for another living being, they may transfer these nurturing behaviours to their interactions with others, thus becoming more socially aware and considerate. AAT sessions also promote important values such as courage (Punzo et al.). It was mentioned in Punzo's study that children had gained courage from riding the horse, and this courage was applied to their daily lives, helping them speak up for themselves and defend their own decisions. This, in turn, enhances their self-esteem and self-confidence. With increased confidence in themselves and their social abilities, those with better relationships with animals are also reported to have better relationships with their friends and family.

These psychological benefits may be particularly valuable for children with social difficulties arising from traumatic experiences, as AAT can help boost their self-confidence in socialising and support them in coping with difficult emotions (Mcfarlane; Germain et al). Trauma researchers argue that trauma does not simply heal with time; instead, individuals may become increasingly vulnerable to everyday stressors if the trauma remains unresolved (Mcfarlane). As a result, trauma should not be left unaddressed but instead managed through effective therapeutic interventions. As a complementary approach, AAT may encourage engagement in treatment (Dravsnik et al.). Therapy animals provide a sense of security, comfort, and unconditional acceptance through their non-judgemental and affectionate behaviour (Rodrigo-Claverol et al.). It was found that during an AAT programme that lasted for eight weeks, with a 1.25-hour session per week, young participants with trauma-related issues completed the equine programme successfully with a sense of accomplishment (Kelly and Cozzolino). It was also observed that they could actually take on the role of caregiver and accepted the responsibility of caring for the dogs. Through the programme, participants learnt patience, emotional regulation and how to interpret subtle social cues, while forming trusting relationships with the other volunteers. This collaborative experience may promote trust and social engagement. Furthermore, this program showed short-term improvements in participants' self-control, self-esteem, and reduced depressive symptoms. However, the study had a relatively small sample size due to the limited number of participants in the programme. Further research involving larger groups of participants is needed to provide more quantitative results and larger numbers of brain scans. Overall, Kelly and Cozzolino suggested that AAT benefits vulnerable populations such as children and youths with various mental conditions and overcome trauma, including the effects of sexual abuse.

The effectiveness of AAT is further supported by long-term programmes implemented in real-world settings. One evidence is the Green Chimneys Children's Services, in which human-animal interaction plays a crucial role in improving the mental state of hundreds of

children and adolescents living in its care (Fine). For more than 60 years, Green Chimneys has provided specialised care for children with psychological needs.There, children who had experienced severe trauma or abuse at home were involved in the training of assistance dogs, where they learnt to be responsible for and protect the dogs. Among the 23 clinicians interviewed, many reported improvements in children' s self-regulation, meanwhile most of them appeared to experience the animals as soothing, comforting and safe (Flynn et al.). Clinicians also observed improvements in students' self-worth, self-efficacy and competence. This provides long-term and more consistent evidence of how AAT can be integrated into our daily lives and what it can bring to the children in need. Furthermore, AAT usually involves enjoyable interactions with animals, which can be particularly attractive to young children (Punzo et al.). Children described horse-riding as "like flying", highlighting the enjoyment and sense of freedom that are not easily found in other forms of therapy. As a result, the enjoyable nature of AAT may encourage children to participate more actively in therapy sessions.

In conclusion, the findings of all these studies reviewed suggest that AAT may help alleviate symptoms of anxiety and depression, improve self-esteem and self-confidence, and support children with social difficulties arising from traumatic experiences. Collectively, these findings highlight the potential benefits of the human-animal bond in enhancing therapeutic outcomes. However, the efficacy of AAT should not be considered in isolation; we should also place emphasis on the welfare of the animals involved in AAT, as well as its limitations and challenges associated.

Ethics, limitations and challenges

While previous studies suggested that canine and equine therapies are efficacious complementary approaches to the conventional therapies due to their physiological benefits and psychological benefits, it is also important to consider the ethics, limitations and challenges of AAT in real-life. Firstly, it is hard to establish consent with a dog or a horse to participate in a therapy programme. Thus, it becomes important to assess their welfare using biological markers such as salivary cortisol, oxytocin concentrations, and heart rate variability to determine whether AAT is a mutually beneficial relationship or places unnecessary stress on the animals. In a study involving 19 therapy dogs from Mayo Clinic's Caring Canine Program, cortisol and oxytocin concentrations remained relatively stable after the dogs completed common AAT activities, such as interacting with strangers in unfamiliar environments (Clark et al.). This suggested that therapy animals were not severely stressed after each AAT session. Even in the absence of positive impacts, no adverse physiological effects were observed in the therapy animals. There was a significant decrease in the heart rate of the dogs at the end of the 20-minute session, suggesting that they were calmer after the session. Thus, it seems that participating in animal-assisted activities did not adversely affect the dogs' physiological state and may even have had positive effects. However, Clark et al. suggested that the absence of negative physiological effects does not necessarily indicate positive psychological well-being. Therefore, further research using more non-invasive physiological indicators, such as

Electroencephalography, which measures brain signals and neural processes, is needed to better evaluate the welfare of therapy animals (Kulgod et al.).

The applicability of AAT sessions is also limited by participants' physiological and psychological conditions. Some may have allergies to animal fur, some may have phobia of animals, and some may even exhibit irritability and aggression (Fine). As allergic reactions can become severe, AAT should not be used for individuals with animal allergies (Shmuel and Cortes). Statistics show that dog bites among children are not uncommon and they are usually sustained in the neck, shoulder and facial regions due to children's heights (Overall and Love). This may cause intense emotional distress and fear arising from the potential risk of being bitten by a dog. Certain behaviours by children may unintentionally provoke a negative response from the dog, leading to injuries like bite or scratch. Therefore, it is important to ensure the safety of both the animals and the patients during the session. In Fine's Handbook on Animal-assisted Therapy, the case of a four-year-old boy, Josh, was discussed. He tended to be overly aggressive towards his peers, and was found by his mother abusing newborn kittens at home. In such cases, it might be unsuitable to use AAT as an approach to help Josh as he was very likely to have negative responses towards the therapy animals. It was later found that it was Josh's father who had treated him roughly, resulting in this behavior. Josh's situation had improved after his parents learned to change. This does not mean that individuals like Josh, who may have a tendency to display violent behaviours towards animals, should be completely prevented from being involved in human-animal interactions. Instead, they can still participate in animal-assisted activities under the strict supervision of professionals to ensure both the safety of the therapy animals and the patients(Fine). However, considering cases like Josh, AAT may not be the most suitable option if a patient has negative responses towards animals. Therefore, therapists should obtain a complete history of the children's experiences with animals and identify the underlying causes of their behaviours to ensure that the therapy process is effective and therapeutic.

Another limitation of AAT is its accessibility to different patient groups. One factor affecting its accessibility is the availability of therapy animals. Among the animals used in AAT, the two most common are dogs and horses. However, the transportation of horses can be limited due to external factors such as diseases. One example is the outbreak of equine influenza in Australia which resulted in the restriction of horse transportation throughout the state of Victoria in 2007 (Davis et al.). As a result, the availability of horses for equine-assisted therapy used in a 10-week therapeutic horse riding programme was reduced, which further limited patients' access to such programmes. This would impact the feasibility of equine-assisted programmes. Apart from the availability of therapy animals, financial constraints are one of the main problems preventing patients from participating in AAT (Hamm). Due to the high start-up costs of operating a facility that provides equine-assisted therapy and the ongoing training of staff, the cost of equine-assisted therapy can be too high for families to cover, especially those without insurance. Therefore, although AAT has demonstrated promising therapeutic benefits, its accessibility remains limited by the limited availability of therapy animals and financial barriers.

In response to these limitations, some researchers have proposed robotic animals as an alternative to real animals (Melson et al.). For example, the use of robotic dog AIBO in interactions with 72 children aged between 9 and 15 years old was studied by Melson et al. It was found that children still treated AIBO as a living being in certain aspects such as giving it commands and talking to it. In fact, more than 60% of the children believed that AIBO possessed mental states, social characteristics, and moral values, and they were equally likely to give commands to AIBO as they were to a living dog. However, it was observed that the children used fewer gentle touches and they recognised that it was not a real dog. Although this might be attributed to immature technology, as AIBO had a hard metal surface and lacked certain characteristics of a real dog, the findings suggest that a robotic dog can still be easily distinguished by young children as an artificial object rather than a real living dog. Therefore, the use of robotic animals as a substitute for real therapy animals does not yet appear to be a realistic approach. In the future, however, as suggested by Melson et al, children may be more receptive to interacting with robotic animals if they evolve to more closely simulate the biology and behaviors of real dogs. This may increase the feasibility of delivering AAT through advanced technologies, allowing more patients to benefit from such interventions.

In conclusion, ethical considerations, limitations and challenges of AAT, such as the physiological and psychological well-being of the therapy animals involved, the applicability of AAT to participants with different physiological and psychological conditions, and challenges such as the cost of running a facility that provides AAT, remain important considerations for its application among children. Nevertheless, these challenges do not outweigh the potential of AAT as a complementary therapeutic approach. Instead, they highlight the need for AAT to be implemented responsibly and ethically. As awareness of AAT continues to grow, it has the potential of becoming a more widely-adopted therapy that supports the psychological and physiological well-being of children in an increasingly stressful society.

Conclusion

This literature review explored the mechanisms behind these therapies and the psychological and physiological effects on children. By analysing literature, canine and equine therapies have shown to be supportive in terms of a range of physiological and psychological benefits they bring to patients. This includes better physical well-being shown by changes in biological markers such as decreased cardiovascular arousal and cortisol levels, as well as reduced anxiety and depressive symptoms, increased self-confidence and better socialising behaviour for children with trauma-related issues (Nagengast et al.; Vormbrock and Grossberg; Brandão et al.; Punzo et al.). Therefore, the findings suggest that AAT, in general, including equine therapy and canine therapy, may help children to improve their physiological and psychological well-being, especially those who have difficulty engaging in social situations.

However, benefits often come with trade-offs. This literature review has also discussed the potential ethical concerns, challenges and limitations behind AAT. AAT must be carried out carefully under professional supervision, with a thorough assessment of patients to ensure a safe

session for both patients and therapy animals (Fine). By looking through all the existing research, it seems like there is a lack of studies focusing on the well-being of the animals involved in AAT. Although the aim of AAT is to help human patients, we should not ignore the importance of ensuring the welfare of the animals involved. Future studies could use more objective physiological measures, including blood-based indicators and non-invasive measures such as electroencephalography (EEG), to provide a better understanding of the effects of AAT (Kulgod et al.). This is important as such indicators provide measurable and can therefore support a more comprehensive evaluation of AAT's effectiveness. In addition, many studies involved relatively small sample sizes, limiting the generalisability of their findings. Many AAT programmes also involve natural environments, which may act as a mediator and contribute to the effectiveness of AAT. Thus, it is also critical to consider the role that nature plays in AAT, as some of the observed benefits may not come solely from interactions with the therapy animals. Future research should consider the influence of nature on AAT and include more randomised controlled trials (RCTs) to allow clearer comparisons between AAT and control groups and provide stronger evidence of its effectiveness.

Overall, AAT shows promising potential in supporting the psychological and physiological well-being of children in an increasingly stressful society. Although AAT may be a relatively new therapeutic approach, we should not underestimate the power of the human-animal bonds formed through the process. We should encourage greater awareness of AAT and more research so that it can be more carefully implemented as an ethical and responsible therapeutic approach.

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Hemodynamics of Coronary Artery Stenosis and Comparison of Two Coronary Revascularization Procedures By Vivian Tong Wang

Abstract

Coronary Artery Disease (CAD) is one of the most common cardiovascular diseases in the world and an important cause of death. The main pathological mechanism of CAD is atherosclerosis, that is, lipids are gradually deposited on the arterial wall, triggering inflammatory reactions and forming atherosclerotic plaques.As the plaque continues to grow, the coronary artery lumen gradually narrows, reducing the oxygen-rich blood supplying the myocardium, thus affecting the normal function of the heart.

Coronary artery stenosis not only reduces blood flow, but also changes important hemodynamic parameters, including blood flow velocity, pressure distribution and wall shear stress. These changes will not only reduce myocardial perfusion, but also may further promote vascular endothelial dysfunction and plaque development. In recent years, as an important tool in biomedical engineering, Computational Fluid Dynamics (CFD) has been widely used to simulate blood flow in coronary arteries to help researchers understand disease mechanisms and optimize treatment plans.

At present, the main treatments for restoring coronary blood flow include stent implantation in Percutaneous Coronary Intervention (PCI) and Coronary Artery Bypass Grafting (CABG). Although both methods are aimed at restoring myocardial blood supply, there are obvious differences in their engineering principles, treatment methods and long-term clinical effects.

This article will explore how atherosclerosis changes coronary blood flow from the perspectives of biology and biomedical engineering, and analyze how coronary stents and CABG restore coronary circulation, while comparing the advantages, limitations and applicability of the two treatments.

1. Introduction

As an important circulatory organ of the human body, the heart needs to continuously obtain oxygen and nutrients to maintain normal contractile function. The coronary artery system is responsible for providing oxygen-rich blood to the myocardium, so the structure and function of the coronary artery are important for maintaining heart health. However, due to factors such as lifestyle changes, aging and metabolic abnormalities, more and more people are affected by coronary artery disease.

The main cause of coronary artery disease is atherosclerosis. Atherosclerosis is not a simple vascular obstruction, but a complex biological process, including vascular endothelial injury, lipid entry into the vascular wall, immune cell aggregation and inflammatory reaction(Fan and Watanabe, 2022). As the atherosclerotic plaque increases, the coronary artery lumen will gradually narrow, limiting blood flow, thus reducing the ability of the myocardium to obtain oxygen(Libby and Theroux, 2005).

In recent years,Computational fluid dynamics (CFD) , as a vital tool in biomedical engineering,can establish a computational model based on the patient's vascular structure, analyze blood flow velocity, pressure gradient and wall shear stress, and provide new methods for disease prediction and treatment design.

At present, coronary circulation is mainly restored through PCI stent implantation and CABG. PCI mechanically expands the stenotic area so that the blood can re-pass through the original blood vessels; while CABG bypasses the obstruction site by establishing a new blood flow pathway. Both methods have their own advantages, so they need to be selected according to the specific situation of the patient (Mohr et al., 2013).

Based on the above background, this article mainly discusses two core questions:
(1) How does coronary artery stenosis caused by atherosclerosis affect hemodynamics?
(2) How can coronary artery stents and coronary artery bypass grafting restore coronary circulation?

2. Biological mechanism of coronary artery disease

2.1 Coronary artery structure and function

The coronary artery is located on the surface of the heart, and its main function is to deliver blood to the myocardium. Because the myocardium needs continuous contraction, it is very sensitive to oxygen supply. When the body is exercising or under pressure, the workload of the heart increases and more oxygen is needed. At this time, the coronary arteries will increase blood flow through vasodilation (Duncker and Bache, 2008).

Under normal circumstances, coronary blood flow is regulated by many factors, including blood pressure, vascular resistance and myocardial metabolic needs. However, when the blood vessels undergo stenosis, the blood flow regulation ability will be limited. Even if the myocardium needs more oxygen, the blood supply cannot be fully increased, eventually leading to myocardial ischemia(Duncker and Merkus, 2004).

2.2 The development process of atherosclerosis

Atherosclerosis usually begins with vascular endothelial dysfunction. Healthy vascular endothelium can regulate blood flow and prevent inflammation, but when affected by hypertension, high cholesterol or other factors, endothelial cells may be damaged.

Damaged vascular walls are more likely to absorb low-density lipoprotein cholesterol (LDL). LDL entering the blood vessel wall will be oxidized and stimulate the immune system to produce an inflammatory response(Fan and Watanabe, 2022). Subsequently, immune cells such as macrophages will gather and form foam cells, gradually forming early atherosclerotic plaques(Libby and Theroux, 2005).

As the disease develops, more lipids and fibrous tissue will accumulate inside the plaque, which will increase the volume of the plaque and lead to a narrowing of the vascular lumen.

The main factors affecting the development of atherosclerosis include:

high cholesterol level, high blood pressure;Smoking, diabetes,obesity, age increase.

Recent studies have found that by reducing LDL cholesterol levels, the further development of plaques can be reduced, and some plaques may be stabilized or reduced (Ueki et al., 2024).

3. The effect of atherosclerosis on coronary hemodynamics

3.1 Changes in blood flow resistance

The diameter of blood vessels has a decisive influence on blood flow. When the coronary artery undergoes stenosis, the blood passage area decreases and the flow resistance increases.

According to the principle of fluid mechanics, a slight decrease in the radius of blood vessels may also lead to a significant increase in resistance. Therefore, as the plaque expands, even if the blood vessels are not completely blocked, it may seriously affect the blood supply(Duncker and Merkus, 2004).

In stenotic areas, blood usually appears: First, the blood flow velocity increases. Due to the smaller effective channel, the blood needs to pass through the narrow area at a higher speed. Second, the pressure decreases. Obvious pressure loss will occur when the blood passes through a narrow position. Third, there is a disturbed flow. The area downstream of the stenosis may produce vortices, affecting the normal blood circulation. These changes will reduce the ability of the myocardium to obtain oxygen (Shaaban and Duerinckx, 2000).

3.2 Changes in wall shear stress

The wall shear stress of the vascular wall is the mechanical force generated by the blood flow on the inner wall of the blood vessel. Shear stress within the normal range can help maintain the health of blood vessels, but abnormal shear stress may lead to vascular endothelial dysfunction (Shaaban and Duerinckx, 2000).

In the atherosclerotic region, the blood flow pattern will also change due to the change in the shape of the blood vessels. High shear stress may occur near the stenotic area, and low shear stress and flow separation may occur in the area after the narrowing area. These abnormal mechanical environments affect endothelial cells, making them more prone to inflammatory reactions, thus promoting the formation of new atherosclerotic plaques(Sun and Xu, 2014).

Therefore, a circulation is formed between atherosclerosis and blood flow changes: Plaque formation → Vascular stenosis → Blood flow change → Endothelial injury → Further plaque formation

4 Application of computational fluid dynamics in coronary artery research

Computational fluid dynamics (CFD) is a method of using computers to simulate fluid motion. In coronary artery research, CFD can establish a patient-specific vascular model and simulate the movement of blood when passing through blood vessels of different structures (Taylor and Steinman, 2010).

4.1 Governing Equations: The Navier–Stokes Equations

The fundamental governing equations for fluid motion are the Navier–Stokes equations, which consist of the mass conservation equation (continuity equation) and the momentum conservation equation (equation of motion). For blood flow in the coronary arteries, the following assumptions are usually made: blood is an incompressible Newtonian or non-Newtonian fluid; the flow is laminar or transitional; gravitational effects are neglected. Under these conditions, the continuity equation and the momentum equation can be expressed as:

Continuity equation (mass conservation): $\nabla \cdot u = 0$

Momentum equation (Navier–Stokes equation): $\rho(\partial u/\partial t + u \cdot \nabla u) = -\nabla p + \nabla \cdot \tau$

Where u is the velocity vector, p is the pressure, ρ is the blood density, and τ is the viscous stress tensor. For non-Newtonian fluid models (e.g., the Carreau model or the Casson model), τ exhibits a nonlinear relationship with the shear rate to more accurately describe the changes in blood viscosity at low shear rates(Morris et al., 2016).

In practical coronary CFD simulations, the Reynolds-averaged Navier–Stokes (RANS) equations combined with turbulence models (e.g., the k-ω SST model) are often used to handle flow instability regions, or large eddy simulation (LES) is employed to capture post-stenotic turbulent details. Because the Reynolds number in coronary blood flow typically ranges between 100 and 2000, the flow is predominantly laminar before the stenosis, while transitional flow or local turbulence may appear after the stenosis.(Morris et al., 2016).

4.2 Key Steps in CFD Simulation

Geometric modeling: Based on coronary CT angiography (CTA) or optical coherence tomography (OCT) images, patient-specific vascular geometric models are generated through image segmentation and three-dimensional reconstruction(Taylor & Steinman, 2010). Sufficient lengths are usually extended both proximal and distal to the stenotic segment to ensure fully developed flow.

Mesh generation: The three-dimensional geometric model is discretized into finite-volume or finite-element meshes. For stenotic regions, bifurcation sites, and areas near stents, local mesh refinement is required to capture the velocity boundary layer and high gradients in wall shear stress. The mesh size typically ranges from hundreds of thousands to millions of elements, and mesh independence verification is required. (Malve et al., 2014)

Boundary condition setting:

- Inlet boundary: Patient-specific flow velocity waveforms (measured via Doppler ultrasound or phase-contrast magnetic resonance imaging) are used, or standardized physiological waveforms (e.g., under resting or hyperemic conditions) are applied.
- Outlet boundary: Lumped parameter models (Lumped Parameter Model) are commonly employed to couple the resistance and compliance of the downstream coronary microcirculation, i.e., each outlet branch is assigned a specific pressure-flow relationship (e.g., resistance-capacitance models) to simulate the flow reserve of the myocardial bed. (Quarteroni et al., 2019)

- Wall boundary: The vessel wall can be assumed to be rigid (simplified computation) or fluid‑structure interaction (FSI) methods can be adopted to account for wall elasticity; the latter provides a more accurate characterization of wall shear stress distribution under pulsatile flow.

Numerical solution and post‑processing: The finite volume method (e.g., ANSYS Fluent, OpenFOAM) or the finite element method (e.g., COMSOL) is used to discretize and iteratively solve the governing equations until the residuals converge to preset thresholds (typically 10^{-4} to 10^{-6}). In the post‑processing stage, hemodynamic parameters such as velocity field, pressure field, wall shear stress (WSS), oscillatory shear index (OSI), and relative residence time (RRT) are extracted(Malve et al., 2014).

Through the above CFD approaches, researchers can obtain quantitative impacts of vascular stenosis on myocardial perfusion, simulate the postoperative blood flow recovery after stent implantation, or evaluate flow distribution under different bypass graft configurations, thereby providing mechanical evidence for individualized treatment decisions (Sun and Xu, 2014).

5. Engineering approaches for coronary blood flow restoration.

When coronary artery stenosis seriously affects the blood supply, drug treatment alone may not be able to completely restore myocardial perfusion, so it is usually necessary to improve blood flow through intervention or surgery. At present, the two most common methods of blood flow recovery are coronary stent implantation in Percutaneous Coronary Intervention (PCI) and Coronary Artery Bypass Grafting (CABG). Although the ultimate goal of both methods is to improve myocardial blood supply, they solve the problem of blood flow obstruction in a completely different way: stents mainly expand the original blood vessels, while CABG bypasses the obstructed area by establishing a new blood flow pathway.

5.1 Coronary Stent Treatment (Coronary Stent)

5.1.1 The basic principle of PCI and stent implantation

Percutaneous coronary intervention (PCI) is a minimally invasive treatment method, which is mainly used to treat local coronary artery stenosis. During the operation, the doctor will send the balloon and stent into the stenotic area through the catheter. First of all, the balloon expansion temporarily opens the narrowing position, and then a metal stent is placed to support the blood vessel wall to keep the blood vessel open, so as to restore blood flow(McKavanagh et al., 2018).

From an engineering perspective, the core function of coronary artery stents is to change the geometric structure of blood vessels. Since atherosclerosis reduces the effective radius of blood vessels, and the radius of blood vessels has an important impact on blood flow resistance, increasing the diameter of blood vessels can significantly reduce blood flow resistance and improve blood supply capacity.

In other words, the stent is achieved by changing the structure of blood vessels:

Increase the cross-sectional area of blood vessels → reduce flow resistance → increase coronary blood flow → improve myocardial oxygen supply.

5.1.2 The development of stent technology

Early coronary artery treatment mainly relied on balloon dilatation, but simple dilation was easy to cause blood vessels to contract again, so metal stent technology was developed later.

Modern coronary stents mainly include:

(1) Bare-metal stent

The bare metal stent can provide mechanical support to prevent the blood vessels from closing immediately. However, because the blood vessels may produce tissue proliferation after mechanical stimulation, the patient may have in-stent restenosis (McKavanagh et al., 2018).

(2) Drug-eluting stent

In order to reduce the risk of re-stenosis, researchers have developed drug-eluting stents. This kind of stent can release anti-proliferative drugs and reduce the excessive growth of vascular smooth muscle cells, thus reducing the probability of blood vessels being blocked again(Fang et al., 2023).

In recent years, the design of the stent has been continuously optimized, including a thinner stent structure, better materials and a more accurate drug release method, which has continuously improved the therapeutic effect of PCI.

5.1.3 Mechanical Analysis of Stent Treatment

From a solid mechanics perspective, the stent implantation process involves complex interactions among the balloon, stent, and vessel wall. When the stent is expanded, its metallic mesh structure exerts a radial supporting force on the vessel wall to overcome the elastic recoil of the vessel wall. This process can be understood via Laplace's law ($\sigma = P \cdot r/t$): the stent must provide sufficient radial stress (σ) to maintain the vessel radius (r); otherwise, the vessel wall (with thickness t and subjected to pressure P) will recoil again.

However, the metallic structure of the stent alters the local stress-strain distribution in the vessel. Stress concentration often occurs at the two ends of the stent (i.e., the transition zones between the stent edges and the normal vessel wall), which is attributed to the much higher rigidity of the stent compared with the vessel wall, resulting in a mismatch in their mechanical properties(Chiastra et al., 2022). This stress concentration can induce excessive stretching and damage to the local vessel wall, activating inflammatory responses and vascular smooth muscle cell proliferation, and is one of the important mechanical triggers for in-stent restenosis (Shafiabadi Hassani et al., 2024).

Furthermore, after stent implantation, the stent struts disturb the blood flow pattern along the inner vessel wall, creating regions of low wall shear stress and flow stagnation points near the struts. These areas are precisely the predilection sites for thrombosis and neointimal hyperplasia (Chiastra et al., 2022).

5.1.4 Advantages and limitations of stent therapy

The biggest advantage of coronary artery stent is its minimally invasiveness. Compared with large-scale heart surgery, PCI usually does not require thoracotomy, so the recovery time of patients is shorter and the hospitalization time is less.

Its main advantages include: First, the trauma of surgery is relatively small. Patients can usually resume their daily activities faster. Second, it is suitable for the treatment of local or single vascular stenosis. Third, for patients with acute myocardial infarction, blood flow can be restored quickly and myocardial damage can be reduced (Fang et al., 2023).

However, stent treatment also has certain limitations. First of all, the stent may lead to renarrowing. Although drug-eluting stents have reduced this risk, blood vessels may still narrow again due to inflammatory reactions or tissue proliferation. Secondly, the stent changes the original vascular structure, which may affect the local hemodynamic environment. For example, the metal structure of the stent may cause changes in the distribution of blood flow, resulting in abnormal shear stress in some areas. In addition, for patients with severe multiple vascular diseases or complex coronary artery lesions, simple stent treatment may not achieve the best long-term effect (Shafiabadi Hassani et al., 2024).

5.2 Coronary artery bypass grafting (CABG)

5.2.1 The basic principle of CABG

Coronary artery bypass grafting (CABG) is a method to improve myocardial blood supply by establishing a new blood flow pathway. Unlike the stent, CABG does not directly open the originally obstructed blood vessels, but establishes a new “detour route” outside the blocked area.

During the operation, doctors usually use the blood vessels of other parts of the patient himself, such as the internal mammary artery and saphenous vein. These blood vessels will be connected to the aorta and the coronary artery behind the blocked area, so that the blood can bypass the stenotic area and reach the ischemic myocardial tissue (Rezaeimoghaddam et al., 2020).

Therefore, the engineering principle of CABG can be understood as:

Create a new flow path → bypass the high resistance area → restore myocardial blood supply.

5.2.2 Hemodynamic changes in CABG

Because CABG changes the flow path of the entire coronary artery system, hemodynamic analysis plays an important role in surgical design. Researchers need to consider: the length of the graft,the angle of vascular connection,blood flow resistance,the distribution of blood in the new path(Rezaeimoghaddam et al., 2020).

From an engineering mechanics perspective, the core challenge of CABG lies in the compliance matching between the graft and the native coronary artery. Compliance (C) is

defined as the relative change in vessel volume per unit change in pressure ($C = \Delta V/(V \cdot \Delta P)$). Arterial grafts (e.g., the internal thoracic artery) have a compliance close to that of the native coronary artery, whereas venous grafts (e.g., the great saphenous vein) have a significantly higher compliance than that of arteries. This compliance mismatch can lead to the following problems:

Uneven blood flow distribution: A highly compliant venous graft may over-distend during diastole, causing hemodynamic instability; during systole, it may recoil, leading to flow "competition" or "steal" phenomena.

Stress concentration at the anastomosis: At the anastomotic site between the graft and the coronary artery, owing to the difference in mechanical properties between the two vessel walls, pulsatile blood flow impact generates local stress concentration and abnormal shear stresses, which stimulate intimal hyperplasia and are among the main causes of anastomotic stenosis and long-term graft occlusion(Takami et al., 2024).

In addition, the length of the graft vessel and the anastomotic angle are also crucial. An excessively long graft increases flow resistance; a too-sharp or perpendicular anastomotic angle causes blood flow to impinge on the opposite wall of the anastomosis, forming regions of low shear stress and flow stasis, which likewise promote intimal hyperplasia(Rezaeimoghaddam et al., 2020).

Therefore, modern biomedical engineering research has begun to use CFD models to simulate the blood flow effect of different CABG schemes to help optimize surgical design.

5.2.3 Advantages and limitations of CABG

The main advantage of CABG is its long-term blood flow recovery effect.

Compared with PCI, CABG is especially suitable for:

- Multivessel coronary artery disease;
- Coronary artery disease of the left main coronary artery;
- Extensive or complex vascular lesions.

Because CABG can bypass multiple blockage areas, it usually has a more stable long-term effect on severe patients.

However, CABG also has obvious disadvantages. First of all, it is a large-scale surgery that requires thoracotomy, so it takes a long time for the patient to recover. Secondly, the risk of surgery is relatively high, including infection, bleeding and postoperative recovery. Therefore, CABG is usually not the first choice for all patients, but needs to be evaluated according to the patient's age, disease severity and overall health(Doenst et al., 2019).

6. Comparison between PCI and CABG

6.1 Comparison of Treatment Methods

Although both PCI and CABG can improve coronary blood flow, they both represent two different engineering solutions. The core idea of PCI is repairing the original channel. It opens

stenotic blood vessels through the stent, so that the blood continues to flow through the original path. And the core idea of CABG is establishing a new channel. It bypasses severely damaged areas by creating new blood flow pathways.

Table 1. Comparison of Treatment Methods

Comparison Item	PCI (Stent Treatment)	CABG (Coronary Artery Bypass Grafting)
Basic Principle	Expand the original blood vessel	Establish a new blood flow pathway
Surgical Method	Minimally invasive intervention	Open surgical procedure
Recovery Time	Shorter	Longer
Local Lesions	Better effect	Can treat complex lesions
Multivessel Disease	Limited effect	Usually more suitable
Main Risks	Restenosis, thrombosis	Surgical risks, longer recovery time

6.2 Clinical selection factors

The choice of treatment method does not simply depend on which technology is more advanced, but needs to consider the specific situation of the patient. For example, for patients with single-vessel local stenosis, PCI can usually quickly restore blood flow and reduce the risk of surgery.

However, for patients with severe multivessel coronary artery disease, especially in the presence of complex vascular structures, CABG may provide better long-term results. In addition, the patient's age, diabetes status, heart function and future quality of life needs will all affect the treatment options(Doenst et al., 2019).

Relevant clinical studies show that in some patients with complex coronary artery disease, CABG may be superior to PCI in terms of long-term therapeutic effect, while PCI has the advantages of fast recovery and small trauma.

7. Discussion

The development of coronary artery disease reflects the close connection between biology and engineering.

From a biological point of view, atherosclerosis is mainly caused by lipid accumulation, inflammatory reaction and changes in vascular walls. However, from an engineering perspective, these biological changes are ultimately manifested as changes in vascular structure and changes in fluid dynamics. Therefore, coronary artery disease is not only a "blockage problem", but also a complex hemodynamic problem (Sun and Xu, 2014).

Traditional medical examinations usually focus on whether the blood vessels are stenotic, but only observing the degree of stenosis cannot fully predict the risk of disease. For example, some mildly stenotic areas may develop rapidly due to the special blood flow environment, while

some severely stenotic areas may remain relatively stable. Therefore, the future research on cardiovascular diseases needs to combine:

- Vascular imaging technology;
- CFD simulation;
- Artificial intelligence prediction model;
- Personalized medicine.

By establishing patient-specific vascular models, doctors may not only observe the current state of the disease in the future, but also predict the long-term effects of different treatment regimens on blood flow.

In addition, there are still certain limitations on the current treatment methods. For stent treatment, the main problems are restenosis and long-term hemodynamic changes. For CABG, the main problems are surgical trauma and long-term patency of the graft (Batchelor and Damluji, 2024).

Therefore, the future research direction may include: First, develop a more intelligent new stent so that it can better simulate the structure of natural blood vessels. Second, use artificial intelligence combined with CFD to improve the accuracy of disease prediction. Third, design the best treatment plan according to the patient's personal vascular structure to achieve real precision medicine(Quarteroni et al., 2019).

8. Conclusion

Coronary artery disease is a complex cardiovascular disease caused by atherosclerosis. Its development is not only affected by biological factors, but also by hemodynamic alterations.

With plaque formation and vascular stenosis, the blood flow velocity, pressure and wall shear stress in the coronary artery will change. These changes not only reduce the blood supply to the myocardium, but also may further promote the development of the disease.

Coronary artery stents and coronary artery bypass grafting are important treatment methods to restore coronary circulation at present. The stent restores blood flow by expanding the original blood vessels, while CABG bypasses the obstructed area by establishing a new blood flow pathway. The two methods have their own advantages and need to be selected according to the specific situation of the patient.

From the perspective of biomedical engineering, the treatment of coronary artery disease not only depends on the development of medical technology, but also needs to combine fluid mechanics, computational simulation and engineering design.

In the future, with the development of CFD, artificial intelligence and personalized medicine, researchers are expected to predict the development of diseases more accurately and design safer and more effective treatment plans to improve the long-term prognosis of patients with coronary artery disease.

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Wildfire and Long-Run Mental Health By Cyrus Chen

Introduction

The summer of 2015 was unlike anything the American West had seen in decades. Fires torched through twelve states, forcing families to flee with hours of warning. Thick smoke filled the air and turned afternoon skies orange. News coverage focused on the immediate physical aftermath: hospitalizations, property loss, acres burned. However, a crucial proponent of the aftermath remained unanswered: what happened to people's mental health after an event like this? Not in the days after, when the trauma is still fresh, but years later, when the cameras have moved on?

That question is harder to answer than it sounds. However, the reasons to expect a mental health impact are not hard to identify. Smoke from large wildfires contains harmful particles that may directly worsen mood disorders and cognitive function. Displacement is psychologically traumatic in ways that extend well beyond the physical act of evacuation. Homes are not the only things lost. Traumatic events can also strip away simple comforts, like daily routines, as well as deeper sources of stability, such as communities and a sense of safety. For residents who depend on forests for their livelihood, including ranchers, loggers, and seasonal workers, a large fire can wipe out a year's income or more in a matter of days. And even people not directly exposed to fire or fire-generated smoke can experience elevated anxiety through media coverage (Currie and Saberian, 1). What is less clear however, is whether the resulting mental health effects of all that exposure are temporary, or whether they compound and persist over years. That is the central empirical question this paper tries to answer.

The 2015 fire season is particularly well-suited to this kind of study. Because the fires spread across multiple independent events in ecologically diverse areas, from the high desert of the interior Southwest to the forested slopes of the northern Rockies, the wildfire exposure is unlikely to have been driven by a single shared regional cause. Counties that burn regularly tend to be more rural. They are more economically marginal and farther from healthcare providers than counties that have lower fire hazard potential. Direct comparison between burned and unburned communities would attribute preexisting declines in mental health to the fire.

This paper tries to solve that problem using a post-matching regression design. Instead of comparing fire-affected counties to all non-fire counties, I restrict the comparison group to counties with similar wildfire risk: counties that have similar fire hazard potential to those that burned but happened not to experience a qualifying fire in 2015. Four years after the fires, wildfire-affected counties showed depression prevalence nearly three percentage points above these similar comparison counties. That gap is large. This gap only increases when I replace the binary fire indicator with acreage of burned land. Counties that lost more land to fire displayed proportionally worse depression.

The paper proceeds as follows. Section 2 reviews the literature and presents hypotheses. Section 3 describes the data and explains methodology. Section 4 presents the main results. Section 5 concludes.

Literature Review and Hypothesis

The mental health consequences of natural disasters have been well documented across a wide range of event types. Norris et al. (1) find that psychological distress is the consistently documented outcome across hurricanes, floods, and earthquakes spanning two decades of study. Neria, Nandi, and Galea (1) find that disaster exposure increases substantial post-traumatic stress disorder (PTSD) risk. A recent meta-analysis in the United States finds hurricane and flood events are associated with increased depression and post-traumatic stress (Miller et al., 107333).

However, Wildfires are harder to study than most other natural disasters. This is because wildfires are often more difficult to recover from. A hurricane moves through a region in a matter of days. Wildfire can burn for weeks, and its effects can reach communities far beyond the fire line through smoke. It also leaves behind a landscape of charred land that remains visible for years.

The research on wildfire and mental health has grown considerably in recent years. Silveira et al. (1) study survivors of the 2018 Camp Fire in Northern California. They find that direct wildfire exposure significantly raises the risk of PTSD, major depressive disorder (MDD) and generalized anxiety disorder (GAD) at six months post-disaster. Brown et al. (1-2) follow 3,070 students in grades seven through twelve after the 2016 Fort McMurray wildfire in Alberta. They find that 37 percent suffered from probable PTSD and 31 percent suffered from probable depression eighteen months after the fire. Mirabelli et al. (1) find that adults exposed to heavy wildfire smoke for six or more weeks had 30 percent higher anxiety symptoms than those with shorter smoke exposure. Jung et al. (1-2) find that wildfire smoke exposure is associated with more emergency department visits for mental health related issues within seven days after exposure following the 2020 California wildfires. Wettstein and Vaidyanathan (1) find that antidepressant and anxiolytic prescriptions rise noticeably in the weeks following fire onset. This pattern is consistent with a real increase in distress rather than a change in help-seeking behavior alone. Currie and Saberian (1) identify four possible mechanisms behind such distress after analyzing Canadian administrative data: local air quality, evacuation orders, local costs of fires, and media-driven climate anxiety. Molitor, Mullins, and White (1) and Persico and Marcotte (23) provide causal evidence that air pollution exposure increases suicide rates in US. Considering all these mechanisms increases the fire impact on mental health by 75%. With these studies in mind, Ye et al. (2), after a systematic review of 139 peer-reviewed studies on wildfire health, identify the need for more studies on long-term effects on public health.

Beyond the mental health literature, economists have documented the economic costs of mental health problems. Greenberg et al. (1) estimate that major depressive disorder costs the United States over $300 billion annually in lost productivity, increased healthcare utilization, and reduced earnings. Those costs would be substantially increased in a region experiencing economic disruption from a natural disaster during the same period. Merdjanoff et al. (3-4) project that changes in mental health in communities following major disasters unfold gradually over time. Acute distress in the first year tends to progressively transition into chronic depression in the second through fifth years, long after disaster response resources have been withdrawn. If

wildfires follow a similar path, the policy window for effective intervention may be longer than what most current disaster mental health programs assume.

These previous studies have established a short-term correlation between mental health and natural disasters (including wildfires). What most of these studies share is the lack of a long-term follow-up window. The wildfire-specific clinical literature generally follows survivors for weeks to eighteen months at most, ignoring multi-year mental health trajectories. County-level population health outcomes, the aggregate signal most relevant to regional mental health policy, remain almost entirely unstudied in the long run. This paper addresses those gaps directly. Given that the aftermath of wildfire has long-term effects, it can be logically assumed that the corresponding mental health effects would also continue long-term. Moreover, wildfires tend to happen in rural areas that have weaker economies and more limited healthcare infrastructure compared to the urban areas. Mental health issues related to wildfires are more likely to persist over time. I hypothesize that a correlation exists between long-term mental health and wildfire.

Data

Wildfire events are recorded in the Monitoring Trends in Burn Severity (MTBS) fire perimeter database, maintained jointly by the U.S. Geological Survey and the U.S. Forest Service. MTBS maps the perimeter of every wildland fire affecting at least 1,000 acres in the Western United States or 500 acres in the Eastern United States. It uses Landsat satellite imagery to outline burned area boundaries. The database covers all fire seasons from 1984 through 2022 and reports each event's fire type (wildfire, prescribed burn, or wildland fire use), ignition year, and total acres burned. I restrict events classified as wildfire, excluding prescribed burns. Prescribed burns are planned events that do not generate psychological trauma, unplanned displacement, and financial loss associated with wildfire exposure.

The 2015 fire season is selected because it is distinctive in both scale and geographic reach. This study covers the eleven-state in Western US: Arizona, California, Colorado, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, Washington, and Wyoming. In 2015, counties hit by wildfires spanned several distinct ecological zones, including California chaparral, Montana lodgepole pine forests, Oregon shrublands, and Arizona grasslands. This ecological diversity is essential for the study's credibility. As such, it is unlikely that any single shared event or regional shock besides wildfires is responsible for the mental health patterns observed four years later.

A wildfire occurred in a county if MTBS burn perimeter polygons intersected the county boundary (2020 TIGER/Line county shapefiles, U.S. Census Bureau).[1] County land area in acres is computed from the TIGER/Line ALAND field (converted from square meters), and wildfire severity of county *c* is measured as the percent of a county's land area burned (PctBurned) in 2015:

$$\text{PctBurned} = (\text{Acres burned}) \div (\text{Land area of the county}) \quad (1)$$

[1] All regressions project county shapes onto the U.S. National Atlas Equal Area projection (EPSG:5070) to ensure area calculations are not distorted by the curvature of the Earth.

This research uses two survey-based mental health prevalence measures from the CDC PLACES database (formerly 500 Cities). This database reports model-based estimates of health behavior and outcome prevalence at the county level. One measure is the percent of adults with depression, which is the estimated percentage of adults who report having been told by a doctor that they have depressive disorder. The other measure is the percent of poor mental health days, which is the estimated percentage of adults who report 14 or more poor mental health days in the past 30 days. These two measures were first included in the CDC PLACES database since 2019. To avoid COVID-19 confounding, only 2019 cross-sectional data is used in this research. The post-2020 data are excluded entirely to avoid the confounding impacts of COVID-19.

Both mental health measures are based on data from the Behavioral Risk Factor Surveillance System (BRFSS) and are estimated using small-area estimation techniques that integrate survey responses with local demographic characteristics. The BRFSS is a nationwide telephone survey of over 400,000 adults annually. Its county-level estimates for depression and poor mental health days are synthesized using multilevel regression and poststratification (MRP) methods that utilize individual survey responses and county demographic data from the Census Bureau. This statistical approach improves direct survey estimates in small counties. The key advantage for this study is that the BRFSS-based measure is available for virtually all counties in the United States, including the small and rural Western counties that are the focus of this analysis. A direct measure based on administrative healthcare records, such as insurance claims or Medicaid data, would exclude uninsured individuals and would vary in coverage across states. By contrast, the PLACES measure is population-based and comparable across counties regardless of insurance status or healthcare utilization. Hence, this measure is well-suited for a study that includes many rural counties with low insurance rates.

Structural wildfire risk is measured using the Wildfire Risk to Communities (WRC) county-level dataset published by the U.S. Forest Service. The primary matching variable, Wildfire Hazard Potential (WHP), is the national percentile rank of each county's mean annual burn probability as simulated by FSim, a physics-based large-fire simulation model that uses LANDFIRE vegetation and fuel data together with historical weather statistics. Because FSim simulates fire behavior from landscape characteristics rather than historical fire occurrence, WHP national rank is plausibly exogenous to realized wildfire events. It serves as an objective measure of structural fire risk. The share of county buildings in the Direct Exposure WHP zone, which is the highest-risk category, also enters the matching procedure.

The 2013 Rural-Urban Continuum Codes (RUCC) published by the USDA Economic Research Service classify counties on a 1–9 scale from large metropolitan areas (RUCC 1–3) to completely rural counties (RUCC 7–9). The 2013 vintage is used to identify counties with similar rurality. It is included as both a matching variable and a control in the cross-sectional regressions.

Other socioeconomic covariates include population and median household income. These data are derived from the ACS 1-year estimates for 2014. Annual county unemployment rates in 2014 come from the Bureau of Labor Statistics Local Area Unemployment Statistics (BLS

LAUS) program. The HRSA Health Professional Shortage Area (HPSA) designation is a binary indicator that equals one if the county is designated a mental health HPSA in any year during the study period. For a county to qualify as a HPSA designation, it must have fewer than one psychiatrist per 30,000 residents. It can also qualify if the county lacks adequate outpatient mental health services relative to the characteristics of its population. Among the 81 burned counties, a majority are designated as mental health HPSAs at some point during the study period. This lack of providers is a relevant feature of many burned counties. In counties where mental health access was already restricted before the wildfires, an increase in depression would typically persist longer: the accessibility barriers prevent early intervention that might shorten recovery time.

Matching and Descriptive Statistics

A county is burned if it experienced its first MTBS wildfire with a burned area more than 1,000 acres in calendar year 2015. There were 94 burned western counties in 2015. Unburned counties are those with no qualifying wildfire in any year from 2015 through 2019. Counties that experienced qualifying fires in 2016–2019 are excluded from both groups to keep the data clean.

Direct comparison between burned and unburned counties can be problematic. Burned counties are typically different from those that never burned. The burned counties are typically more rural, more economically marginal, and more exposed to other environmental stressors. It is plausible that these differences can result in diverging mental health trajectories, such as declining provider access in rural areas or worsening economic conditions in resource-dependent counties. The matching procedure restricts the sample to include only unburned counties that are structurally similar to the burned counties.

The most common alternative to this kind of structural matching is to use nearby counties, often in the same state or region as comparison counties. However, a county in western Oregon that burned in 2015 should not be compared to a nearby county with low fire risk. These counties were already on very different trajectories in terms of forest management, timber industry health, and long-run economic prospects. The WHP-based matching approach addresses this issue by selecting unburned counties based on predicted fire risk from landscape characteristics. Counties matched within the same WHP quintile face the same structural wildfire threat.

In addition to the WHP, mental health trends may be different because of county differences in vegetation type, climate zone, topography, and rural economic profile. To control these differences, within each quintile, I match each burned county to unburned counties using Mahalanobis distance computed over six pre-2015 covariates (see Panel A of Table 1). The one-to-one matching makes the burned and unburned counties similar in both fire risk and the socio-economic-demographic conditions that likely drive mental health trends.

A 50-kilometer minimum separation is imposed between each burned county and its matched counterpart (Zhang et al., 1991). This prevents matching a burned county to bordering county that may share the same fire plume, evacuation area, or media market. If no unburned county within the same WHP quintile satisfies the distance constraint, the nearest-quintile

unburned county is used as a fallback. This occurs in less than 5 percent of unburned counties. Out of the 94 burned counties in 2015, 81 appear in the matched dataset.

Table 1 presents summary statistics for the primary matched panel. Panel A shows that the matching achieves close balance on all pre-2015 covariates. WHP national rank differs by 0.013 between burned and unburned counties ($p = 0.70$), rural-urban continuum codes differ by 0.21 ($p = 0.81$), and income differs by $3,600 ($p = 0.34$). None of these differences are statistically different from zero. The close balance on income and RUCC is critical because these two covariates are most likely to predict diverging mental health trajectories independent of wildfire. Counties with lower income and higher RUCC codes tend to have less access to mental health providers, higher rates of chronic economic stress, and less capacity to recover from minor shocks. The $3,600 income difference and 0.21 RUCC difference between burned and unburned counties are both small relative to the cross-county variation in these variables. The matching selects socio-economically and demographically comparable comparison counties.

Panel B reports the summary statistics in 2019. Four years after the 2015 fire season, depression prevalence is 21.2 percent in burned counties and 18.0 percent in similar unburned counties. The poor mental health days are 15.1 for burned counties and 13.2 percent for unburned counties. The low p-values show that the burned counties had significantly higher depression prevalence and poor mental health days than the similar unburned counties.

Table 1. Summary Statistics: Matched Panel (Wildfires ≥1,000 Acres, 2015)

Variable	Burned Mean (SD)	Unburned Mean (SD)	Difference	p-value
Panel A: County Characteristics (2014)				
WHP national rank (0.0–1.0)	0.863 (0.161)	0.876 (0.100)	-0.013	0.701
Rural-urban continuum code (1–9)	4.5 (2.1)	4.2 (2.8)	0.2	0.808
Share of buildings in direct exposure zone	0.477 (0.201)	0.402 (0.258)	0.075	0.351
Population (thousands)	46.9 (51.1)	49.9 (56.6)	-3.0	0.914
Median household income ($1000)	44.5 (8.8)	48.2 (7.3)	-3.6	0.344
Mental health HPSA (0/1)	0.00 (0.00)	0.00 (0.00)	0.00	—
Unemployment rate (%)	8.9 (2.7)	8.6 (3.3)	0.3	0.779
Panel B: Post-Treatment Outcomes (2019)				
Depression prevalence (%)	21.2 (3.6)	18.0 (2.4)	3.2***	0.001
Poor mental health days (pct 14+)	15.1 (2.0)	13.2 (1.4)	1.9***	0.001

*Notes: Burned counties experienced a wildfire ≥1,000 acres in 2015. Similar unburned counties had no qualifying wildfire 2015–2019. Standard deviations in parentheses. Panel B outcomes from CDC PLACES 2019. *** p<0.01, ** p<0.05, * p<0.10.*

Methodology

Because CDC PLACES provides depression data starting from 2019, I estimate a cross-sectional OLS regression using the matched data:

$$Y = \alpha + \beta \cdot \text{Burned} + X'\gamma + \varepsilon, \quad (2)$$

where Y is a 2019 outcome variable for county c. Burned is a binary variable indicating whether the county c was burned in 2015. X is a vector of baseline controls included in Panel A of Table 1. ε is a random shock. First, the depression prevalence in county c is used as the outcome variable. Then, the percent of poor mental health days (≥14 days per month) is used as the outcome. Standard errors for both cases are heteroskedasticity-robust.

To evaluate whether fire severity affects the outcome, I replace the binary indicator Burned with the log of percent of land area burned in 2015:

$$Y = \alpha + \beta_D \log(1 + \text{PctBurned}) + X'\gamma + \varepsilon. \quad (3)$$

β_D shows how depression varies when percent burned area (PctBurned) increases by 1%.

Results

Table 2 presents the key findings. At the threshold (burned area ≥ 1,000 acres), counties that experienced a 2015 wildfire report depression prevalence 2.76 percentage points higher than matched unburned counties in 2019. The difference is statistically significant ($p = 0.004$). The effect is large relative to 18.0 percent, the mean of the unburned counties.

It is important to consider what a 2.76-percentage-point increase in depression prevalence means at the county level. The burned counties in the matched sample had a combined adult population of roughly 1.5 million in 2019. A 2.76 percent increase corresponds to approximately 40,000 more adults living with a diagnosed depressive disorder four years after the fires**.** These counties already reported above-average depression rates before any fire occurred. Depression at this scale has real consequences for families and communities. It reduces labor force participation, increases emergency healthcare utilization, and raises the risk of more severe behavioral health outcomes in the coming years. The burden suggested by this estimate is both substantial and long-lasting.

One possible mechanism that may explain why the depression effect persists four years after the fires is the structural shortage of mental health providers in Western rural counties. Among the 81 burned counties, most were designated as Health Professional Shortage Areas (HPSA) for mental health at some point during the study period. When depression onset occurs in a county where health care access is limited, the likelihood that an acute stressor becomes clinically diagnosed is decreased. Obtaining a diagnosis can be difficult to achieve and navigate. This structural barrier could explain why the depression prevalence gap is still visible in 2019.

The variable “poor mental health days” is used as an alternative measure of mental health outcome. That measure utilizes different survey questions and captures different aspects of mental health. Column (1) of Panel B in Table 2 shows that wildfire-burned counties report poor mental health days 1.63 percentage points higher in 2019 ($p = 0.002$). The effect is pronounced

relative to the mean of 13.2 percent for the unburned counties. This result is consistent with the results using self-reported depression diagnosis.

The clinical significance of a 1.63 percentage point increase in poor mental health days deserves attention. Poor mental health days is defined as the share of adults reporting 14 or more days of mental distress in the past 30 days. It suggests that adults experienced clinically significant levels of mental distress for around half of a month. In unburned counties, 13.2 percent of adults already report this level of distress. This figure is greater than the national average and is consistent with the mental health challenges documented in rural Western communities. An additional 1.63 percentage points represents a 14 percent relative increase. For a county with 30,000 adults, that corresponds to roughly 490 more adults experiencing frequent psychological suffering. These are adults who, by this measure, are functionally impaired by their mental state for at least two weeks out of a month. The similar estimates of depression diagnosis and the mental distress days strengthen the conclusion that wildfire burned counties face more severe mental health burden.

Column (2) of Table 2 replaces the binary treatment indicator with the log of percent land area burned in 2015. The dose-response coefficient is positive and statistically significant: a one percent increase in burned area is associated with 1.18 percentage points increase in the depression prevalence. The difference is statistically significant ($p = 0.020$). The result for poor mental health days is still positive but is statistically insignificant.

Table 2. Treatment Effects on Depression Prevalence and Poor Mental Health Days: 2019

	Burned (Binary)	**Percent Burned (Dose-Response)**
Panel A: Depression Prevalence (%)		
Estimate β	2.758***	1.180*
(SD)	(0.94)	(0.50)
Panel B: Poor Mental Health Days (%)		
Estimate β_D	1.634**	0.198
(SD)	(0.51)	(0.27)

*Note: Heteroskedasticity-robust SEs in parentheses. *** p<0.01, ** p<0.05, * p<0.10.*

In summary, the results are generally consistent and complementary. The depression finding is large and statistically significant. It also appears in two independent survey measures. Because burned and unburned counties are similar in WHP national rank and social-economic-demographic characteristics, these differences are associated with the wildfire incidences in 2015.

Conclusion

Four years after the 2015 wildfire season, burned counties showed worsened mental health conditions than unburned counties. That gap is significant. That gap appears in two independent survey measures of mental health and is wider in counties with increased land loss due to fire. Hence, county-level public health data strongly indicate that wildfires are associated with a mental health mark that persists long after the smoke clears.

Most policy tools that currently exist for wildfire mental health response are designed based on the acute phase. FEMA's Crisis Counseling Program, the primary federal mechanism for post-disaster mental health support, provides funding for community-based counseling in the immediate months following a federally declared disaster. The program typically lasts up to nine months following disaster declaration. It is focused only on psychological first aid. For communities continuing to experience elevated depression for four years post-disaster, that kind of short-term analyses may be insufficient. Wildfire mental health needs are not a short-term emergency problem. It requires effort and can be labeled as a medium-term infrastructure problem. Addressing these needs demands a combination of immediate crisis response and sustained investment in telehealth capacity, community health worker programs, and rural mental health provider recruitment in the years following a major fire.

The result has a direct policy implication. The mental health burden from wildfire appears to persist rather than fade over the four-year window. This suggests that disaster mental health programs (like the Crisis Counseling Assistance and Training Program) that conclude within the first year are likely leaving the period when the harm is greatest unaddressed. A straightforward response is to extend behavioral health resources into the medium term by increasing sustained telemedicine access and community mental health investment in fire-affected counties. The affected population in this study, roughly 1.5 million adults across 79 Western counties, is large enough that even a modest per-person increase in treatment access could offset a meaningful share of the depression burden identified.

The mechanism most consistent with the evidence is not simply that wildfires are traumatic events. What drives the persistent mental health effect is the combination of scale and aftermath. A wildfire that burns a significant amount of a county's land area does not just create a short-term emergency. It can damage livelihoods, reduce economic activity, strain local resources, and weaken county services over time. These are slow-moving stressors that continue to generate psychological harm long after the acute emergency has passed.

One limitation of this research is the use of cross-sectional analyses due to the limitation of depression data. Because CDC county-level depression estimates are only available beginning in 2019, pre-fire county-level depression measures are unavailable for a full-blown causal inference. Including more recent wildfire seasons like 2020 is a natural extension to the current analysis. However, the coincidence with COVID-19 complicates the research. It is worth future investigation. Three directions for future work emerge from the current findings. First, extending the analysis through 2023 would allow direct tests of whether the depression gap observed in 2019 widened further, stabilized, or began to close. That trajectory matters for policy timing. If the gap is still growing at year eight, the argument for sustained behavioral health investment is

substantially stronger. However, the breakout of COVID-19 creates a big challenge for the analysis.

Wildfires are becoming more frequent and more severe across the American West, and there is no credible projection that suggests this trend reversing in the near term. The physical costs are well reflected in insurance premiums, emergency budgets, and infrastructure planning. The mental health costs have been harder to quantify. They do not show up in satellite imagery or fire perimeter maps. They accumulated quietly in the households and clinics of communities that were living with wildfire long before it became a national news story. This paper is an attempt to make those costs visible in the data. It is an attempt to provide a number that planners and policymakers can use when deciding whether to fund a mobile mental health clinic in a fire-affected rural county, to extend a telemedicine waiver for another year, or to invest in community mental health infrastructure in places that need it most. This paper provides a solution to this dilemma. The need is real, it is large enough to measure, and four years after the fires, it has not been resolved on its own.

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Selective Alteration of SST-Associated Inhibitory Transcriptional Signatures in the Alzheimer's Disease Hippocampus By Hannah Kim

Abstract

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases worldwide, characterized by cognitive impairment and neural network dysfunction. It has been shown that excitatory-inhibitory (E/I) imbalance and altered GABAergic signaling cascade contribute to AD pathophysiology. However, which inhibitory neuron subtypes are vulnerable remains poorly understood. In this study, we analyzed a publicly available bulk hippocampal RNA sequencing dataset from patients with AD and age-matched normal controls and investigated transcriptional changes associated with two main inhibitory neuronal populations in the hippocampus: somatostatin (SST)-expressing interneurons and parvalbumin (PV)-expressing interneurons. Differential gene expression analysis indicates that expression of SST, a key marker of hippocampal SST-expressing interneurons, is significantly reduced in patients with AD, whereas expression of PV, a marker of PV-expressing interneurons, was not different between normal samples and patients with AD. We further examined additional SST-associated genes and found significant alteration of multiple SST-associated genes. In contrast, co-expression analysis between SST/PV and other interneuron markers was preserved, and differences in interneuron-associated gene correlation between AD and control samples were not statistically significant. Taken together, this study provides evidence of the selective vulnerability of SST interneurons in AD pathophysiology and highlights SST-related inhibitory circuits as potential targets for future investigation.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder and a leading cause of dementia worldwide (Scheltens et al.). The progressive decline in cognitive function, particularly episodic memory impairment, is closely associated with dysfunction in hippocampal circuits essential for learning and memory formation(Breijyeh and Karaman). Although extensive research has focused on amyloid-beta accumulation, tau pathology, and neuronal loss as major pathological features of AD, growing evidence suggests that alterations in neural circuit function also play a critical role in disease progression (Grieco et al.; Breijyeh and Karaman; Scheltens et al.).

The current treatment options for AD are limited, and the lack of effective treatment highlights the importance of identifying new therapeutic targets. The relative contribution of excitatory and inhibitory synaptic inputs is called the Excitation-Inhibition (E-I) balance, a promising therapeutic target for AD (Ghatak et al.).

Maintaining normal brain function requires a balance between excitatory and inhibitory neurotransmission(Sears and Hewett). Beyond canonical molecular pathways, including amyloid-beta accumulation and Tau phosphorylation, E/I imbalance contributes to cognitive impairment in AD (Ghatak et al.). The hippocampus, a core brain region for learning and

memory, contains different types of inhibitory interneurons that regulate the activity, timing, and synchronization of excitatory principal neurons (Pelkey et al.). Inhibitory interneurons in the hippocampus are not a homogeneous population; they comprise multiple molecularly distinct subtypes that regulate hippocampal information processing. Within in the hippocampus, somatostatin (SST)-expressing interneurons and parvalbumin (PV)-expressing interneurons form two major subtypes of interneurons with distinct physiological functions (Pelkey et al.). PV interneurons preferentially target perisomatic regions of pyramidal neurons and contribute to the generation of neural oscillations, including gamma-frequency activity(Penttonen et al.; Sohal et al.). In contrast, SST interneurons target distal dendritic regions of pyramidal neurons and regulate dendritic integration, synaptic plasticity, and network excitability(Scheyltjens and Arckens). Because these interneuron subtypes contribute differently to hippocampal function, they may show differential susceptibility to AD.

Recent studies indicated that there is impaired GABAergic signaling and interneuron function in both animal models of AD and patients with AD (Xu et al.; Bai et al.; Verret et al.). However, whether these changes reflect broad impairment across all inhibitory subtypes or selective vulnerability in specific interneuron subtypes remains unclear. Understanding subtype-specific alterations of interneurons may provide important insights for the mechanisms underlying hippocampal dysfunction in AD.

Recent advances in transcriptomic technologies have enabled investigation of the complete set of transcripts in various cell types and tissues (Wang et al.). Researchers have used bulk RNA-Seq to identify differentially expressed genes (DEGs) and study biological pathways implicated in various neurological disorders (Wang et al.). DEG analysis can identify genes that may be overexpressed or underexpressed in disease conditions. Transcriptomic datasets offer valuable opportunities to investigate disease-associated molecular mechanisms and gain new insights into cellular vulnerability. In particular, examining expression patterns of cell-type-specific genes can reveal whether disease progression preferentially affects specific neuronal populations.

In this study, we analyzed a publicly available RNA-sequencing dataset from the hippocampus of patients with AD to examine alterations in inhibitory interneuron-associated genes. We focused on two major inhibitory interneuron populations, SST- and PV-expressing interneurons, to assess whether they show differential transcriptional vulnerabilities. We first examined SST and PV expression as representative markers and then evaluated a broader set of SST-associated genes identified in previously published single-cell transcriptomic data. We also examined co-expression relationships among interneuron-associated genes. Through these analyses, we aimed to characterize subtype-associated transcriptional alterations in inhibitory interneurons and identify molecular signatures that may contribute to AD pathology.

Method

Identification and analysis of RNA-Sequencing dataset

A bulk RNA-sequencing dataset was obtained from the Gene Expression Omnibus (GEO) stie (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15969; GSE159699) (Nativio et al.). Differentially expressed genes between AD and control were used using LIMMA. The dataset included hippocampal tissue samples from patients with AD, age-matched older controls, and young controls. Diagnostic classification was obtained from the corresponding processed dataset. For this paper, we compared samples from patients with AD with age-matched older control samples and excluded young control samples from this comparison. A processed, log-transformed counts-per-million (logCPM) gene expression matrix was used for subsequent analyses.

Differential Expression and Individual Marker Analysis

Differential expression analysis was performed to investigate changes in gene expression between control samples and samples from patients with AD. Differential expression was assessed via linear modeling using the limma package in R(Ritchie et al.). For genome-wide differential expression analysis, statistical significance was checked based on p-values, and adjusted p-values were calculated to account for multiple comparisons. A volcano plot was generated to visualize genome-wide differential expression patterns, including SST and PV. Heatmaps were generated to visualize expression patterns of selected genes across individual samples. Data visualizations were performed in R using dplyr, ggplot2, and pheatmpa packages. Expression of individual markers was extracted from each sample, and differences in their expression between control samples and samples from patients with AD were evaluated using the Wilcoxon rank-sum test. Results were considered statistically significant when the p-values were less than 0.05.

Selection of interneuron-associated genes

To investigate whether AD affects different types of inhibitory interneurons, two major hippocampal inhibitory populations, SST- and PV-expressing interneurons, were examined. SST and PV were initially evaluated as individual marker genes for SST and PV interneurons. To extend the analysis for SST interneurons, additional genes representing SST-associated transcriptional signatures were also examined. Previously reported single-cell transcriptional signatures of SST interneurons(Fisher et al.) were used to find additional SST-associated genes. Additional GABAergic inhibitory neuronal markers, including GAD1, GAD2, and other genes, were examined to characterize gene correlations.

Correlation and differential co-expression analysis

Pearson correlation analysis was used to examine transcriptional relationships among inhibitory interneuron-associated genes. A set of genes associated with inhibitory interneurons and neuronal signaling, including PV, SST, VIP, GAD1, GAD, SLC32A1, GRIN, SLC17A7 and CAMK2A, was selected, and correlation matrices were calculated separately for control samples and AD samples using Pearson's correlation coefficient. For selected gene pairs of interest,

correlation coefficients between control and AD groups were directly compared using Fisher's r-to-z transformation. To examine group differences in correlation, a permutation test was performed and two-sided p-values were determined from 10,000 permutations.

Results

Transcriptomic analysis workflow identifies altered inhibitory neuronal signatures in the Alzheimer's disease hippocampus

To investigate how AD alters gene expression patterns, we analyzed a publicly available bulk RNA sequencing dataset from human hippocampal tissue samples from patients with AD and cognitively normal controls. We performed differential gene expression analysis to identify genes with altered expression patterns in the hippocampus during RNA-sequencing preprocessing and normalization (Figure 1). To test whether specific GABAergic neuronal subtypes show differential vulnerability in AD, we focused mainly on inhibitory neuronal markers and interneuron subtype-associated genes.

The hippocampus contains different interneuron populations with distinct properties and molecular identities. SST-expressing interneurons and PV-expressing interneurons are two main inhibitory subtypes in the hippocampus. Therefore, we examined SST and PV expression patterns to determine whether specific interneuron subtypes showed differential transcriptional changes in patients with AD. With this approach, we expected to identify potential subtype-specific changes within inhibitory neuronal populations.

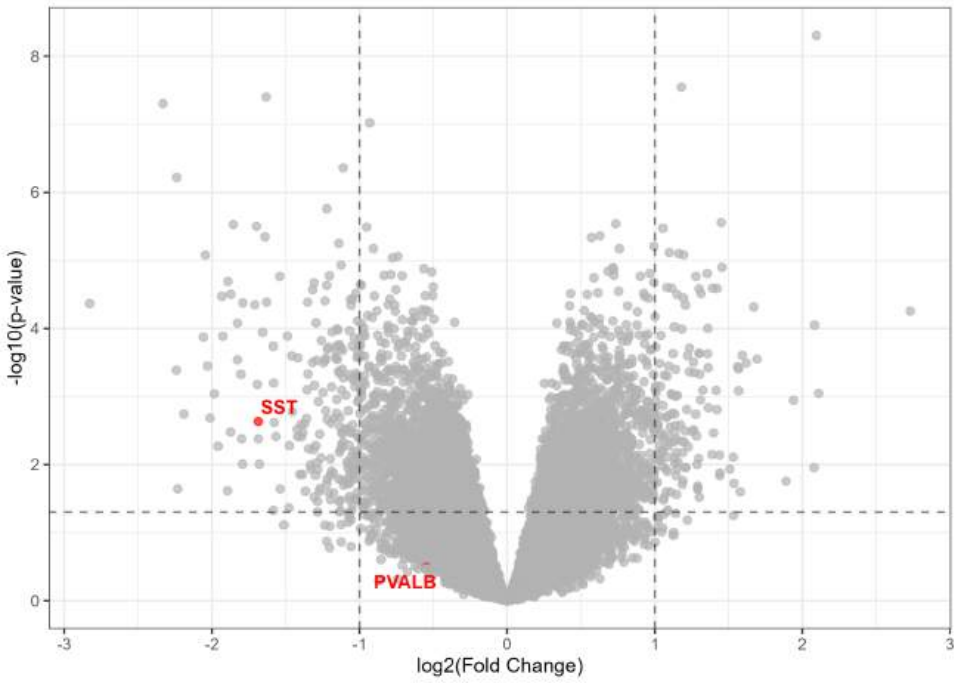

Figure 1. Volcano plot demonstrating differentially expressed genes, including SST and PV.

Selective Reduction of SST Expression in the Alzheimer's Disease Hippocampus

To determine whether inhibitory interneuron populations exhibit differential expression patterns in AD, we first examined the expression of two major interneuron markers in the hippocampus: SST and PV. Differential gene expression analysis of bulk RNA-sequencing data from human hippocampal tissue indicates that SST expression was significantly reduced in samples from patients with AD compared with normal controls ($p = 0.0044$) (Figure1). In contrast, patient samples and control groups showed no statistically significant difference ($p = 0.54$) (Figure 1). These data indicate that AD exhibits cell-type-specific alterations in interneuron expression patterns. The selective reduction of SST interneuron expression suggests that differential gene expression associated with inhibitory interneurons is distinct for specific interneuron subtypes. Instead, our findings indicate that AD pathophysiology preferentially affects SST-positive interneurons, whereas the PV-associated inhibitory interneuron population is preserved during disease progression.

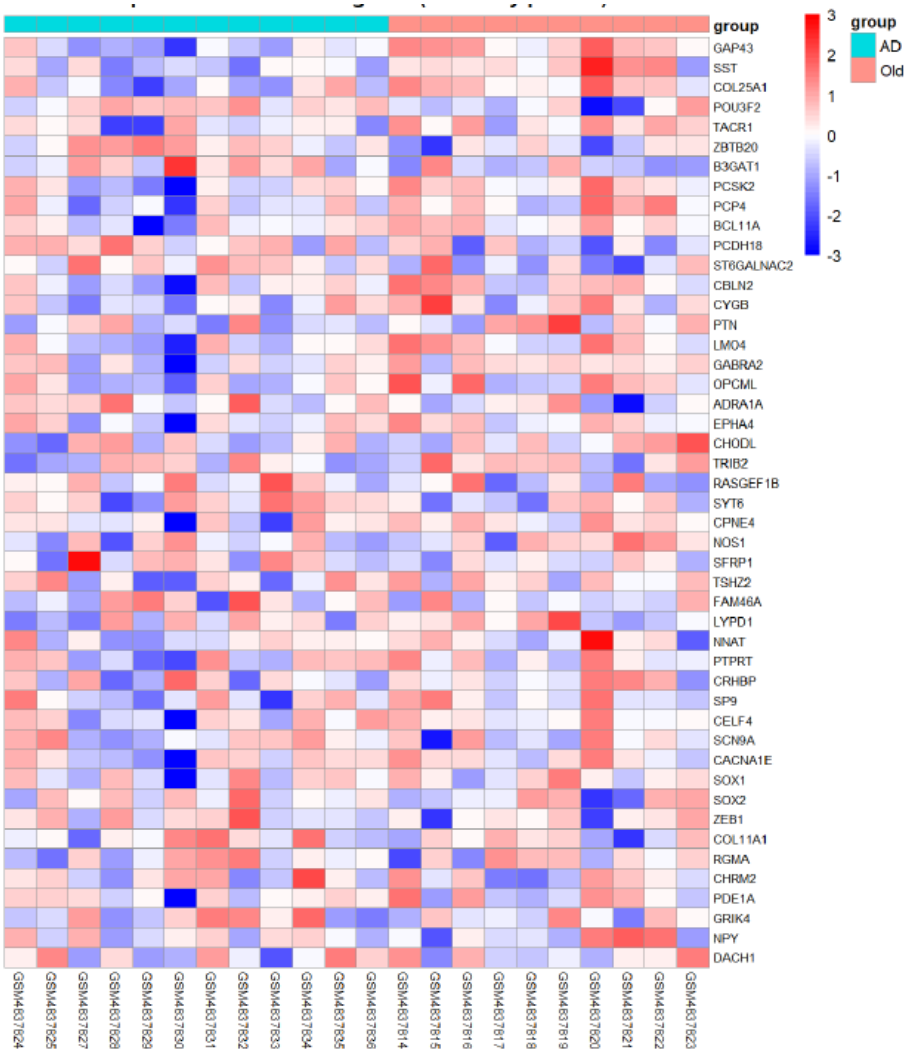

Figure 2. Heatmap showing expression patterns of SST-associated genes.

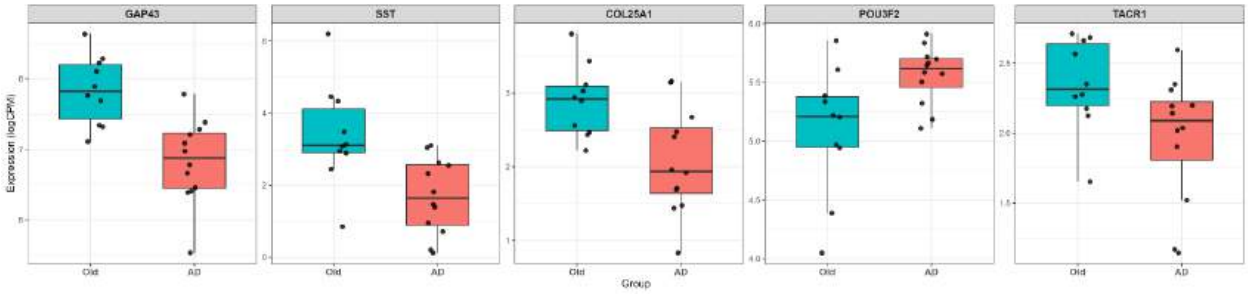

Figure 3. Expression changes of SST-associated genes. Top 5 most significant SST-associated genes.

Reduced SST-Associated Inhibitory Transcriptional Signatures

To further investigate SST-associated inhibitory transcriptomic changes, we analyzed the expression of 50 SST neuronal markers identified in a recent single-cell transcriptomic study (Fisher et al.).

We compared gene expression patterns of 50 candidate genes between age-matched control subjects and patients with AD and found significant downregulation of multiple SST-associated genes in AD brain tissue (Figure 2). In addition to significant changes in SST expression, we found 8 additional genes that differed significantly between healthy controls and patients with AD (Figure 3). Growth-associated protein 43 (GAP43) is a neuronal growth cone marker and synaptic-plasticity-related gene and exhibits the most significant reduction among all tested genes. In addition, collagen type XXV alpha-1 (COL25A1), involved in neuronal cell-cell adhesion and synaptic organization, was also significantly reduced in brain samples from patients with AD. These results indicate that AD affects a broad inhibitory transcriptional network, and the altered SST-associated gene signature suggests an impaired molecular network for maintaining SST interneuron identity and inhibitory signaling within hippocampal circuits. One important point is that we use bulk RNA-seq data and measure tissue-level signals, not individual cells. Therefore, we cannot rule out the possibility that some genes come from other cell types.

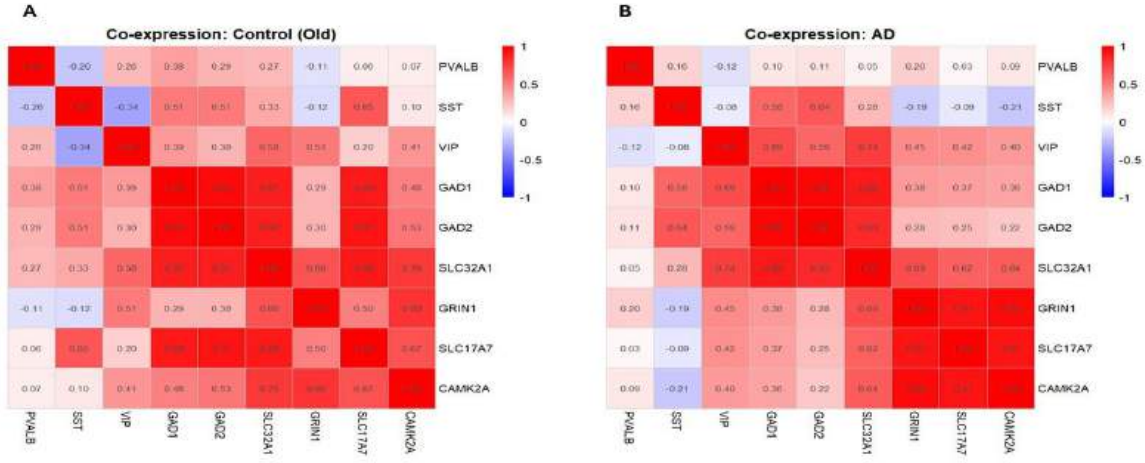

Figure 4. Heatmaps representing correlation analysis of interneuron-related genes. A) age-matched old sample; B) samples from patients with AD.

Correlation analysis of interneuron-related genes

In the differential expression analysis, we found that SST expression was significantly reduced in samples from patients with AD, whereas PV expression did not change significantly. To further examine whether AD alters transcriptional coordination of inhibitory interneuron-related neuronal markers, we performed co-expression analysis comparing interneuron-associated genes between control samples and samples from patients with AD (Figure 4). We found a positive correlation between SST and GAD1 in both control samples and samples from patients with AD, with a slight increase in the correlation coefficient from control to AD samples (control: 0.51; AD: 0.59). In addition, the correlation between SST and GAD2 showed a similar pattern (control: 0.51; AD: 0.64). However, those differences are not significant (Fisher's r-to-z test, P = 0.838 and P = 0.698, respectively; permutation test, P = 0.76 and P = 0.56, respectively). In contrast, the correlation between PV and GAD1 showed a disruption (control:0,38; AD: 0.10). However, this difference was also not statistically significant (Fisher's r-to-z test, P = 0.56; permutation test, P = 0.78). Together, these results suggest that co-expression relationships among interneuron-associated genes showed different directional patterns between control and AD samples, but these differences were not statistically significant. Therefore, these correlation analyses do not provide sufficient evidence of altered co-expression in AD.

Discussion

In this study, to investigate the role of a specific inhibitory neuronal population in AD, we analyzed a publicly available RNA-sequencing dataset from a hippocampal brain sample from patients with AD. Our analysis suggested a selective reduction in SST expression and in SST-interneuron-associated genes, whereas PV expression remained relatively unchanged. Interestingly, correlation analysis indicated directional differences in relationships between SST/PV and interneuron-associated genes across control and AD samples. However, those results were not statistically significant between groups. Together, our findings suggest that AD pathophysiology may not uniformly impair interneuron subpopulations, but instead may selectively target specific interneuron subtypes.

The hippocampus is a core region important for memory formation and information processing and has been shown to be affected by AD pathology(Rao et al.). Amyloid-beta accumulation and Tau pathology are well-known major contributors to cognitive impairment in AD(Scheltens et al.). However, growing evidence indicates that neural circuits become functionally disrupted during disease progression(Grieco et al.). Among these circuit-level disruptions, E-I imbalance has received increasing attention as a potential mechanism for cognitive impairment in AD (Ghatak et al.).

Our data indicate that PV expression did not differ significantly between control and AD samples. In addition, although the PV-GAD1 correlation was lower in AD samples than in controls, this difference was not statistically significant. Therefore, our data do not provide

sufficient evidence of disruption in PV-associated co-expression. However, the directional trend may warrant further investigation with a larger dataset.

Results from SST-associated genes showed a different pattern. We found a significant reduction in SST expression and alteration in SST-associated genes. However, the correlation between SST and GAD1/GAD2 was preserved and did not differ significantly between controls and AD samples. Our data suggest that SST- and PV-associated inhibitory transcriptional programs may undergo distinct forms of molecular reorganization in AD pathophysiology. Taken together, our results suggest distinct inhibitory interneuron alterations in AD.

Our findings may also help explain the heterogeneity of inhibitory circuit dysfunction in AD. Instead of having a uniform transcriptional response across GABAergic interneurons, our data indicate that different interneuron markers may exhibit distinct patterns of alteration. Reduced SST expression and altered SST-associated transcriptional signatures, along with relatively preserved PV expression, suggest subtype-specific differences in transcriptional vulnerability. This distinction needs to be considered for therapeutic strategies aimed at restoring inhibitory circuit function in AD.

Identifying subtype-specific molecular alterations may offer better opportunities to restore hippocampal circuit function therapeutically. SST-associated signaling pathways may be therapeutic targets, given that multiple SST-associated genes are affected in AD. Therefore, future studies should investigate whether normalizing SST-associated signaling can improve hippocampal circuit activity and cognitive function.

This study is not without limitations. Our data analysis was based on bulk RNA-sequencing data. Therefore, our findings represent tissue-level transcriptional signaling rather than cell-specific measurements. For example, reduced SST expression may reflect cell abundance, tissue-level transcriptional regulation, or both. To determine whether the observed transcriptional patterns originate specifically from SST- or PV-expressing interneurons, additional validation using single-cell RNA sequencing datasets will be necessary. Similarly, gene-gene correlation results should not be interpreted as changes in functional connectivity. In addition, the relatively small sample sizes may affect statistical power to detect differences in co-expression patterns. Larger datasets will help determine whether gene-gene correlations differ between control and AD samples.

Despite these limitations, our findings provide evidence that inhibitory interneuron-associated transcriptional programs would be differentially changed in the AD hippocampus. These findings support a model in which inhibitory circuitry dysfunction may not be uniform across interneuron subtypes in AD, but involve distinct molecular changes in SST-associated inhibitory programs.

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Investigating Voltage Production in Laboratory-Made Microbial Fuel Cells Using *Chlorella vulgaris* Algae By Caden Kim

Abstract

Microbial fuel cells (MFCs) are electrochemical systems that utilize microorganisms to convert organic matter into electricity. In contemporary society, the issues of climate change and global warming warrant an investigation into alternative energy technologies; MFCs allow for the production of electrical energy in an environmentally friendly manner as they utilize bacteria to break down the organic matter in wastewater. In this study, two MFCs were fabricated at the Missouri University of Science and Technology Sustainable Materials and Manufacturing Lab to investigate whether or not laboratory made MFCs could generate measurable voltage. Both cells were constructed with a PVC pipe outer layer, a 3D printed clay proton exchange membrane, and a soil and compost mixture slurry. Furthermore, the cells featured graphite rods and carbon felt to act as electrodes. These cells produced a maximum of 0.722 V, indicating that MFCs generate far lower levels of voltage compared to the amount that practical applications use. This study ultimately displays that although MFCs do produce sustainable electricity and measurable voltage, this energy is inadequate to replace existing energy sources. Additionally, since the two MFCs were constructed with the same design parameters, this study does not explore the impact of individual design elements on voltage generation. However, the study can guide scientists in efficiently constructing MFCs that do produce some voltage, allowing future research to discover the impact of various design elements on MFC voltage generation.

Keywords Microbial fuel cells, electrochemical, microorganisms, climate change, sustainable electricity, voltage

Introduction

Microbial fuel cells (MFCs) are an electrochemical system that utilizes bacteria to turn wastewater into electricity; they are currently utilized to clean wastewater while producing sustainable energy at the same time (Mercer). This energy technology is currently in high demand due to a greater need to produce renewable energy, because traditional energy is produced by burning fossil fuels (*United Nations*). A microbial fuel cell is divided into two chambers: an anode and cathode chamber, which are separated by a membrane. The bacteria is inserted into the anode side, and this bacteria is responsible for breaking down organic matter and ultimately generating current (Mercer). This idea supports the fact that MFCs are a sustainable way to produce energy because there will continuously be energy produced as long as there is still organic matter for the bacteria to break down.

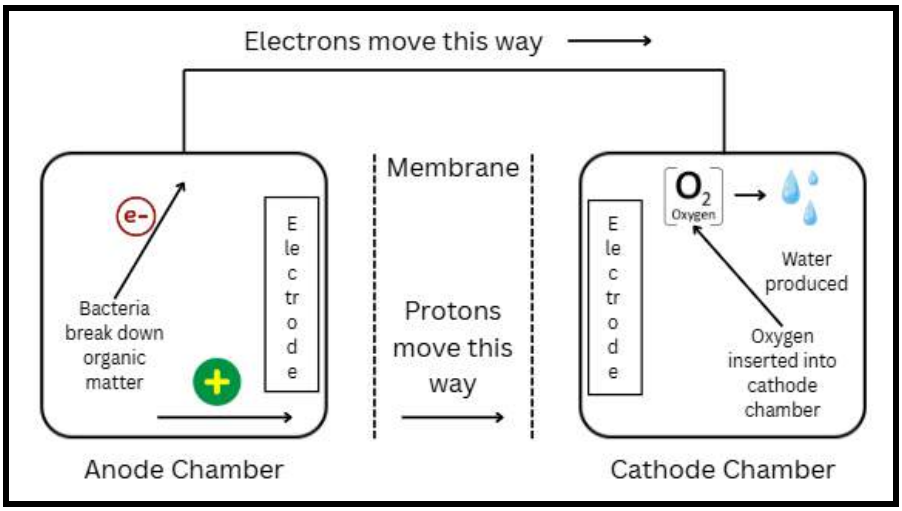

Figure 1: Graphic illustrating the functionality of a microbial fuel cell

Figure 1 displays how a MFC functions. When the bacteria in the anode chamber break down organic matter, there are both protons and electrons that are released; these electrons and protons have differing paths to get to the cathode chamber. This is because electrons will be transferred to the anode electrode;to clarify, the anode electrode is not the same as the anode chamber (Vishwanathan). There is an external wire that is connected to the anode electrode that allows these electrons to transfer to the cathode electrode, allowing data to be collected regarding voltage or current by inserting a measuring device in this wire. Furthermore, this movement of electrons generates current, and current is a result of the potential difference between the anode and cathode, therefore generating voltage (Vishwanathan).

However, the protons have a different path; they do not go to the anode electrode. Unlike the electrons, these protons go through the membrane that separates the anode and cathode chambers. Ultimately, the cathode chamber will receive protons, electrons, and also oxygen, causing the chemical reaction (Logan).

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \text{ (eq 1)}$$

As a result, it is necessary for both protons and electrons to make it to the cathode chamber. The oxygen is supplied into the cathode chamber from an external source, or, scientists utilize algae and rely on photosynthesis to create oxygen, which is what this study did when constructing the two MFCs. Additionally, the water that is produced is left inside of the cathode chamber because this chamber typically will already have a fluid inside, such as the leftover algae or another substance.

Despite the potential that MFCs have to produce energy in a sustainable manner, previous research has introduced a few limitations. For instance, previous studies have found that MFCs often face cost challenges regarding their cost-effectiveness (Chakma, et al.). Previous studies have also found that there is a high level of difficulty scaling MFCs to a large scale (Chakma, et

al.).This study will therefore include a cost analysis and investigate whether or not MFCs can generate measurable voltage.

Research Question

Can laboratory made microbial fuel cells assembled with a clay membrane, carbon felt, graphite rods, and a soil-compost slurry generate measurable voltage?

Hypothesis

It is hypothesized that laboratory-made MFCs assembled with a clay membrane, carbon-felt electrodes, graphite rods, and also a slurry based on a soil and compost mixture will produce measurable voltage because the organisms in the mixture will break down organic matter, releasing electrons to the wire in the external circuit and therefore generate current and voltage.

Methodology

Using laboratory resources and materials, two microbial fuel cells were fabricated at the University of Missouri Science and Technology's Sustainable Materials and Manufacturing Lab. Both MFCs followed similar processes of construction, and there was no intention to explore how the differences in design impacted the voltage output between the two cells.

The MFCs were both five inches long in length because of two 5 inch PVC pipes that were readily available in the lab. The tube was open on both sides, so a sheet of acrylic plastic was utilized to cut circular endcaps. These endcaps were later sealed onto both sides of the PVC pipe.

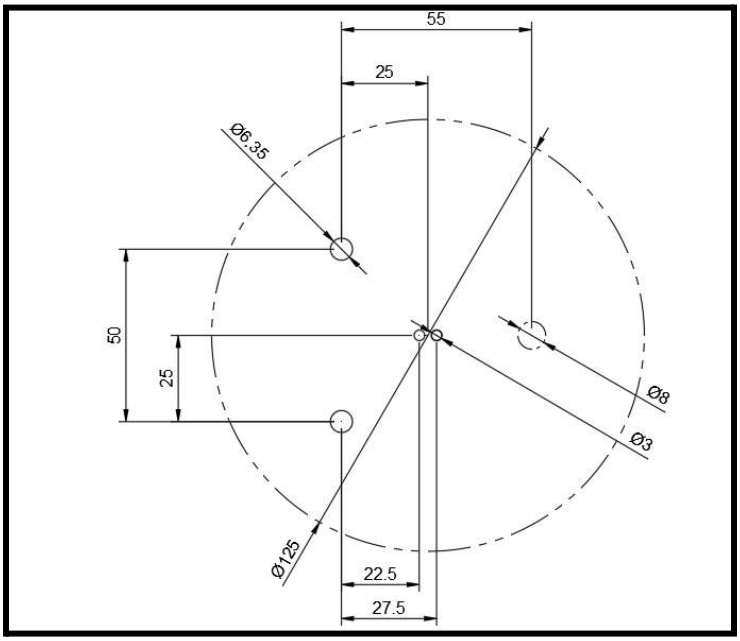

Figure 2: Fusion360 drawing file of PVC endcaps. All measurements are in millimeters.

Since the middle of each MFC was going to have a clay membrane, the two holes in the center of Figure 2 were inserted as a place for the graphite rods. The initial plan was to have a 5 inch by 5 inch sheet of carbon felt on each side of the membrane, and then stick graphite rods into the carbon felt. The material choices were intentional; carbon is the most popular anode material because of its high conductivity and thermal stability, in addition to the fact that it is very cost effective (Roy, et al.). Furthermore, graphite rods are also popular anode materials, and they maintain high conductivity while being a relatively inexpensive material (Roy, et al.).These graphite rods will ultimately allow the electrons on the anode side to reach the external circuit, thereby creating current.

The CAD drawing file also features two holes on the left half of the circle and one larger hole on the right half of the circle. These holes were all intended to be utilized for tubing, with the left two holes being for the anode side while the right hole being for the cathode side.

It was also decided that algae would be utilized on the cathode side, specifically *Chlorella vulgaris* algae. By inserting algae into the cathode chamber, there is no need for an additional oxygen supplying mechanism (Aiyer). Through photosynthesis, oxygen will be released from the *Chlorella vulgaris* algae, which will be the source of oxygen in order for the chemical reaction described in equation 1 to finish (Aiyer).

Next, the clay based membrane was designed. A CAD model for the membrane was created on Fusion360 and then uploaded into a Moore 1 Tronxy Machine, allowing a membrane to be 3D printed with clay. Our target dimensions for the membrane were 5” by 4 mm by 5”, but because it was found that using the Tronxy Machine could potentially shrink 20% of the print, the CAD model was created with 20% larger dimensions. It was decided that if the membrane printed too big, the necessary amount of clay could be later cut off.

Figure 3: A piece of cardboard and glove boxes were placed on top of the membranes to prevent any warping

Unfortunately, numerous problems were encountered while attempting to print the 3D clay membrane. The extrusion rate of the Tronxy Machine was very inconsistent, meaning there were frequent gaps in the clay while printing. Numerous attempts were conducted, and the extrusion rate was constantly monitored and adjusted to ensure that there would be no air pockets in the clay when firing it.

Once the membranes were done printing, they were cooled for approximately one week in preparation to fire them. Figure 3 showcases the process of cooling, as a piece of cardboard

and some boxes were placed on top of the membranes to prevent further warping. After three days, the membranes were moved to the top of a faraday cage, allowing these membranes to be exposed to the air on both sides.

However, dealing with warping and the inconsistent machine were not the only encountered problems. A week later, after examining the membranes, there were minor cracks inside of the clay. Although these were small cracks, they would still potentially cause the membrane to crack while firing them. Therefore, leftover clay sludge was utilized to repair these cracks. Since this meant more clay and water were being added to the membranes, the membranes were weighted each hour. When both membranes had stopped decreasing in weight, it was assumed that all of the water had dried out and the membranes were ready to be fired. The membranes were ultimately fired in a box furnace. The ramp rate was 150 °C/h with holds at 90 °C and 570 °C for 60 and 30 mins, respectively, so that the water could freely evaporate. Then, the membranes were held for 10 minutes at the bisque temperature, which was 1005°C.

Figure 4: This soil and compost mixture was created for the anode side. A 1 to 1 ratio of soil and compost were added and mixed, and distilled water was added as needed in the process.

While the membranes were being fired, the soil and compost mixture was created; this mixture is to fill the anode side with organic matter so that microbial activity would occur. After measuring the internal dimensions of the tube, the CAD model was utilized to find the internal volume of the anode chamber, so that the necessary amount of mixture could be made. The total internal volume of the total PVC pipe was measured to be 95.107 in^3, which is equivalent to 1558.525 mL. Since the membrane was 5 inches by 4 millimeters by 5 inches, this volume came out to 64.516 mL (the amount of space that the membrane takes up in the cell). After subtracting the 64.516 mL from the 1558.525 mL, this means that the remaining space inside of the chamber is 1494.009 mL. This number was divided in half because the anode and cathode chamber will have equal volumes, meaning the anode chamber's volume is 747.005 mL. Using this number, approximately 800 mL of the soil and compost mixture was created to account for some of the

mixture remaining in the measuring container. The ratio of soil to compost was 1:1, and distilled water was added as needed in order to get the mixture to a moist and loose consistency.

After the soil and compost mixture was created, half of each side of the MFCs were spray painted black. The black paint ensures no light reaches the anode chamber, and the other side is not painted black because the cathode chamber has the algae; algae require light due to them being photosynthetic, and the clear PVC side would later allow for light to be shined into the MFC.

Ultimately, the porcelain clay membranes were fired, and it was time to assemble the cells together. Initially, a sealant from Walmart was utilized to seal the PVC endcaps and the membranes into the tube. However, this sealant took too long to dry for the timeframe of the project, so JB Weld marine-based sealant was used due to its curing time of only one hour. Additionally, a marine-based putty from JB Weld allowed for any potential leaks to be fixed, as a layer of this putty was attached onto the outer perimeter of the endcaps. After the sealant and putty dried, distilled water was pumped into both chambers to ensure there were no leaks. When it was apparent that no leaks were in either cells, the fabrication process was finished.

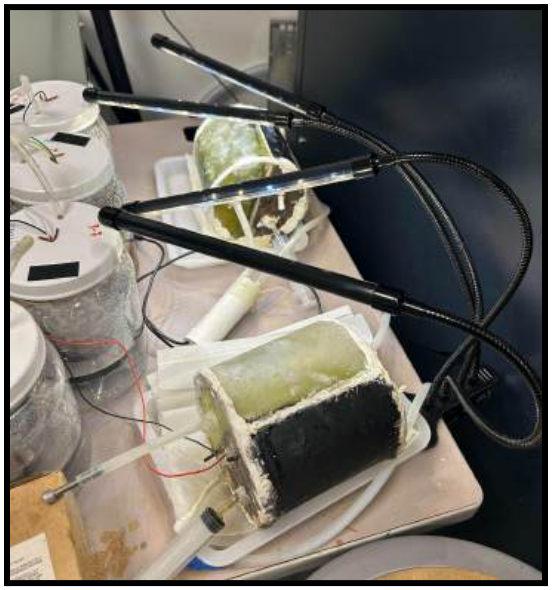

Figure 5: Implementation of MFCs under lights. Both cells are wired to a multimeter to measure voltage and current output.

Figure 5 showcases the implementation process for both cells. There are lights hovering over the cathode chambers so that the algae can undergo photosynthesis and release oxygen. The figure also showcases the spray painted anode side to prevent light from entering through the anode chamber, in addition to the white putty that was attached to the outer perimeters of the cells.

To measure the voltage output of the MFCs, the potential difference between the two electrodes was monitored. To do this, a battery tester was used; this tester acted as a multimeter, which is a device that can measure voltage and current. The setup included connecting wires to

both graphite rods (there is one graphite rod in each chamber), and then connecting this multimeter to the external wire so that voltage could be found and graphed.

Results

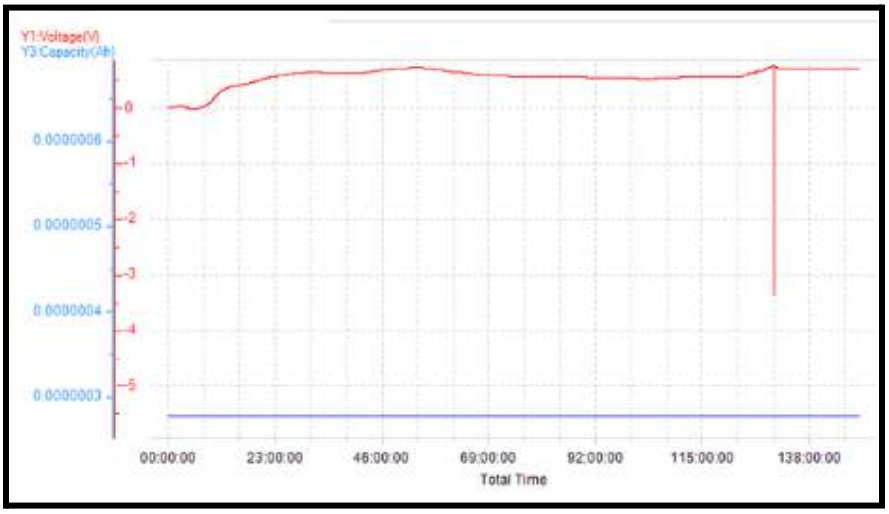

Figure 6: Voltage output of MFC 1 over time

The maximum voltage that MFC 1 got to was 0.722 V, which is illustrated through Figure 6. Data collection started on August 7th, and ended on August 14th, which means the data collection lasted just around a week. There is a very sharp drop for a very brief moment just around the 138:00:00 time frame which is likely due to internal shorting within the MFC or a loose connection with the wire.

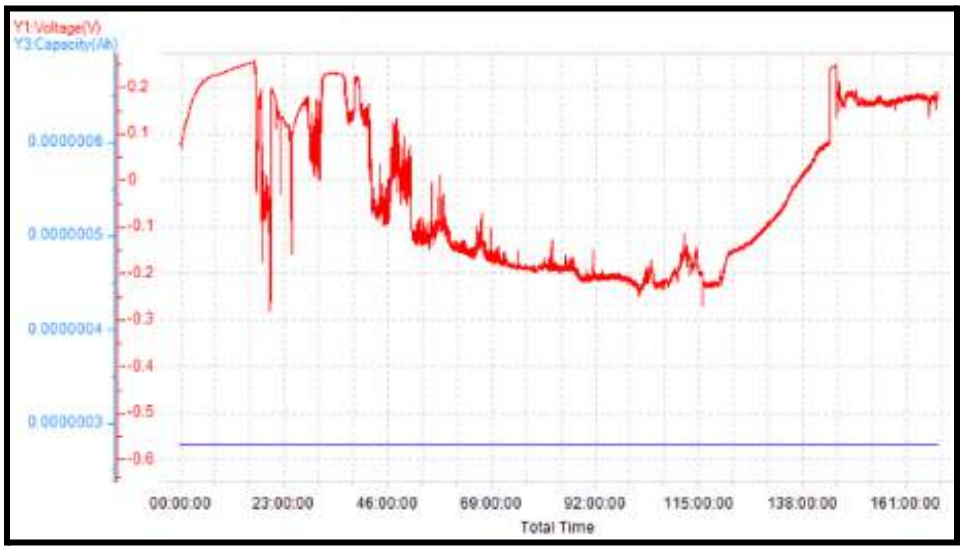

Figure 7: Voltage output of MFC 2 over time

The maximum voltage that MFC 2 hit was 0.258 V, which is displayed through Figure 7. This data reflects the span over a week; the data collection started on August 6th, and ended on August 14th. There are numerous sharp decreases in voltage but also time intervals where the voltage steadily increases. The sharp decreases in voltage may potentially reflect loose connections in the wiring, but internal shorting may have occurred within the cell, which would also cause a steep drop in voltage (these are similar reasons listed under Figure 6 that account for the sharp drop in the voltage of MFC 1). The steady increase in voltage starting at the 115:00:00 time frame reflects the time where pumpkin puree was added into the anode chamber; the pumpkin puree acted as a feeding mixture that would provide extra food for microorganisms to break down and cause electrons and protons to be released.

Discussion

Both cells were able to produce positive, measurable voltage. This supports the hypothesis that was created before the MFCs were fabricated, as the hypothesis predicted that both MFCs could produce measurable voltage. The reasoning stated in the hypothesis further parallels the results; the measurable voltage that was found is a direct result of the organisms in the soil-compost mixture breaking down organic matter and therefore releasing electrons to the external wire, thereby creating a potential difference between the anode and cathode. Ultimately, it can be said that the results support the hypothesis.

For MFC 2 specifically, which the voltage over time graph can be seen on Figure 7, it was found that adding pumpkin puree as a feeding mixture around the 115:00:00 mark caused the voltage to steadily increase. This is likely due to how MFCs work. Adding in pumpkin puree means there is more available food for the bacteria in the anode chamber, meaning the bacteria can break down the organic matter in the pumpkin puree and therefore release more electrons and protons. The increase in electrons being released will likely lead to a greater potential difference in the external circuit.

Furthermore, it is vital to account for the sudden drops and several fluctuations. As described earlier, these likely reflect noisy signals from loose wire connections. Internal shorting is another possibility of why the voltage suddenly dropped. Internal shorting means that another circuit that was unintended forms within the MFC. This causes some electrons to go into the intended external circuit in the external wire, but at the same time, other electrons go into the unintended, newly formed circuit. Ultimately, as less electrons are going through the intended circuit, the potential difference between the anode and cathode will be less, therefore causing less voltage to be measured.

However, despite producing measurable voltage, it is clear that this voltage is inadequate for MFCs to be considered a practical way to produce energy sustainably. The maximum voltage is only 0.722 V, which is substantially lower than the voltage that devices like computers and cellphones typically use. To further investigate whether MFCs were cost-effective, a cost-analysis was performed to figure out the cost of producing each MFC.

Cost Analysis

A table was utilized to estimate how much each MFC took to fabricate. Some costs were approximated and therefore have a "~" symbol in front of the cost. If the cost was approximated, the item was averaged between the prices that Walmart, Menards, and Lowe's were charging online.

Item	Cost	Quantity	Total
5" clear acrylic pipe	~$3.49	2	$6.98
Acrylic sheet	$4.98	1	$4.98
Black spray paint	$6.94	1	$6.94
Walmart sealant	$8.97	1	$8.97
JB weld sealant	$5.77	3	$17.31
Siphon pump	$15.99	1	$15.99
JB marine putty	$5.70	2	$11.40
Carbon felt	~$9.32	1	$9.32
Graphite rods	~3.00	4	$12.00
Wires	~$0.25	4	$1.00
			Total: $94.89 94.89 / 2 = $47.45 per MFC

Table 2: Cost Analysis of Laboratory-Made MFCs. The parts and items that were used to construct the cells are broken down in this table to ultimately analyze the cost of fabricating this cell.

Table 2 ultimately showcases that it cost approximately $47.45 to assemble one MFC. Moreover, it is important to note that the analysis accounts for the materials that were already available in the lab, because these supplies still cost money to acquire in the first place. Considering the low voltage output that these cells produced, the cost analysis indicates that MFCs are not cost-effective and that there still is a lot of future research to be conducted for the MFCs to be seen as a practical alternative to produce sustainable energy.

Limitations & Future Outlook

There are a few limitations that must be considered in this study. For instance, as only two MFCs were constructed, the results from the study cannot represent MFCs on a large scale. Additionally, the soil-compost mixture that was utilized in the anode chamber may have consisted of differing compositions of bacteria and organic matter, affecting how quickly electrons are produced at a given time interval.

It is worth mentioning that previous studies with MFCs have found similar results to this study. One past investigation indicated that the voltage output of MFCs is nowhere near sufficient for a practical level, including powering cell phones or computers (Chakma, et al.). Furthermore, MFCs are difficult to scale on a large scale because voltage output and scale do not have a linear relationship (Chakma, et al.). This means that a bigger MFC or more MFCs does not necessarily equate to more voltage output.

However, there is potential to multiply the voltage output from MFCs, which is a topic a future study can explore. A past study has attempted to multiply the voltage output of MFCs (Koffi, et al.). The study first placed numerous MFCs both in series and in parallel, but found that this still could not produce sufficient voltage due to voltage reversal, which means one or more of the MFCs opposes the voltage from other cells, and thus the total net voltage is decreased. However, the investigation developed a low voltage booster multiplier (LVBM), which uses MFC and boosts its voltage. As MFCs create direct current (DC), meaning electrons only flow in one direction, a LVBM uses a transistor-based self oscillating low-voltage booster to turn the DC into alternating current (AC); the transistor switches the direction of the current back and forth (Koffi, et al.). As the current direction keeps switching back and forth, the AC multiplies, and is later turned back into DC current, ultimately allowing for far more voltage to exist (Koffi, et al.).This research with low voltage booster multipliers are continuously ongoing and offer a potential way to utilize the sustainable energy produced from MFCs.

Conclusion

This study illustrates that laboratory-made microbial fuel cells can produce measurable voltage, but this voltage is insufficient to be utilized for practical everyday applications. Both MFCs were fabricated utilizing the exact same design parameters, utilizing laboratory materials such as PVC pipes, marine-based sealants, and carbon felt. Although the laboratory-made cells could produce up to 0.722 V, the low voltage generation and the fact that it cost $47.45 just to assemble one MFC supports the idea that these cells are currently not cost-effective. However, MFCs still have potential to be applicable to contemporary society as future research can potentially investigate whether this voltage can be multiplied so that the power output can be utilized as an alternative way to produce sustainable energy.

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Music Therapy Effect on Elderly Individuals With Neurodegenerative Diseases
By Catherine Lee

Introduction

As the aging population continues to rise in today's society, so does the number of elderly individuals suffering from neurodegenerative diseases. In fact, an estimated 6.9 million Americans alone are currently living with Alzheimer's dementia (Alzheimer's Association). According to the Cleveland Clinic, a highly respected medical institution, neurodegenerative disorders are "chronic conditions that damage and destroy parts of your nervous system over time, especially your brain." These diseases result in a wide range of symptoms, including memory loss, difficulties with concentration, and behavioral changes. Although neurodegenerative conditions remain permanent and incurable, advancements in medicine have produced several treatment options aimed at slowing disease progression and managing symptoms to improve patients' quality of life ("Neurodegenerative Diseases…"). While traditional medical treatments have long been viewed as the most effective for addressing these symptoms, recent studies suggest music therapy as a promising complementary approach, particularly for its effects on memory restoration in dementia patients. Music therapy is defined as the "use of music interventions in a clinical and evidence-based manner to achieve specific goals, which are tailored to the individual" (Bleibel et al.). In these sessions, licensed professionals implement various strategies based on each patient's unique needs to help minimize their struggles ("Music Therapy"). This high level of personalization distinguishes music therapy from conventional approaches, which often follow standardized protocols regardless of the difference in severity of symptoms. Given the increasing need for alternative treatment options for elderly individuals struggling with basic daily functions, the potential of music therapy has become a crucial area of exploration. With growing recognition of its cognitive, emotional, and social benefits, music therapy has emerged as a highly personalized and impactful treatment within the field of neurodegenerative care. Therefore, music therapy can be considered a highly effective alternative to traditional therapy methods in treating neurodegenerative diseases in elderly individuals because it enhances cognitive function, improves emotional well-being, and fosters social connectedness.

Cognitive Function Restoration

One of the most devastating consequences of neurodegenerative diseases is the gradual deterioration of cognitive functions. As symptoms progress, the ability of these elderly patients to retain memories and maintain focus diminishes. However, nostalgic music has been shown to stimulate such impaired cognitive functions, resulting in greater brain activity associated with memory recall. Researchers at the University of Toronto conducted a study of 14 individuals where they listened to a playlist with both self-selected music and unfamiliar pieces with no personal ties to it over a period of three weeks. The study found that when participants listened to unfamiliar music, they exhibited activity primarily in the auditory cortext. In contrast, when long

known music was played, the prefrontal cortex was activated, an area crucial for deep cognitive functions such as decision-making, reasoning, and personality (Goodyer). This finding indicates a higher level of cognitive engagement when patients are exposed to music with personal significance, as it activates areas linked to memory and self-awareness. Because music therapy intentionally incorporates songs that are personally meaningful for each patient, it becomes especially effective in restoring cognitive functions weakened by neurodegenerative diseases. In support of these findings, Dr. Assal Habibi, director of the University of Southern California Dornsife Center for Music, Brain, and Society, led a different research using MRI scanners to observe brain activity during music-evoked memory recall. Habibi found that nostalgic music activated patients' default mode network, which is typically engaged during introspective or daydreaming states, as well as visual areas that normally process what you see in front of you ("Nostalgic Music…"). Despite having their eyes closed throughout the entire experiment, participants showed activity in both these areas of the brain meaning that nostalgic music allowed participants to recall and visualize memories from past experiences related to the song. This further suggests that music can facilitate the retrieval of vivid personal memories. While music therapy helps to regain lost memories, it also has a significant impact in storing information more efficiently than traditional therapeutic methods in neurodegenerative disease patients. The structure of music composed of repetitive songs, chants, and rhymes acts as a template to organize and chunk information into more manageable units within the brain (Thaut). These rhythmic elements of music contribute to regulating brain functions as well as activating neural networks involved in language processing (Bleibel et al.). Altogether, these components allow elderly patients to comprehend and retain information throughout their day in a more orderly way. Since music therapy emphasizes songs that hold personal meaning, it consistently demonstrates the ability to support memory and cognitive clarity, making it a valid alternative to traditional approaches that may overwhelm patients with complex verbal instructions.

Emotional Well-Being

While enhancing cognitive function is a crucial step to take for regain practical skills, it is equally important for therapeutic approaches to address the emotional toll neurodegenerative diseases impose on elderly individuals. Mark Travers, a PhD-level psychologist specializing in emotional behavior, argues that music-evoked nostalgia can act as a "form of self-regulation, offering emotional comfort and buffering against stress" as cognitive decline often exacerbates feelings of stress and anxiety. The opportunity that patients are given during their sessions to reflect on their personally meaningful and cherished memories based on different songs is crucial in helping patients cope with stress (Heshmat). Music therapy often leads to music-evoked autobiographical memory, which occurs when songs trigger recollections of events, people, and places from the past (Jakubowski). It often occurs as an involuntary memory, meaning that the individuals would make no effort to intentionally try and recall such memories, but they still come to mind spontaneously. Oftentimes, these sudden involuntary memories are able to spark a feeling of joy and in turn, they lower stress and anxiety levels without patients having to

consciously recall them. Simultaneously, music therapy also releases dopamine and endorphins, both hormones that contribute to happiness and pain relief (Zoppi). Additionally, it can reduce the amount of stress hormones that the body produces, such as adrenaline and cortisol, which directly impacts feelings of anxiety that an elderly patient may be experiencing. Thus, music therapy helps patients achieve greater emotional stability by lowering stress and anxiety levels while boosting satisfaction levels. This balance between minimizing negative symptoms while enforcing positive symptoms has earned it widespread attention as a promising intervention for building emotional resilience (Feng and Wang). Based on this multi-faceted emotional benefit, music therapy can be recognized as a form of therapy qualified enough to be an effective alternative to traditional therapy because it is extremely attentive to the emotional well-being of all the elderly patients.

Social Connectedness

Due to the emotional burdens and fragile mental health neurodegenerative diseases place on patients, elderly individuals tend to isolate themselves from their surrounding communities (Peralta). However, music therapy is able to recognize the connection between emotional distress and social withdrawal, and fosters renewed social connectedness by rebuilding patients' sense of belonging and engagement with their surrounding communities in ways traditional therapy often fails to achieve. Traditional therapeutic methods often rely primarily on verbal communication, which becomes increasingly difficult as symptoms worsen. On the other hand, music therapy offers a nonverbal approach in communicating, which makes it easier for patients to freely express their thoughts (Peralta). With the opportunity of learning to interact with others without the pressure of verbalizing their feelings, neurodegenerative disease patients are able to grow a stronger sense of belonging to the world around them. In fact, individuals who experience musical nostalgia within their sessions tend to express greater levels of social connectedness than those who do not (Travers). The lyrical aspect of music helps these patients express their internal struggles without stressing about putting these feelings into spoken words, allowing for a space where patients can connect through shared emotional experiences. As music therapy takes a unique approach in letting patients express themselves compared to traditional therapy, it presents itself to be even more unique by integrating various types and genres of music from all sorts of cultures. Through this, patients develop a shared understanding of historical meanings behind pieces of music among themselves and with those who do not have neurodegenerative diseases as well, which gives them the perfect opportunity to reconnect with all groups of people (Zoppi). A broader sense of shared emotional experiences is seen in *Selenidad: Selena, Latinos, and the Performance of Memory* by Deborah Paradez, where Selena Quintanilla's music is shown to transcend individual memory and build a sense of collective cultural remembrance. Paradez discusses how Selena's music allowed the Latinx community to mourn, celebrate, and reconnect with their cultural identity in the wake of her tragic death, highlighting how music can serve as a vessel for preserving memory and fostering social connectedness. This phenomenon of music's potential uniting people emotionally reflects similarly to the role of nostalgic music in

therapeutic settings for individuals with neurodegenerative diseases. Just as Selena's music helped Latinx listeners collectively recall their shared experiences and identities, music therapy uses personally meaningful songs to bring back forgotten memories, create emotional resonance, and break free from the isolation that often follows cognitive decline. In both contexts, music is the key for communities to connect with each other despite verbal limitations, and regain a sense of belonging that traditional therapies may struggle to offer. Since music therapy does not rely on spoken language to communicate opinions, but rather uses a shared sense of emotions attached to certain pieces of music to help reconnect them into the rest of the community, this makes music therapy an easier approach in helping elderly individuals adjust to their surroundings and people again compared to traditional therapeutic methods that heavily rely on spoken words.

Limitations

In recent years, traditional therapy has improved the lives of many elderly individuals with neurodegenerative diseases, but with the rise of music therapy, debates surrounding its potential as an alternative therapeutic approach have become prevalent. However, like any other therapeutic method, music therapy comes with a possible limitation. Although many consider the flexible nature of music therapy as a significant strength, critics argue that it lacks the standardization necessary for consistent implementation across clinical settings. This lack of regulation causes hesitation among patients' families or even healthcare providers regarding its legitimacy in being classified as a genuine medical intervention to neurodegenerative diseases. Without an implementation of a set of strict standards for all music therapists to follow regardless of their patient, critics may continue to disregard music therapy as a genuine alternative to the traditional therapeutic methods they have stuck with throughout the past.

Conclusion

Nevertheless, both traditional methods of therapy and music therapy have significantly improved the lives of elderly individuals with neurodegenerative diseases with its own unique approaches. Traditional therapy focuses more on speech practices and verbal communication while music therapy emphasizes each patient's personal needs and desires through nonverbal expression of feelings, marking their key differences in their approach. While both have their features that make it a reliable approach to treating these diseases, music therapy stands out to be more effective for its holistic and personalized treatment. Its ability to reignite memory through familiar melodies, regulate emotions through nostalgic recall, and foster social connections through nonverbal, shared understanding of music makes it uniquely qualified to address the complex challenges these elderly patients face daily (Bleibel et al). Unlike traditional therapies that often rely on standardized routines, music therapy aims to adapt to each individual's personal history, preferences, and emotional state to make it as impactful as it could be. Though it may not replace traditional methods entirely, its growing support from scientific research suggests its vast potential for the future. All in all, music therapy is a highly effective alternative to traditional therapy methods in treating neurodegenerative diseases in elderly individuals

because it restores necessary cognitive functions, regulates emotional stability, and helps build social connections to other patients and the rest of society. As society continues to experience rapid growth in its elderly population, acknowledging music therapy as an appropriate and effective treatment intervention is crucial to meeting the complex cognitive, emotional, and social needs of aging individuals with neurodegenerative diseases.

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Hereditary Breast Cancer Mutations in High-Penetrance Genes By Esha Varma

Abstract

Breast cancer is the most commonly diagnosed cancer in the world, with over 2.3 million cases reported annually. Although often sporadic, 5-10% of all breast cancer cases are hereditary, arising from inherited genetic mutations. Specific mutations in high-penetrance genes (e.g., BRCA1, BRCA2, PTEN, TP53) result in 25% of all hereditary breast cancer cases. These mutations significantly increase the lifetime risk of breast cancer by disrupting DNA repair mechanisms and other critical cellular functions. The aim of this paper is to detail the inheritance patterns of typical high-risk mutations on high-penetrance genes. Also emphasized is the importance of genetic testing and risk assessment and clinical strategies for cancer management.

Introduction

A recent publication estimated that there were 464,000 cases of female breast cancer and 131,000 deaths in Europe in 2012 [1]. Breast cancer arises from abnormal cell division within the breast tissue with tumors metastasizing to other regions of the body. While environmental and lifestyle factors contribute to breast cancer risk, hereditary predisposition plays a significant role in a subset of cases. Inherited mutations in specific genes can markedly increase an individual's susceptibility to breast cancer [2]. Understanding the genetic foundations of hereditary breast cancer is essential for efficient risk assessment, early detection, and management. Advancements in screening and treatment offer improved survival rates within individuals susceptible to hereditary breast cancer [2]. To better understand hereditary risk, it is necessary to examine the genetic basis underlying these inherited mutations.

Genetic Basis of Hereditary Breast Cancer

Breast cancer can be broadly categorized into two very distinct forms, sporadic and hereditary. Sporadic cancer arises from random genetic mutations acquired throughout an individual's lifetime through environmental exposures, lifestyle factors, or errors during DNA replication. Conversely, hereditary breast cancer is caused by inherited mutations from specific high-penetrance genes, a term that describes the likelihood of a specific phenotype being expressed. A study found that out of 496 breast cancer patients who underwent surgery between January 2002 and December 2017, 95 were found to have 48 deleterious germline mutations in 16 cancer susceptibility categories [3]. Of these 95 patients, 63.2% had *BRCA1/2* mutations, which are high-penetrance tumor suppressor genes. These findings emphasize the importance of specific high-risk genes, particularly BRCA1 and BRCA2.

Key Genetic Mutations Associated with Hereditary Breast Cancer

BRCA1 and BRCA2 Mutations

Tumor suppressor genes are integral for tissue growth control. Both BRCA1 and BRCA2 are crucial in repairing double stranded DNA breaks through homologous recombination. Disruption, deletion, or loss of these tumor suppressor genes promotes the growth of cells that can become cancerous. BRCA1 mutations are seen in about 7% of families with multiple breast cancer cases and in about 40% of families with ovarian and breast cancer. BRCA2 mutations are found in 20% of families at high-risk for ovarian and breast cancers but in less than 3% of women with early onset breast cancer [4]. Individuals carrying BRCA1 or BRCA2 mutations exhibit poorer breast cancer specific survival compared to noncarriers. Furthermore, BRCA1 mutation carriers demonstrate poorer overall survival outcomes than those with BRCA2 mutations. [5]

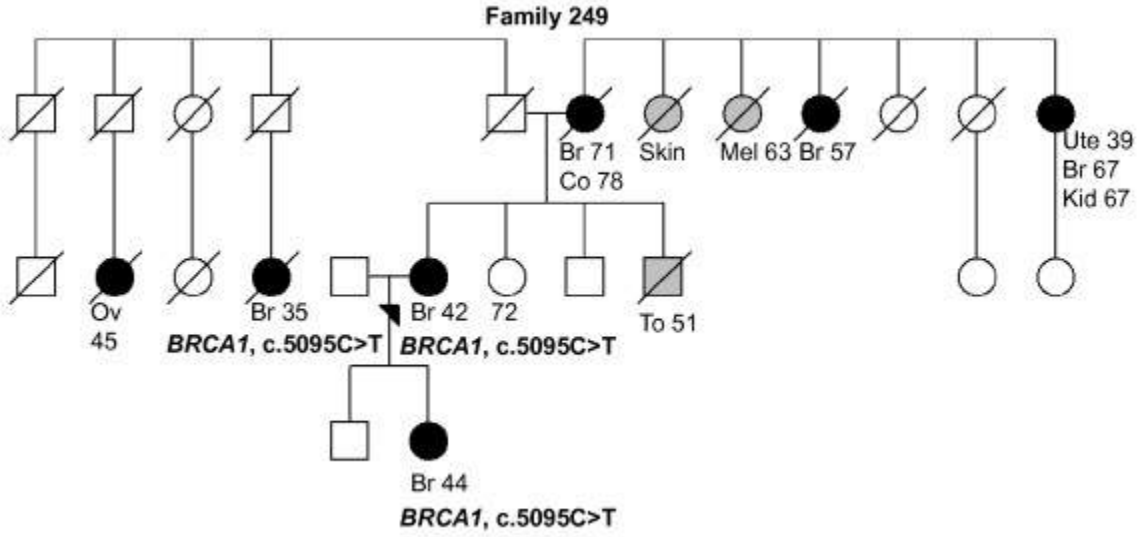

Figure 1 [13]. Family pedigree of the index individual with the identified BRCA1 c.5095C > T variant [13]. Individuals affected by cancer are indicated by filled symbols, with cancer type and age of diagnosis shown beneath the symbol (e.g., Br 44 = breast cancer, age 44). Genetic testing in several relatives revealed a shared BRCA1 pathogenic variant, c.5095C>T, which appears in affected individuals across more than one generation and along both maternal and paternal branches.

This pedigree illustrates a hereditary breast and ovarian cancer family carrying the BRCA1 c.5095C>T variant. The shaded symbols denote individuals who developed cancer; they are not necessarily proven mutation carriers, since genetic testing was performed only in a subset of affected family members. As seen in several pedigrees of highly penetrant genes, breast cancer appears at relatively young ages (e.g., 35, 42, 44), and cases occur across both maternal and paternal lineages. A confirmed BRCA1 variant is shown in three affected individuals, supporting the presence of a shared pathogenic allele segregating in the family, although incomplete testing means that some cases may represent untested carriers or sporadic disease. Unaffected relatives may still carry the mutation but have not developed cancer due to age, variable penetrance, or chance, while individuals who do not inherit the variant are not at

increased hereditary risk [5]. The overall pattern of early onset and multiple cancers in successive generations is characteristic of families with pathogenic BRCA1 variants.

BRCA1 and BRCA2 expression is high in the S phase of the cell cycle, suggesting a key role in maintaining genomic stability during DNA replication. As noted, BRCA1 and BRCA2 participate in double-strand break repair via homologous recombination. If DNA is damaged, BRCA1 associates with RAD51 and localizes to the damaged region, where BRCA1 is phosphorylated. Mutated BRCA1 and BRCA2 genes lead to error prone non-homologous end joining, restraining cells from repairing their DNA breaks [6].

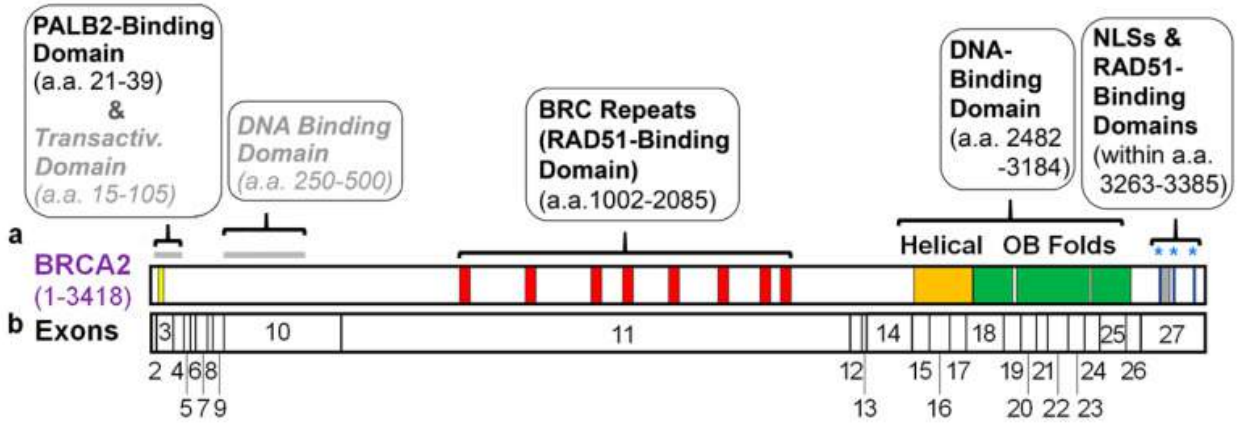

Figure 2 [7]. Schematic representation of the positions of identified domains in human BRCA2 and of the 27 exons that encode this protein. This diagram highlights the protein's DNA binding domain (amino acids 2482-2184), which consists of its helical domain and oligonucleotide/oligosaccharide-binding (OB) folds. The diagram also shows a secondary N-terminal DNA-binding region (a.a. 250–500, indicated in gray in the original figure), which has been reported to bind DNA but is less well characterized than the C-terminal DNA-binding domain (a.a. 2482-2184). Each domain is critical for BRCA2's role in DNA repair and homologous recombination [7].

Earlier work assumed that germline BRCA1 and BRCA2 variants explained most hereditary breast cancer. More recent studies show that, while BRCA1/2 are still the single most frequently mutated genes, together they account for only about 25–28% of the overall hereditary breast cancer risk [6]. The remaining risk is distributed across germline mutations in other high-penetrance genes.

TP53 (Li-Fraumeni syndrome)

The TP53 gene encodes the protein P53, which is another tumor suppressor used in regulating cell cycle arrest and apoptosis. Mutations in the TP53 gene are closely associated with the Li-Fraumeni Syndrome, a rare disorder that increases the risk of developing various cancers. Breast cancer associated with TP53 mutations is often diagnosed before age 35 and is frequently hormone receptor-negative, increasing its aggressiveness and limiting treatment options [8].

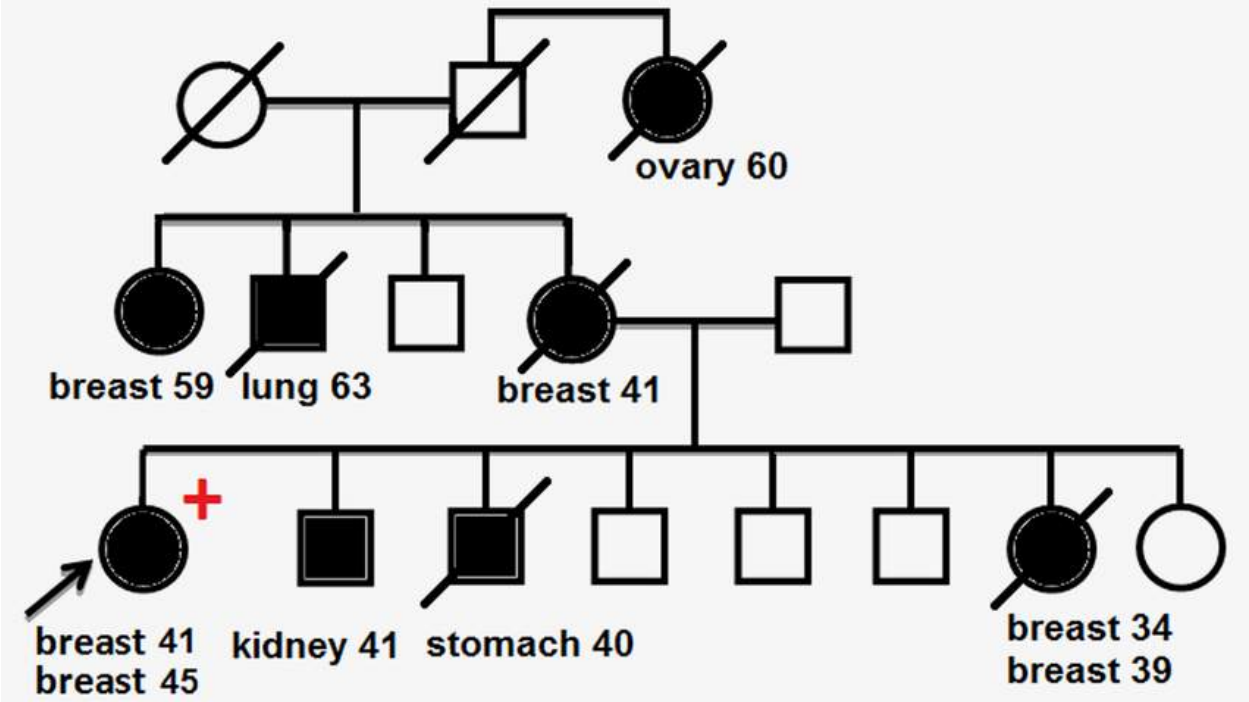

Figure 3 [14]. Pedigree consistent with Li-Fraumeni Syndrome, showing early onset breast cancers and diverse tumors.

Figure 3 depicts the vertical transmission of the autosomal-dominant TP53 variants. TP53-associated families typically show very early-onset cancers, a mix of tumor types among close relatives, and multiple primary malignancies in the same individual. These patterns are evident here, with breast cancers in the 30s–40s alongside lung, kidney, and stomach tumors.

Mechanistically, p53 acts as the "guardian of the genome" by sensing DNA damage and halting the cell cycle to enable repair or trigger apoptosis if the damage is irreparable. When TP53 is mutated, this protection is lost and cells then accumulate DNA damage without repair or programmed cell death. This mutation also disrupts homologous recombination, similar to BRCA1 and 2, creating genomic instability [9].

PTEN (Cowden syndrome)

PTEN, also known as phosphatase and tensin homolog, is a tumor suppressor gene that regulates the Pl3K/AKT signaling pathway. This pathway overlooks many significant functions, including cell growth, proliferation, and the enhancement of the cell's sensitivity to apoptosis [10]. Loss or mutated PTEN function has been identified in a wide spectrum of neoplasms, being considered a founder genetic event for tumorigenesis and tumor progression [11].

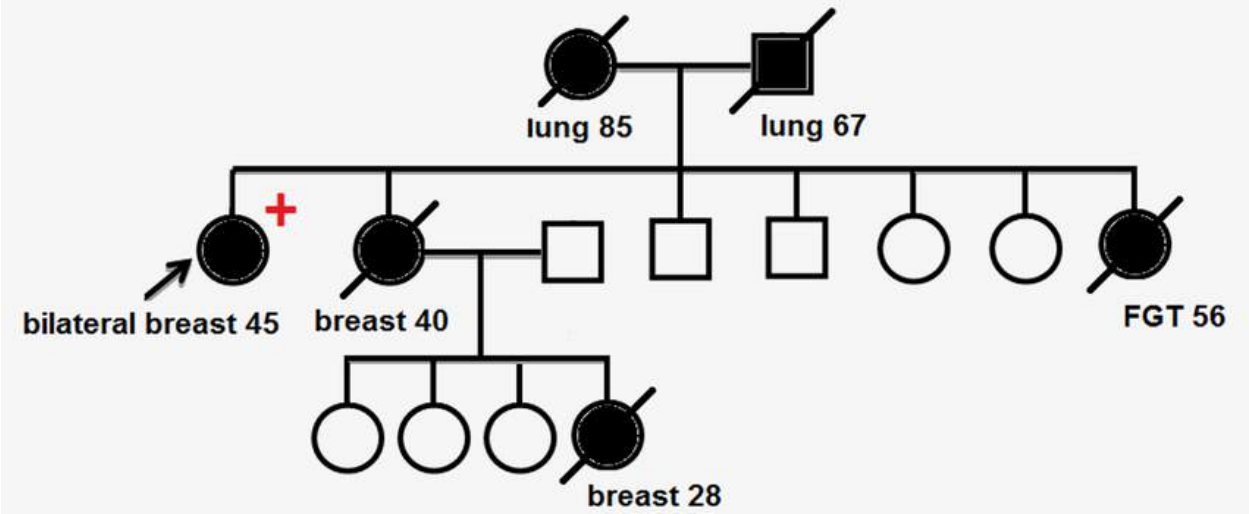

Figure 4 [14]. Pedigree of a Polish family with a mutation on the PTEN gene.

This pedigree is most consistent with autosomal-dominant inheritance of a high-risk tumor-suppressor mutation such as PTEN. The pattern shows vertical transmission across three generations with both sexes able to pass on risk, ruling out X-linked inheritance and favoring an autosomal locus. Unaffected relatives alongside affected ones at similar generational positions suggest a roughly 50% transmission risk with age-dependent, incomplete penetrance.

Genetic Testing and Risk Assessment

Mitigating breast cancer risk is essential for individuals, both male and female, who may carry mutations in high-penetrance genes such as BRCA1, BRCA2, TP53, PTEN, and PALB2. The cornerstone of this risk prevention is genetic testing, which screens individuals at high hereditary risk even before symptoms appear. This enables early surveillance, risk-reducing interventions, and earlier treatment should cancer be found. Multi-gene panel testing is now common, allowing simultaneous assessment of a wide range of high-risk and moderate-risk genes [17]. For example, as shown in Table 1, syndromes such as HBOC, Li-Fraumeni, and Cowden syndrome are tied to specific gene mutations, each associated with a spectrum of malignancies beyond just breast cancer.

Syndrome	Gene(s)	Malignancies
HBOC	*BRCA1* *BRCA2*	Breast (female and male), fallopian tube, ovarian, peritoneal, prostate, pancreatic
Li Fraumeni syndrome	*TP53*	Adrenal, breast, colon, glioma, leukemia, lymphoma, neurofibrosarcoma, osteosarcoma
Cowden syndrome	*PTEN*	Breast, endometrial, non-medullary thyroid, kidney, colorectal
Ataxia telangiectasia	*ATM*	Breast, endometrium, gastric, glioma, skin
Peutz–Jeghers Syndrome	*STK11*	Colorectal, gastric, pancreatic, breast, ovarian
Neurofibromatosis 1	*NF1*	Peripheral nerve sheath tumor, astrocytoma (optic nerve), high-grade astrocytomas, glioma, pheochromocytoma, possibly breast
Bloom syndrome	*BLM*	Breast, colon, cervix, esophagus, larynx, lymphoma

Table 1 [17]. Genetic disorders related to increased breast cancer risk [17].

Early detection of harmful gene variants allows doctors to sort patients into different risk levels and choose the most appropriate steps along the cancer prevention and care pathway. Figure 5 shows this pathway, which stretches from prevention all the way to survivorship, and includes major strategies such as risk-reducing surgeries, regular mammograms, MRI screening, and newer targeted treatments. Genetic risk assessment influences decisions at every stage, from considering preventive mastectomy to deciding whether to use PARP inhibitors in patients with BRCA mutations. Even when testing does not reveal any known pathogenic variants, people with a strong family history of cancer may still need closer and more frequent screening [18].

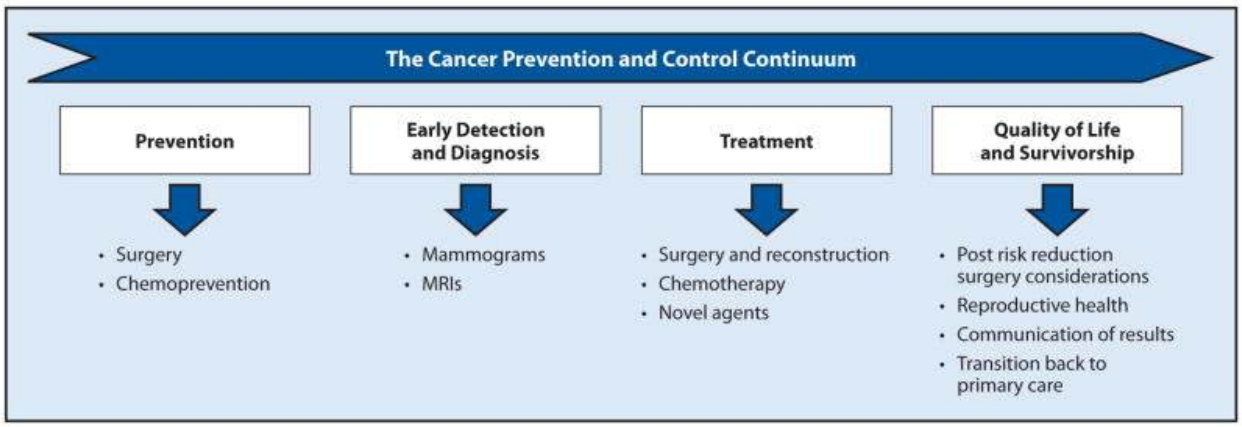

Figure 5 [18]. The Cancer Prevention and Control Continuum [18].

Risk assessment is not just a medical process; it is also highly personal. Patients benefit from thorough genetic counseling both before and after testing so they can understand what positive, negative, or uncertain results might mean for them and their families [19]. Counseling also helps people cope with the emotional impact of learning they may have inherited a cancer risk, and it raises important ethical questions about whether and how to test relatives. In the end,

combining genetic information with thoughtful, patient-centered care allows for customized monitoring, prevention, and treatment plans, which can greatly improve outcomes for those at risk of hereditary breast cancer.

Conclusion

High-penetrance mutations in BRCA1, BRCA2, TP53, PTEN, and PALB2 destabilize genome integrity, bias repair to error-prone mechanisms, and combine with growth cues to initiate and promote tumor development. These molecular features carry direct implications for patient care. Germline multigene panel testing identifies carriers for individualized screening, risk-reducing interventions, and informed family counseling. Tumors with homologous recombination deficiency are sensitive to PARP inhibitors and can be platinum chemotherapy-sensitive. Alterations that activate PI3K–AKT signaling, like loss of PTEN, predispose to the use of pathway-targeted agents and logical combinations. As results can include variants of unknown significance and gene-specific penetrance varies, management must integrate personal and family history with expert interpretation and shared decision-making.

Linking genetics to mechanism and mechanism to management simplifies a pathway for recognizing hereditary risk, selecting the appropriate tests, and translating molecular vulnerabilities into therapies. Future advances will come from enhanced risk models that integrate single-gene findings with polygenic background, more comprehensive catalogs of actionable repair defects, and broader availability of counseling and targeted therapies that improve outcomes for patients and families.

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A Proposed Framework for Integrating Productivity and Niche-Based Measures in Functional Diversity Analysis: Simulation-Based Characterization

By Aryan Ayyanger and Ahdav Vijay

Abstract

Functional diversity indices provide quantitative approaches for describing ecological differences among organisms, but many existing frameworks rely on predefined traits or abundance distributions. This study proposes a Functional Diversity Index (FDI) that integrates oxygen consumption and dietary niche composition within Rao's Quadratic Entropy structure weighted by Pianka-based niche dissimilarity. The proposed index was examined using a NetLogo simulation designed to generate randomized distributions or organisms, oxygen consumption, and dietary resource use. Across 91 randomized distributions, FDI values ranged from 0.1807 to 0.3151. Regression analysis explained 84.13% of the variation in simulated FDI values (adjusted $R^2 = 81.5\%$) demonstrating strong internal consistency, while Classification and Regression Tree (CART) analysis identified threshold-like transitions associated with dietary composition within the randomized ecosystem. Additionally, K-means clustering was used to explore patterns among simulated ecological variables, and an optimization procedure was developed to prove the concept of the FDI. These analyses demonstrate how the proposed FDI responds to changes in consumption distributions under the specific simulation conditions. Because the variables used to construct the FDI are also present among the simulated predictors, the present analysis characterized the mathematical behavior of a novel index rather than independently establishing its ecological validity. The framework therefore provides a preliminary model for integrating energy-related consumption and dietary niche structure into functional diversity analysis.

Introduction

The present world is marked by exponential technological growth, and one major consequence of these technological advancements is the rapid environmental changes occurring across the planet. These shifts are driving deep structural changes in our ecosystems. We are beginning to see system-level shifts in productivity, resource availability, and species interactions. Ecologists have emphasized that older methods of simply counting species, without examining how each species uses resources, processes energy, and interacts functionally, are no longer sufficient. There is an immediate need for a precise way to measure changes in biodiversity that can drive meaningful actions to preserve ecosystem stability—an index that can take advantage of the current growth in data science and machine learning.

A major challenge is that functional roles in our ecosystems are extremely dynamic. Resource use shifts with environmental conditions, metabolic demands vary across individuals, and niche overlap can expand or contract depending on productivity levels. Traditional trait-based frameworks, including entropy-based measures such as Rao's Quadratic Entropy, provide valuable structure but depend heavily on predetermined trait matrices that do not adjust

when organisms alter their consumption patterns. Similarly, niche-overlap models like Pianka's Index quantify shared resource use but are seldom linked to energy-flow variables such as Gross Primary Productivity (GPP), even though metabolic theory shows that energy processing is fundamental to ecological organization.

Current functional diversity metrics struggle to represent ecosystems with flexible resource consumption, variable energy flow, and functional distance shifts as environmental conditions change. As a result, they often fail to detect the environmental transitional threshold and nonlinear responses that characterize many ecological systems. These limitations are especially important in contexts where productivity and resource partitioning—not species richness alone—govern resilience.

The motivation for this study arose from this gap. Instead of frameworks developed on static, empirical traits and taxonomic richness, this study works to model continuous, dynamic scenarios within an agent-based architecture. The goal was to create a diversity index capable of capturing how ecosystems reorganize when resource use shifts, even when species identities remain the same. By integrating oxygen-based productivity measures with multi-resource dietary niches, the study attempts to develop a functional index that is sensitive to ecological shifts and clearly identifies tipping points and plastic transformations.

This perspective guided the development of our Functional Diversity Index (FDI), which combines an evenness term derived from oxygen consumption with a quadratic-entropy structure weighted by Pianka-based niche dissimilarity. The index was designed to evaluate functional structure under conditions where organisms differ not only in abundance, but also in how they partition resources and contribute to overall energy flow. Simulation-based testing and statistical analysis helped examine whether such an index could identify functional shifts and reveal the underlying drivers of ecosystem organization.

Literature Review

Limitations of Traditional, Trait-Based Functional Diversity Metrics

Traditional biodiversity metrics primarily focus on species abundance as a descriptive measure of ecological diversity rather than the ecological roles these organisms possess. Many of the most established indices and models, such as species richness and Shannon diversity, quantify the mathematical distributions of these ecosystems in the context of evenness, but they do not capture how organisms function within their environment through distinct roles.

Rao's Quadratic Entropy, introduced by C.R. Rao (1982), measures diversity using pairwise functional differences between species weighted by abundant counts (3). Earlier work by Laszlo Szeidl and Carlo Ricotta (2009) found that traditional indices have shortcomings when representing ecological variation and that entropy-based frameworks like Rao's better represent functional variation by incorporating dissimilarity (12). However, the results of this study also show sensitivity in these indices to the definitions of ecological distance, meaning results dependent on predefined trait structures.

Botta-Dukát (2005) tested Rao's index across multiple simulated communities and found that the model's sensitivity to both trait differences and species abundance satisfied the core criteria for functional diversity (1). However, he also found that Rao's Quadratic Entropy has diminishing interpretive success when many correlated traits are included, since redundancy falsely magnifies rather than clarifying functional separation for ecological representation.

Many works extend Rao's applications beyond ecological representation into external scientific analysis. For example, Smouse (2017) expanded the framework for analyzing genetic diversity where Rao's index had success in capturing the hierarchical variation across species levels (16). However, the study also noted that the model depends strongly on defined matrices, which limits the flexibility in more dynamic ecosystems. This indicates a key limitation in the literature: although trait-based diversity indices elevate ecological representations, they remain constrained by static assumptions rather than modeling consistently changing ecosystems with highest accuracy.

Niche Overlap and Species Interactions

Another approach to understanding biodiversity is based on species interactions, shared resource usage, and ecological similarity. The Pianka's Index has been traditionally used for quantifying the niche overlap present in an ecosystem by comparing the amount of resources used by different species (11). This helped provide a mathematical approach for observing the extent of competition between species within a community. Furthermore, MacArthur and Levins found that coexisting species are strongly shaped by the amount of overlap in resource use, especially concerning ecological niches (9).

Other work has found that niche overlap is not a static characteristic. Fattorini and other researchers found that niche overlap can shift significantly, especially after environmental disturbances, which can change species interactions and the ecological makeup of a community (5). Additionally, Schoener (1974) established that niche overlap is context-dependent and can differ across many environments based on the availability of resources and other constraints (15).

Considering all of this together, these findings indicate that niche overlap shouldn't be perceived as a static aspect of species pairs. Instead, they should be viewed as a dynamic result of differing environmental conditions and the activities within communities. Therefore, biodiversity doesn't only result from species and their traits but also actively changing networks in the ecosystem.

Energy Flow, Productivity (GPP)

Optimizing ecological efficiency and relationships not only focuses on traits and niche overlap between species, but also requires analysis on energy-processing systems that constrain ecological flow and productivity. Ecosystems can be modeled through these energy-based frames though Gross Primary Productivity, metabolic rates, and respiration where limited resources satisfy ecological demands.

Odum (1969) also established that ecological stability is influenced by energy flow pathways: he argued that succession and ecosystem maturity are influenced by energy availability and respiration balances (10). His work demonstrated that developed ecological systems consistently have greater stability in energy efficient scenarios rather than purely high species richness states.

Another study by Lindeman (1942) quantified the efficiencies in energy transfer between trophic levels, and what ideal ecological distributions are for maintaining sustainability (8). His findings showed how productivity losses constrain food web structures, so maintaining plant biomass is crucial for sustenance. Scientists have worked to measure these energy changes and interactions through dynamic and functional manners. Similarly to the research done by Lindeman (1942), Running et al. (2004) models energy through measurable ecological parameters (13). Specifically, the study utilized satellite-derived productivity to demonstrate that GPP has the capacity to model activity and carbon exchange. Due to the relationships between oxygen exchange and photosynthetic productivity, these findings support the use of oxygen-based variables in ecological models.

The relationship between energy-processing and ecological organization was also expanded by the Metabolic Theory of Ecology (2). Their study supported that metabolic rates scale in predictable manners with temperature across organisms and body size. These organism specific energy transfers allow ecological processes such as growth, respiration, and consumption to be mathematically modeled. The authors found that metabolic scaling laws influencing ecological functions is strongly relevant, and that this perspective allows for dynamic ecosystem representation.

Nonlinear Ecosystem Dynamics

Beyond productivity itself, dynamic ecosystem representation involves analysis on the nonlinear responses that ecological systems have to environmental disturbances and functional imbalances. Holling (1973) introduced resilience theory and demonstrated that ecosystems remain stable across a range of environmental conditions before rapidly shifting into alternative stable states once critical thresholds are exceeded (6). This work posited a novel perspective on previous ecological assumptions by proposing that resilience depends on a system's ability to absorb disturbances with functional collapse.

Scheffer et al. (2001) further expanded this concept by focusing on catastrophic regime shifts in defined ecosystems such as lakes and coral reefs (14). The study's findings suggested that small environmental pressures may trigger disproportionately large structural changes once tipping points are exceeded. This suggests that ecological transitions are frequently abrupt rather than gradual, making threshold-sensitive models essential for monitoring purposes. Furthermore, Dakos et al. (2012) formally identified measurable warning signals preceding these ecological tipping points: these included increased variance, stronger correlation patterns, and compromised recovery rates (4). This study's efforts to quantify these thresholds in an analytical system suggest that nonlinear instabilities may be detectable before complete ecological collapse occurs.

Collectively, these studies indicate that ecosystem stability is not purely dependent on biodiversity itself, but also resilience to threshold-sensitive interactions. However, many traditional biodiversity indices and models primarily measure static abundance distributions while overlooking the nonlinear ecological transitions present in realistic environments. Consequently, integrating a productivity-derived perspective with dynamic resource-consumption topping points may provide a more responsive framework for representing instability and thus assists in ecological optimization.

Current Gaps

Traditionally, biodiversity is measured through abundance-based indices such as species richness, Shannon's Diversity Index, and others, which focus on finding how many species exist and how evenly they are distributed. However, a disadvantage of these indices is that they often miss functional roles a species has within an environment – roles that elucidate efficiency, fluidity, and overall health of the biosphere. These functional roles describe how species contribute to energy flow and the environment (through activities like pollination or seed dispersal).

Currently, advanced trait metrics like Rao's Quadratic Entropy and Pianka's Index pursue incorporating a dissimilarity factor and niche overlap. Although this does improve the representation of the functions within a species, current models primarily treat traits as static and fail to capture multi-resource consumption spanning multiple trophic levels.

These limitations pose multiple gaps in measuring functional diversity. There is a lack of rigorous, clear, mathematically based indices that numerically model functional diversity based on energy and resource consumption. Additionally, most indices also exclude GPP (Gross Primary Productivity), which is derived from oxygen or energy flow variables. Due to these factors, current models struggle to detect threshold-driven shifts within an ecosystem's functionality and may fail to fully understand the ecosystem's progression.

Ultimately, our goal was to develop a Functional Diversity Index which incorporates multi resources consumption and oxygen consumption rates, which are derived from GPP. The model combines Rao's Quadratic Entropy (functional dissimilarity weighted by abundance) along with Pianka's Index (niche overlap) to construct a testable framework that can model functional shifts in a simulated terrestrial environment. This leads to the research question: How would a Functional Diversity Index based on multi-resource consumption and oxygen consumption rates derived from GPP identify threshold-driven shifts in ecosystem functional structure within a simulated terrestrial ecosystem?

Functional Diversity Index

The Functional Diversity Index was an integrated model of multiple ecological aspects to cover the diversity of an ecosystem through both interspecies and intraspecies analysis. The first summation term is a measure of functional evenness based on the proportion of oxygen consumed by a species from all of the photosynthetic production by a simulated plant biomass.

Instead of focusing on the raw abundance of a species as a proportion, the oxygen consumption evenness component sets the base for the ecosystem's functional biodiversity. As shown in Figure 1, each of the proportions p_i for (i=1..n) is squared and subtracted from 1, so a higher value for the term occurs when the sum of squared proportions are minimized.

The second term of the index was created with quadratic entropy structure and Pianka's Index for the dissimilarity value. As shown by Figure 1, each term in the summation is the product between the consumption proportions of two distinct organisms and a dissimilarity value based on the organisms' dietary niches The proportions used are the relative oxygen consumption for both the first and second organism, which is defined from the randomization process in the simulation. It's worth noting the multiplier at the front being by one-half mathematically avoids overcounting when distinguishing between permutations and combinations.

$$\frac{1}{2}\left(1-\sum_{i=1}^{n}p_i^2\right)\cdot\left(\sum_{i=1}^{n}\sum_{j=1}^{n}p_i\cdot p_j\cdot d_{ij}\right)$$

Figure 1. Novel functional diversity index: combines evenness, Rao's Quadratic Entropy, and Pianka's Index

The d_{ij} value determined by the dietary composition of the two distinct organisms is calculated by the use of Pianka's index, as shown in Figure 2. This niche value is calculated by first determining the niche similarity between two species (o_{ij}), and then subtracting the value from 1 to measure dietary diversity. This calculated term is repeated for all pairs of organisms. It is worth noting that the niche similarity between the same organisms is exactly 1, so the term d_{xx} is equal to 0. Additionally, the ½ multiplier at the front is to avoid overcounting between the cases d_{ab} and d_{ba} where the niche values are identical and counted twice.

$$o_{ij}=\frac{\left(\sum_{i=1}^{n}p_{ik}\cdot p_{jk}\right)}{\left(\sqrt{\left(\sum_{i=1}^{n}p_{ik}^2\right)\left(\sum_{j=1}^{n}p_{jk}^2\right)}\right)}$$

Figure 2. Piankas Index for measuring niche diversity between two organisms. It is calculated based on dietary consumption distributions.

Methodology

The derived functional diversity index was implemented in a NetLogo-based simulation to characterize the behavior of the proposed mathematical framework under controlled conditions. The index integrates an evenness term based on oxygen consumption, a quadratic

entropy-based structure, and Pianka's niche dissimilarity for dietary compositions. The framework was designed to accommodate variation in species richness and resource types and was evaluated using simulated variables constrained by the parameters of the model. The one-half multiplier in the quadratic entropy component prevents double-counting when distinguishing between pairwise permutations and combinations. Together, these components provide a mathematical framework for examining functional variation across simulated ecosystem distributions.

The simulation development began with defining the fundamental variables and parameters required for randomized ecosystem generation. These attributes, developed in version 1.0, included oxygen output, established user control, and defined important variables, including Net Primary Productivity (NPP) and Gross Primary Productivity (GPP). In version 1.1, distinct organisms were visually represented with unique sizes and colors for a more interactive user-defined interface. Later updates incorporated user-input variables including sample size of organisms from 2 to 1000, and multiple resource types with randomized distributions. The final simulation generated output variables including NPP, GPP, average-consumption, and the Functional Diversity Index value, all of which are displayed in the final interface.

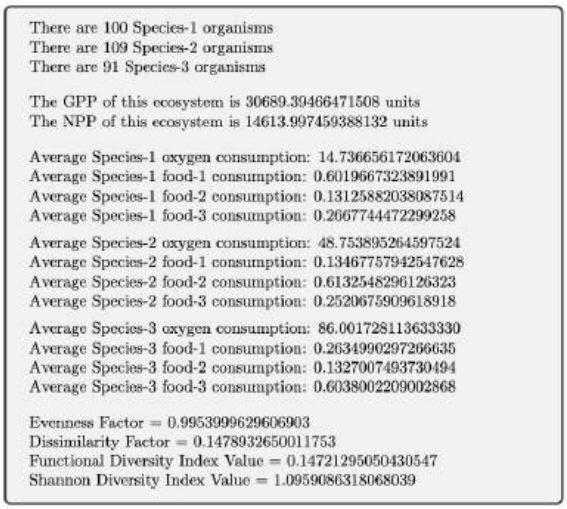

Figure 3. Sample output of the simulation software. Contains variable types, species amounts, and output values.

Procedure

The algorithmic workflow integrates user-defined population parameters with automated stochastic assignments. First, the user entered the number of organisms into the simulation interface. The program then would conduct the random assignment procedures: randomly assigning oxygen and dietary compositions to each of the distinct organisms. This randomization generated varied distributions without assigning organisms to predetermined combinations or traits, allowing characteristics to be classified after assignment. These classifications were in three main categories: Species-1, Species-2, and Species-3, each with defined ranges across the modeled variables. For example, Species-1 consumed between 0 and 30 units of oxygen,

whereas Species-2 consumed between 30 and 70 units, while allowing variation within each category. After these preliminary steps, the algorithm calculated the evenness term based on the oxygen proportions as a guiding value and the pairwise quadratic entropy component using Pianka's dissimilarity value. The simulation also calculated the Shannon diversity index for each specific ecosystem distribution for the purposes of comparing with a conventional diversity measure. This process occurred 91 times for distinct distributions where the statistical values would be derived.

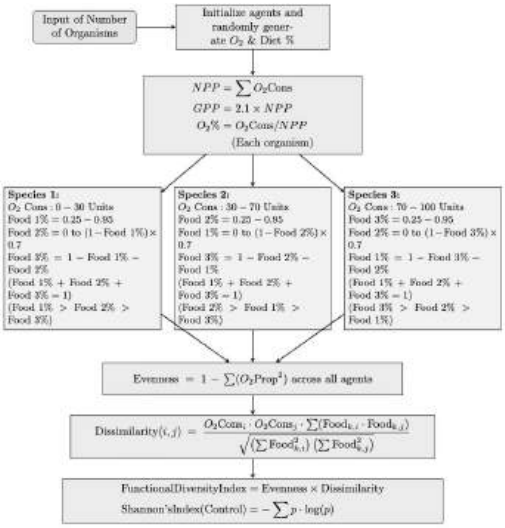

Figure 4. Algorithmic framework for simulation creation. Includes crucial steps and the species classification: Species 1, Species 2, and Species 3.

The primary analysis focused on the resulting values of the Functional Diversity Index and their variation across simulated ecological distributions. Within the wide varieties of randomized distributions, two hypothetical cases established reference conditions for interpreting the randomized results. The first was a baseline scenario defined by a singular organism per species, fixed oxygen and dietary constraints, and isolated calculation of the index. This case represented maximum differentiation under equal representation, which was a core simulation standard for future analysis. The second scenario represented the maximum FDI value obtainable under the practical constraints of the simulation. Although this value was not a formal theoretical maximum, the value represented the maximum under the model's specified restrictions. In this scenario, 1000 organisms were randomized across specific species clusters, with populations generally exhibiting greater diversity. These two hypothetical scenarios served as a reference for examining the range of FDI values and central to subsequent optimization analysis.

Due to the multivariable composition of the functional diversity index and the various degrees of influence amongst categories of a specific variable, regression analysis was used to characterize relationships between the simulated variables and the FDI. Species-based variables, including oxygen consumption and dietary composition, as well as broader ecological parameters were grouped according to their statistical significance across three categories: statistically

significant predictors, suggestive near-significant predictors, and non-significant variables. These classifications provided insight into how the index responded to variation within the simulated ecosystem. However because oxygen consumption and dietary composition are incorporated directly into the construction of FDI, their statistical relationships cannot independently establish ecological validity. Instead regression was used to characterize the sensitivity of the proposed index to simulated variables. Several of the variables exhibited high VIF values, indicating multicollinearity and limiting the interpretation of individual relationships. Due to severe multicollinearity among abundance metrics (elevated VIF > 1000), non-parametric Classification and Regression Tree (CART) analysis was conducted to isolate nonlinear patterns demarcate functional regime shifts. The resulting trinodal identified points at which simulated functional-diversity regimes changed under different combinations of resource-use variables. These thresholds characterize behavior within the model and are not interpreted as empirically established ecological tipping points. Relative variable importance of these distinct variables was compared in standardized graphs for enhanced comparison.

Although regression, CART, and FDI analyses provided information about relationships among the defined variables, they did not fully capture multidimensional patterns across changing ecosystem distributions. For example, the regression analysis considered the importance of the pre-determined variables and their classification among the species categories, including food and oxygen consumptions. To examine greater multidimensional complexity, a K-means clustering approach was integrated as an exploratory method for organizing simulated distribution according to similarities across variables. K-means clustering organizes the data points into distinct variable clusters, indicating repeating patterns or trends that may represent underlying relationships among dimensions. This approach allowed for multidimensional representation by using integrated Euclidean distances to update the center point; thus, the approach was critical for the procedure's practical use across distinct distributions since the variables can be ecologically specific. Within functional ecology, K-means clustering provides a rigorous method for partitioning multi-dimensional trait spaces and organizing species into functional groups (7).

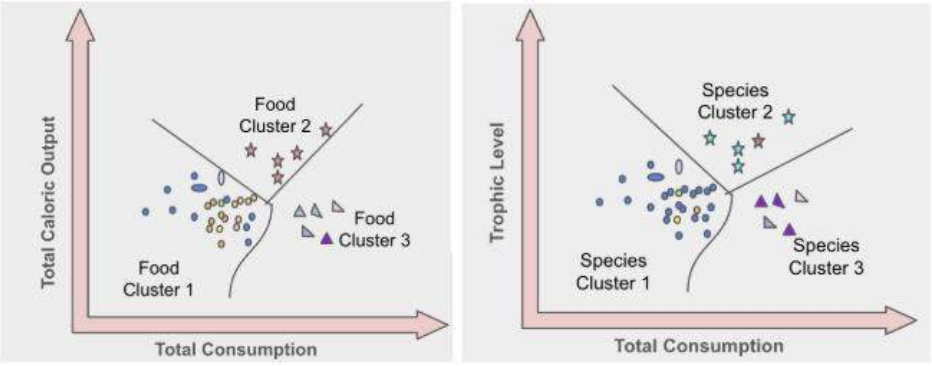

Figure 5. K-means clustering approach for two different dimensions, being the Total Calorie Output and the Trophic Level. The data in these graphs are organized into three clusters.

The preceding analyses provided a proof-of-concept optimization procedure using oxygen consumption rates. Rather than establishing an empirically validated sustainability intervention, the optimization model detailed how the proposed framework could respond to simulated changes in species dominance. The model used a recursive procedure involving the two most dominant species within a given cycle. After calculating the ratio between the most dominant to the second-most-dominant, the algorithm adjusted the dominant proportion according to mathematically defined conditions. The casework tree considered three possible adaptations: maintaining the dominant species-proportion, applying a smoothening function, or multiplying by 0.75 to produce a less abrupt change. The smoothing function itself was incorporated to allow continuous species regulation and was determined using established parameters in the model. Specific conditions and guidelines governing this procedure are presented in **Figure 6.** This optimization therefore demonstrates a potential computational application of the FDI framework within a simulated ecosystem rather than establishing that the procedure would increase sustainability in natural ecosystems.

Definitions

1. Define P_1 as the proportion of holistic consumption of the species with the highest oxygen consumption
2. Define P_2 as the proportion of the holistic consumption of the species with the second highest oxygen consumption
3. Define r = dominance ratio as P_1/P_2
4. Smoothening function: component of multiplier for P_1: $p(r) = 1/(1 + 5(r-1)^2)$
5. Final modifier value $\alpha = 0.3$ is adjustment index for smooth calculation

Algorithm The model will calculate a gradual optimization value for the larger population and will choose the minimum between the generated value, $0.75 * P_1$, and the original value.
Note: The value of $0.75 * P_1$ will only be considered when $0.75P_1 > P_2$
Final Function: Final optimization multiplier: $f(r) = 1 - 0.3 + 0.3 * (p(r))$
Formula : $f(r) = 0.7 + 0.3 * (1/(1 + 5(r-1)^2))$

Approach

Three values will be considered as the possible final optimization value for the highest population P_1: $0.75 * P_1$, $P_1 * f(r)$, and P_1.

Process

1. The value $0.75 * P_1$ and $P_1 * f(r)$ will only be considered if it is greater than P_2
2. $\min(0.75 * P_1, f(r) * P_1, P_1)$ will be selected as the new value
3. This will be repeated recursively for all the populations by shifting the higher population to the next highest: the analysis will occur for P_2 and P_3, P_3 and $P_4, \ldots, P_{n-1}$ and P_n for all n where n is the species richness
4. This recursive analysis occurs for the first few dominant populations: can range from 1 to 3 depending on ecosystem distribution

Reasoning

1. The value of $\alpha = 0.3$ represents the minimum multiplier. The value is chosen for moderate changes in the ecosystem that avoid abrupt ecosystem adaptations
2. The value of 5 in the denominator of the smoothening function allows for more rapid changes to occur at moderate values of r. This is important for the nature of the multiplier: drastically decrease higher ratios of r while preserving lower values

Figure 6. Definitions, algorithm, approach, process, and reasoning for the optimization model. Contains the smoothing function, important parameters, and the tested procedure.

Results

In our simulation results, there were 91 randomized ecosystem distributions, which allowed us to examine the behavior of the Functional Diversity Index (FDI) under multiple different combinations of the oxygen consumption rate, species abundance, and the composition of the diet.

Baseline and Maximum Scenarios

In the base scenario, the model initialized one organism for each species cluster in order to isolate the differences in functionality without the influence of the population structure. Under the scenarios with minimal conditions, the ecosystem produced an FDI value of 0.1807 **(Figure 7)**. This value represents the lower range of FDI values observed within the simulated conditions, where species are equally represented, and resource consumption patterns are relatively constrained. Under the maximum scenario with 1,000 organisms distributed across three species clusters with random trait assignments, the model gave an FDI value of 0.3151, which showed a greater functional differentiation under the larger population distributions **(Figure 7).** This increase from the baseline also shows that the FDI increases under the greater organism representation and randomized resource-use configurations used in the maximum scenario. Altogether, these scenarios helped create an estimated operational range of 0.1807 to 0.3151 for the FDI, which helps provide a reference for later interpretation.

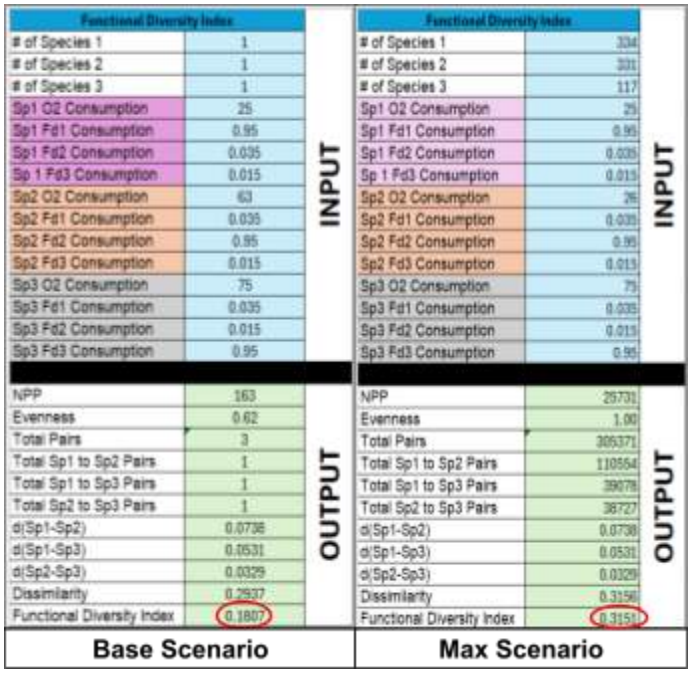

Figure 7. Simulation results showing the baseline and maximum ecosystem configurations used to establish the range of the FDI.

Regression Analysis

Multiple regression analysis was used in order to better determine which ecosystem variables had the strongest prediction of variation in FDI within the simulated ecosystems. The model helped explain the variation, with an R-squared value of 84.13%, and an adjusted R-squared value 81.45%, which shows it's a strong predictor in the simulated model (internal validation in the software). **(Figure 8)**. Most statistically significant predictors observed, which were p values less than 0.001, were most seen in the dietary composition variables such as

Species2_Food2, showing a strong positive relationship with FDI at a coefficient value of +0.217, and Species3_Food2 showing a strong negative relationship with FDI at a coefficient value of −0.195 **(Figure 8)**. These observed results show that the variation happening in food resource allocation to different species is a major contributor to variation in FDI within the simulated model. However, many variables are also able to show close significant trends, which include the Species2_Food1 at a p value around 0.068 and Species2_O2 consumption at a p value around 0.087 **(Figure 8)**. However, the species count and oxygen consumption-related variables were not statistically significant predictors, as seen with the p-value being greater than 0.10. Additionally, these results furthermore show that functional diversity in the model is influenced more strongly by how species use resources than by differences in abundance. The variation inflation factor, which is also known as VIF, is extremely high in some abundance-related variables. This indicates the strong multicollinearity among correlating species metrics. Therefore, it was concluded that the individual coefficients for these variables may be less reliable, but the holistic model still remains strong.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0053702	84.13%	81.45%	47.84%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.0421	0.0538	0.78	0.436	
Species1	0.000136	0.000081	1.68	0.097	200.47
Species2	0.000148	0.000213	0.70	0.489	1443.31
Species3	-0.000069	0.000360	-0.19	0.848	4018.06
gpp	-0.000001	0.000002	-0.31	0.761	12367.87
Species1_O2	-0.000414	0.000574	-0.72	0.473	1.47
Species1_Food1	-0.0239	0.0224	-1.07	0.289	3.92
Species1_Food2	0.0662	0.0614	1.08	0.284	4.02
Runs	0.00045	0.00107	0.42	0.676	1.49
Species2_O2	0.000452	0.000261	1.73	0.087	2.22
Species2_Food1	0.0998	0.0539	1.85	0.068	4.36
Species2_Food2	0.2173	0.0297	7.31	0.000	4.51
Species3_O2	-0.000459	0.000477	-0.96	0.339	1.43
Species3_Food1	0.0279	0.0205	1.36	0.177	1.44
Species3_Food2	-0.1948	0.0380	-5.12	0.000	1.37

Figure 8. Regression model summary with coefficient analysis for identifying the most statistically significant factors in predicting FDI variation.

Cart Threshold Analysis

In order to analyze the nonlinear ecosystem effects, a Classification and Regression Tree (CART) analysis occurred. The analysis showed that the Functional Diversity Index's variation was mainly affected by the two food composition variables of Species1_Food1 and Species2_Food2 **(Figure 9)**. The CART analysis shows the clear threshold tipping points that can affect the functional diversity in the model. For example, if the Species1_Food1 variable is over 0.74, the FDI value experiences a drastic drop, which shows the functional degradation.

However, in contrast, when the Species2_Food2 goes over 0.60, the FDI increases, which helps produce the highest FDI values as seen in Figure 8. Due to these thresholds, there are three distinct functional regimes: the baseline regime, which is a moderate FDI value with resources being used equally, a high performance regime, which is an increased FDI that is mainly influenced by the Species2_Food2 niches, and also a degradation regime, which is reduced FDI because of the overpresence of Species1_Food1. These findings illustrate that dietary composition is the main factor associated with variation in FDI within the simulated model. Because the variables examined in these analyzes also contribute to the formulation of the FDI, these results characterize the internal behavior of the index across randomized ecological configurations rather than providing independent ecological validation.

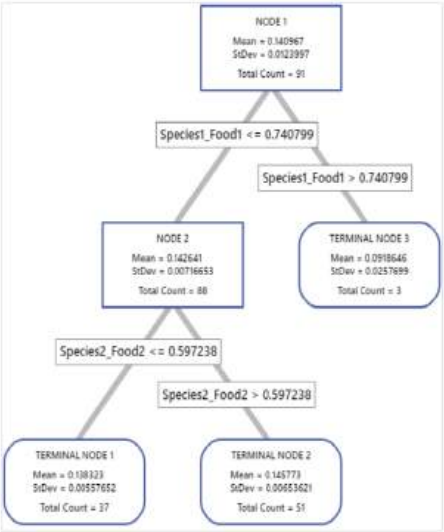

Figure 9. Trinodal CART tree showing non-linear thresholds for certain important variables. The values determine points where the ecosystem could perpetually destabilize.

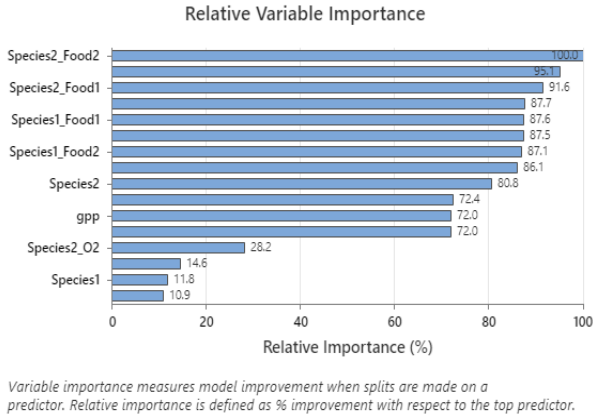

Figure 10. The graph covers the relative importance of simulative variables with respect to the top predictor of the functional diversity index.

K-Means Clustering Analysis

When representing complex ecological settings over multiple variable types, ensuring that the modeling systems are accurate and maximize representational utility is important. In terms of K-means clustering, it is crucial that the appropriate number of clusters is chosen to represent the data because issues like overfitting and underfitting can discard the data's applicability. Using a widely established method called the elbow method, which represents a point of diminishing returns, the optimal number of clusters identified was 3. When increasing the number of dimensions to practically large sets, the optimal cluster amount remained at 3, illustrating strength at that range for representation. In addition, the centroid aspect of this method was rudimentary for the intended type of centralized fluidity.

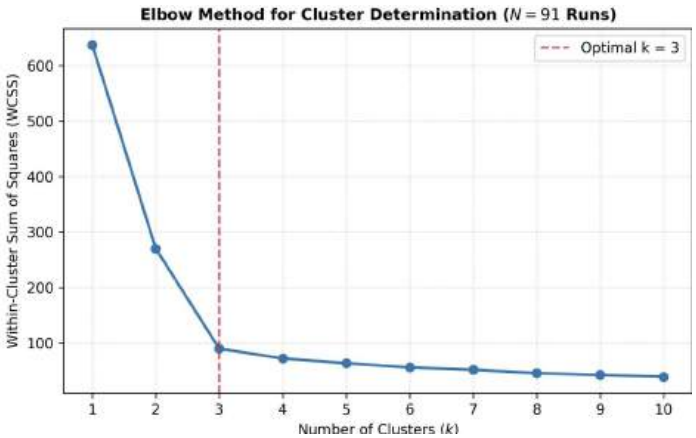

Figure 11. WCSS vs Number of Clusters (k). The elbow point indicates k=3 as the optimal amount.

Discussion

The results show that the FDI model is able to capture differences in modeled functional structure under the simulated conditions, which is based mainly on the consumption patterns of resources and O2-based productivity variables. Throughout the trials and simulation, it was seen that the index consistently was affected by the diet niche composition, which highlights how functional diversity seen in an ecosystem is based more on the ways organisms use resources rather than their sole species abundance.

Threshold-Driven Ecosystem Behavior

The CART analysis helped show how the ecosystem's responses were mainly following the nonlinear thresholds, but not the anticipated gradual changes. Also, when Species1_Food1 went over the value 0.74, the system behaved like a degradation regime in which it had a lower functional diversity in contrast to the high-performance regime achieved once Species2_Food2 was above the 0.60 value, subsequently giving the highest FDI value.

These results help support that the simulated ecosystem configurations can exhibit threshold transitions and distinct regimes within the FDI framework, where ecosystems can face volatility once those limits are reached. Traditional diversity indices, like the Shannon Diversity,

continuously measure the changes within a population's diversity and therefore may miss the nonlinear transitions outlined in our model. Therefore, the given FDI identifies numeric thresholds within the simulated framework that may warrant further investigation using empirical ecological data.

Our FDI and other Biodiversity Metrics

Current biodiversity indices focus on taxonomic richness measures and evenness, omitting the functional roles that a species plays in its habitat. For example, Rao's Quadratic Entropy does have trait differentiation considered by them, oftentimes treating it as stable or stationary characteristics rather than as a variable for resource usage.

Our FDI model combines the O2 consumption from GPP (gross primary productivity) with the dietary niche overlap, sourced from Pianka's Index. This new model helps connect the functional diversity of an ecosystem to the energy flow, which helps provide a holistic and also a more thorough understanding of ecosystem function when paired with the multiresource consumption that may also be prevalent.

Model Limitations

Although a randomized simulation procedure with 91 scenarios is widely regarded as statistically reliable, there are many limitations on what these results mean and can safely suggest based on the project prototype. Firstly, the method is purely an online simulation procedure over manually developed ecosystem ranges and attributes; thus, the findings here, although they may hint at broader truths, must be verified before real-life generalization. Concurring with that same idea, the model does not explicitly target more complex ecosystem functions like predation, reproduction, and migration, which hinders its usage in more surface-level contexts – contexts that don't have permissible access to oxygen and resource consumption rates. This is also seen in the fact that defining functionality by energy may narrow more complex ecological functions, like certain species being keystone or generalist populations. This means that further analysis is required to prove associations between the energy-weighted definitions and complex phenomena. In regards to limitations caused by the simulation assumptions, the most relevant is the fact that GPP and oxygen consumption are both simplified for traceability within the setting. That is, there is an assumed relationship between productivity and metabolism, which may be more complicated in real life.

Ecological Implications

The results of this study also suggest, within the simulated framework, FDI is more sensitive to how species use resources and their functional roles within the environment rather than to species abundance. Many traditional biodiversity indices, like the Shannon Diversity Index mentioned before, measure species richness and evenness, but may indicate that an ecosystem is diverse from the outside, but may not be functioning properly.

Being able to detect early on threshold shifts functionally, as seen in the CART analysis, scientists and ecologists could detect warning signs of an ecosystem about to collapse or degrade. In real life, these sudden changes in the functionalities of populations and species can help indicate the declining state of an ecosystem before species become endangered. With the current discussion of climate change, food security, and ecosystem stability, being able to detect the clear tipping point can help remove any ambiguity about when an ecosystem is about to fail.

Conclusion

Through multi-method statistical analysis and simulation testing, this study establishes a preliminary framework for evaluating functional diversity; under the tested and randomized conditions, the model variation was primarily driven by dietary resources rather than GPP dynamics. Additionally, the CART analysis illustrated that although variables may stay constant, their distribution among ecological populations is a key determinant in the functional diversity value. Moreover, the regression analysis clearly determined that the resource consumption variables had stronger associations with the functional diversity value, supporting the idea that dietary consumption is the most relevant factor. Additionally, as shown by the 91 random simulative distribution including the guiding base/max scenarios, the combination of Rao's Quadratic Entropy and Pianka's index jointly covered interspecies and intraspecies analysis, allowing for holistic ecosystem representation. This fact, in combination with the logical consistency and optimization model that demonstrates the functional diversity index's rudimentary behavior, supports the plausibility of using proposed frameworks to represent functional differences in increasingly complex ecosystems. Such models suggest ecological shifting between states rather than gradual change, which is a pattern that indices like the Shannon-diversity index overlook. Finally, the integration of K-means clustering used in marketing contexts into an ecological context allowed the model to be applied to various variable sets – sets that require multidimensional analysis and machine learning assistance. All in all, the empirical findings and algorithmic structures in this project may be vital in the contemporary era, classified by environmental degradation and biodiversity loss.

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The Effects of Achondroplasia on Quality of Life By Georgia Wei

Abstract

Achondroplasia is a heritable condition that affects human limb development. The condition is caused by a genetic mutation in the fibroblast growth factor receptor 3 (*FGFR3*) gene. This genetic mutation causes hyperactivity of the FGFR3 protein, which results in slowed bone growth. Many individuals with achondroplasia have disproportionate limb growth, resulting in shorter arms and legs but average-sized torsos. Numerous studies throughout have found achondroplasia to be associated with a lower quality of life. In this study, the quality of life of individuals with achondroplasia and those without it are compared using a modified Quality of Life in Short Stature Youth (QoLISSY) survey. The survey focused on physical, social, emotional, and future categories. From the results, the study found that individuals with achondroplasia report experiencing a lower overall quality of life when evaluated with a convenience comparison group, particularly in the social, physical, and future categories. These results suggest future treatment options to look more into targeting improvements in quality of life for individuals with achondroplasia in these categories specifically, including providing both physical and mental support.

Keywords achondroplasia, quality of life, survey, physical quality of life

Introduction

Achondroplasia is a form of skeletal dysplasia that affects bone growth and development. It is characterized by shorter limbs (arms and legs), smaller hands and feet, short stature, a larger head, and a flat nose (Cleveland Clinic). Along with these characteristics, individuals with achondroplasia may be more likely to develop breathing problems (such as sleep apnea), obesity, bowed legs, extra fluid in the brain, and hearing problems (McDonald and De Jesus). Globally, around 250,000 people are affected by achondroplasia (Waller et al.).

Achondroplasia is caused by mutations in the fibroblast growth factor receptor type 3 (*FGFR3*) gene, which plays a crucial role in bone development. The FGFR3 protein regulates bone growth by inhibiting the proliferation of cartilage cells, thereby restricting the length of bones (MedlinePlus). In people with achondroplasia, mutations in the *FGFR3* gene cause the receptor to become overactive, leading to excessive inhibition of bone growth, resulting in the characteristic short stature.

Over 99% of achondroplasia cases are caused by one of two mutations in *FGFR3*, both of which result in the same protein change (MedlinePlus). About 80% of cases result from spontaneous mutations, while the remaining 20% are inherited from a parent with the condition (McDonald and De Jesus).

Achondroplasia can be diagnosed through various methods, including a physical examination, prenatal examination, genetic testing, and other x-rays or imaging scans (Cleveland Clinic). Currently, there are no cures for achondroplasia. However, there are still many different

treatment options available targeted towards specific symptoms or other complications caused by achondroplasia. Current treatment options include surgical procedures to help with excess fluid in the brain, a narrower foramen magnum, a compressed spine, and bowing in the legs; and treatments to help with hearing problems (McDonald and De Jesus). For targeting the limbs specifically, limb-lengthening surgeries could be considered (Paley). There are also a few ongoing drug trials targeted towards increasing growth in children with achondroplasia. Currently, vosoritide is the first approved medication option for treating achondroplasia, which limits signaling from the mutated FGFR3 gene (McDonald and De Jesus).

Although achondroplasia has been characterized for over 50 years, there is still a lot to learn about this condition, including exploring treatment options that can further improve the wellbeing and livelihood of individuals with achondroplasia (Pauli). In this paper, how achondroplasia can affect individuals and their quality of life across social, physical, and emotional aspects is discussed in hopes to inform future treatment options.

Quality of life is used as a way to measure an individual's well-being through evaluations of one's physical, emotional, and social health. While there are numerous ways to measure quality of life, it is typically assessed through standardized questionnaires. Standardized questionnaires enable researchers to compare results to a reference population and draw conclusions on how social, economic, and cultural factors can significantly affect one's quality of life.

The burden of living with medical conditions can result in changes to individuals' quality of life. Achondroplasia is a condition that affects physical appearance through its impacts on bone growth, which could also impact the wellbeing of individuals. As a result, several studies have examined the quality of life of those affected by achondroplasia.

Literature Review

Quality of Life in Children with Achondroplasia and their Parents

One study conducted in Germany examined the quality of life in children with achondroplasia, as well as their parents. The children that participated in this study ranged from ages 5 through 14 and had a medical diagnosis of achondroplasia but did not have any other significant medical conditions that could affect the quality of life assessment (Witt et al.). They were recruited through the German Association for People of Short Stature and their Families. In the study, quality of life was measured through two standardized questionnaires. The first type of questionnaire was called the Pediatric Quality of Life Inventory version 4.0 (PedsQL 4.0) questionnaire, which was completed by children ages 8-14 or by the parents of children ages 5-7. The second type was called the Short-Form-8 (SF-8) questionnaire, which was completed by all parents of patients participating in the study.

The PedsQL 4.0 questionnaire is designed to evaluate the physical, emotional, social, and school functioning in children and adolescents, through either self-reported or parent-reported responses. The SF-8 questionnaire is used to measure parental quality of life by focusing on

overall health, physical activity limitations, daily work difficulty, bodily pain, energy levels, social activity limitations, and emotional difficulties.

In total, 73 families participated in this study, with 73 responses received from the parents and 47 responses received from the children. Based on the PedsQL 4.0 responses, it was found that children with achondroplasia experience a significantly lower total quality of life relative to the healthy reference population based on the reports of the parents (M = 63.70, SD = 15.83 compared to a reference value of 82.70, $t(72) = -10.25$, $p \leq .01$) and the children themselves (M = 73.76, SD = 18.04 compared to a reference value of 83.84, $t(46) = -3.83$, $p \leq .02$) (Witt et al.). From the SF-8 responses, it was found that parents in the study similarly experienced a lower quality of life mentally in comparison to the reference population (M = 46.51, SD = 10.22 compared to a reference value of 53.25, $t(72) = -5.64$, $p \leq .01$), though their quality of life physically was the same as the reference population's (M = 50.50, SD = 8.49 compared to a reference value of 50.30, $t(72) = .20$, $p = .85$) (Witt et al.).

Physical and Mental Health in Adults with Achondroplasia

A different study conducted in Portugal examined the quality of life of adults with achondroplasia, specifically focusing on their physical activity, physical health, and mental well-being (Alves et al.). There were a total of 16 adults with achondroplasia, ten women and six men, who participated in this study. The participants ranged from ages 20-57, and all of them were medically diagnosed with achondroplasia.

It was noted that across the participants, 88% had no children, 44% lived with parents, 69% had a university degree, 63% had a job, 31% had received limb-lengthening treatments, and 25% experienced sleep apnea. The body compositions of the participants were measured by a Tanita MC780-PMA bioelectrical impedance analyser (a tool that analyzes body composition through 31 collected measurements). The participants also completed various questionnaires, including a socio-demographic questionnaire, the Short Form-36 (SF-36) version 2, the Brief Symptoms Inventory (BSI), and the International Physical Activity Questionnaire (IPAQ). This study found there was a strong correlation between the participants' general health and their M_MET (moderate-intensity activities), with M_MET being a huge indicator of a participant's general health. However, with regards to mental well-being, they did not find a significant correlation between physical activity and mental health. Lastly, in comparison to the general population of Portugal, it was found that adults with achondroplasia typically experience lower health-related quality of life and greater psychological distress. Specifically, the SF-36 scores for pain were much lower for adults with achondroplasia (48.69 ± 13.8) versus the general population (71.44 ± 24.27), and it was found that adults with achondroplasia experienced phobic anxiety 40% higher, psychoticism 32% higher, and anxiety 20% higher compared to the general population.

Impacts of Achondroplasia on Health-Related Quality of Life

A study across multiple countries in Latin America (Argentina, Brazil, and Colombia) examined the quality of life in both children and adults. The 172 participants of this study were all diagnosed with achondroplasia (Llerena Jr. et al.). Of the participants in this study, 7% had undergone a limb-lengthening procedure.

Different questionnaires were sent to the children and adults, with the Pediatric Quality of Life Inventory (PedsQL), Quality of Life in Short Stature Youth (QoLISSY), Pediatric Functional Independence Measure (WeeFIM), and Adolescent Pediatric Pain Tool (APPT) used for children and the 5-level EQ-5D version (EQ-5D-5L), Nottingham Health Profile (NHP), Brief Pain Inventory-Short Form (BPI-SF), and Work Productivity and Activity Impairment (WPAI) used for adults. The questionnaires were completed by the participants themselves or by their parents if the patient was a minor.

From the results of this study, the QoLISSY scores for individuals with achondroplasia were on average between 60-70, which was much lower than the average score of 82 for individuals with average stature (Llerena Jr. et al.). Among these scores, participants scored the lowest in the physical and social categories. The WeeFIM scores also increased with age, suggesting that individuals with achondroplasia gain more independence as they age, which increases their quality of life. In regards to pain, over 50% of adolescent participants reported they experience pain in at least one site, and 10% of adult participants experience at least three pain sites. The EQ-5D-5L Visual Analogue Scale (VAS) scores of the participants were also lower than those of the reference population, but their overall health status was similar. The participants also had high NHP scores, suggesting they have poorer subjective health (measured based on the sleep, energy, mobility, pain, isolation and emotional responses of the participants) which was more severe for participants who had limb-lengthening procedures. Overall, individuals with achondroplasia have a lower quality of life and are linked to various complex medical conditions, pain, discomfort, and less independence.

There are many existing studies regarding quality of life in people with achondroplasia. However, current questionnaires for those with achondroplasia focus on aspects regarding one's size and height. These questions make it difficult to compare to a reference population due to their bias towards these aspects that predominantly affect those with achondroplasia. To circumvent this, this study was conducted to examine how having achondroplasia can impact an individual's quality of life across multiple aspects (social, emotional, physical, etc.), refraining from including questions that specifically measure an individual's feelings toward their height.

Methods and Materials

To better quantify the impact of achondroplasia on one's quality of life across multiple aspects, the Quality of Life in Short Stature Youth (QoLISSY) questionnaire (Bullinger et. al) was modified and used to develop a survey focused on social, physical, and emotional health, and prospects for the future. Survey topics from the modified survey came from the QoLISSY questionnaire, but any questions regarding height or size were removed to allow for better

comparison to those without achondroplasia (Appendix A). The modified survey was reviewed by a mentor.

Survey participants were recruited through the Achondroplasia (https://www.facebook.com/groups/61999603825/) and the Dwarfism Facebook Group (https://www.facebook.com/groups/dwarfismworld) as well as broadcasting to peers and colleagues. All survey responses were completed between July and September of 2025. Both those with and without achondroplasia were encouraged to complete the survey. The participants were aware that participation was voluntary and that all survey responses would be anonymous, collected through an online Google Form. As the surveys were anonymous, names, location, or other personal identifying information were not collected to ensure confidentiality. The survey consisted of 12 quality of life questions and 4 demographic questions (Appendix A). The quality of life questions asked participants to respond how frequently they experienced the statements in the past 30 days using a Likert scale, a tool used to quantify the beliefs and experiences of others using a scale. The Likert scale used was from 1 to 5, with 1 representing an individual who never experiences the statement, and 5 representing an individual who experiences the statement very often. All quality of life questions except for one were positively worded, with a higher score suggesting a higher quality of life. As question 1 "I get teased and bullied by others" is phrased negatively, negative reverse scoring was used to ensure that higher mean scores across all quality of life survey questions suggest a higher quality of life. For responses that were left blank, overall mean scores were calculated for those questions using the remaining responses. The 4 demographic questions collected data regarding the participant's age, race/ethnicity, current gender identity, and whether or not they are diagnosed with achondroplasia. For the questions regarding race/ethnicity and current gender identity, the participants had the option to choose "Prefer not to answer".

In order to see whether the collected data is significant, two-tailed *t*-tests were run. A significance threshold of $p \leq 0.05$ was used.

Results

46 people completed the survey, though one person was excluded due to uncertainty in achondroplasia diagnosis. Of the remaining 45 participants, 24 (53.3%) have been diagnosed with achondroplasia, 34 (75.6%) identify as women, and 28 (62.2%) are white (Fig 1). The age of participants ranged from 5 to 61, with a mean age of 35.19 ± 15.96 for participants without achondroplasia ($n = 21$, 95% Cl [28.36, 42.02]) and 31.17 ± 13.83 for participants with achondroplasia ($n = 24$, 95% Cl [25.63, 36.70]) (Fig 2). The majority of participants completed the survey themselves, but anyone under the age of 10 had a caregiver complete the survey on their behalf.

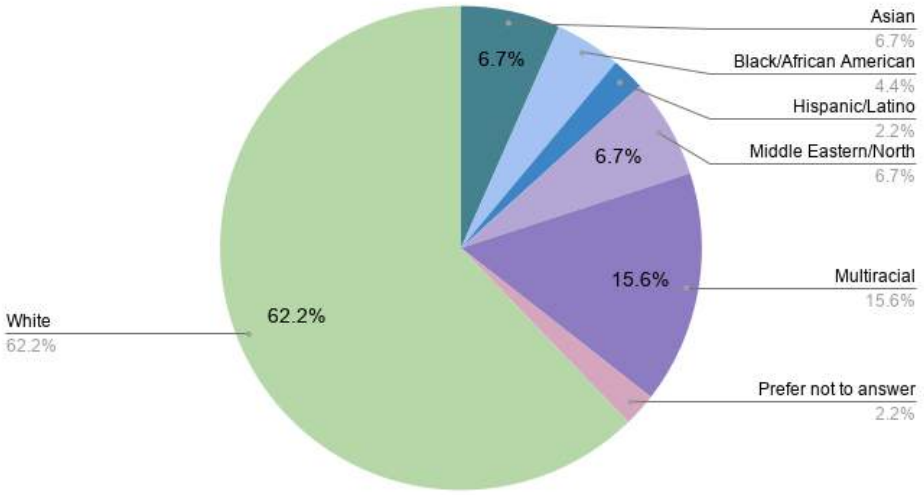

Fig 1. The self-reported racial/ethnic background of study participants.

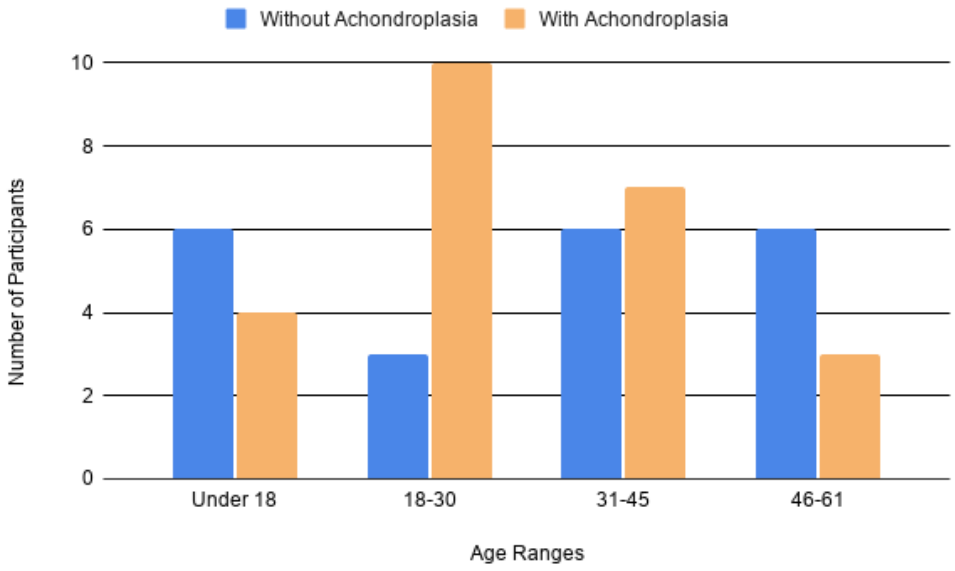

Fig 2. The age of participants from this study represented by different age ranges, separated by participants with and without achondroplasia.

Across all quality of life questions, the average scores of participants without achondroplasia from each category ranged from 3.87 to 4.46 with a mean score of 4.21 ± 0.85 (95% Cl [4.01, 4.42]), while average scores of participants with achondroplasia from each category ranged from 3.33 to 3.49 with a mean score of 3.42 ± 1.02 (95% Cl [3.32, 3.67]). The mean scores of participants without achondroplasia were also greater than the mean scores for those with achondroplasia across each question individually (Appendix B).

To get a better understanding of which aspects of quality of life most affected those with achondroplasia, the survey questions were grouped into four categories: social, physical, emotion, and future (Appendix A). Those with achondroplasia reported a lower score for each category, with the greatest difference in physical quality of life aspects (score difference of 0.99, $p \leq 0.0001$) (Fig 3).

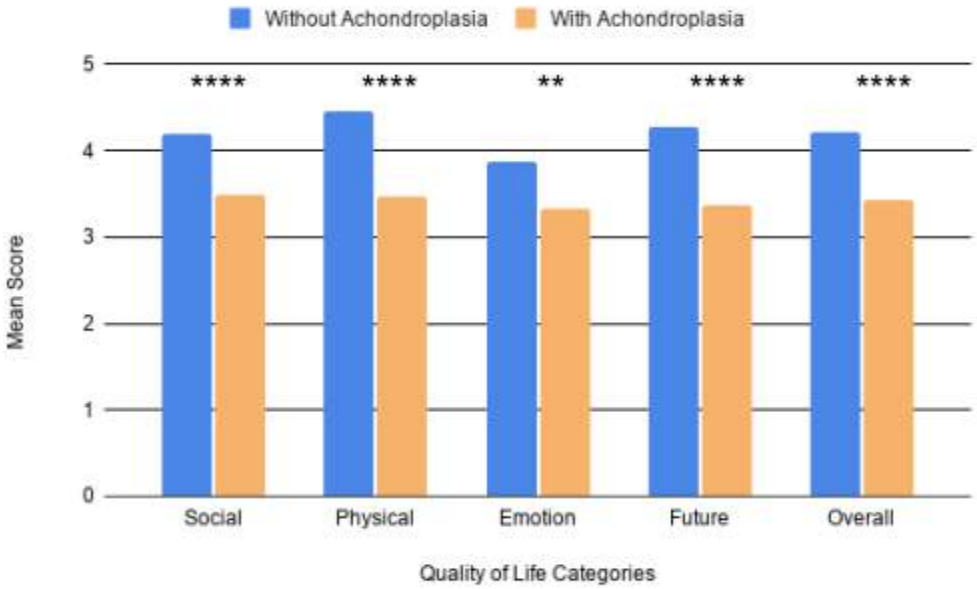

Fig 3. The mean scores of participants across the four assessed quality of life categories and across all quality of life questions overall, separated by participants with and without achondroplasia. Two-tailed unpaired *t*-test, **: $p \leq 0.01$, ****: $p \leq 0.0001$.

When broken down by individual questions, those with achondroplasia reported the greatest difference in quality of life scores in questions regarding energy (difference of 1.13, $p \leq 0.001$) and clothing (difference of 1.29 , $p \leq 0.0001$) (Fig 4). In contrast, the difference in scores between participants with and without achondroplasia were the smallest for the questions regarding confidence and appearance satisfaction (difference < 0.5, $p > 0.05$) (Fig 4).

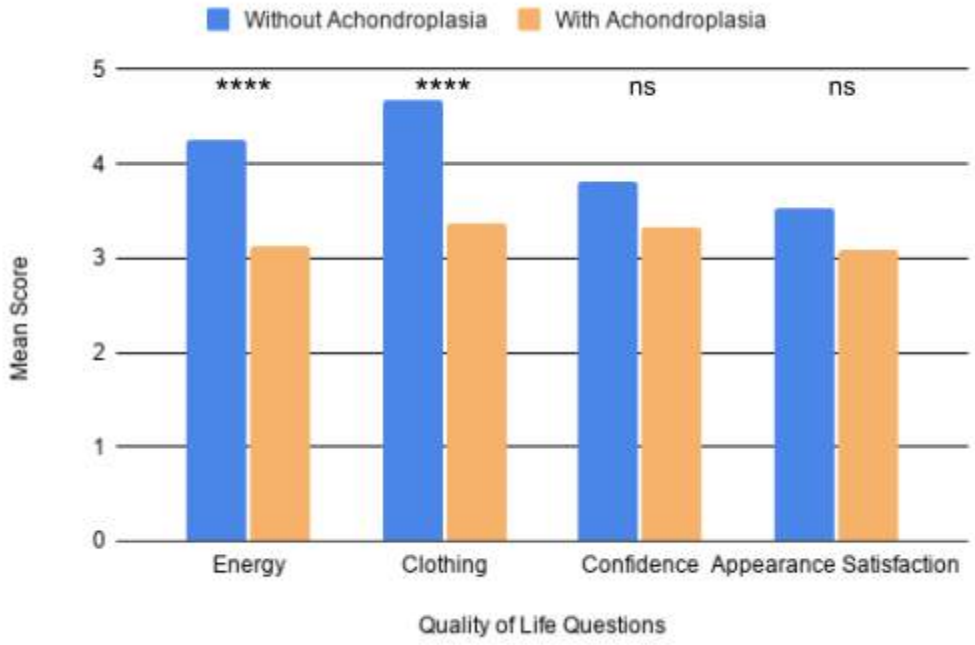

Fig 4. The mean scores of participants for the specific quality of life questions regarding energy, clothing, confidence, and appearance satisfaction, separated by participants with and without achondroplasia. Two-tailed unpaired *t*-test, ***: $p \leq 0.001$, ****: $p \leq 0.0001$, ns (not statistically significant): $p \geq 0.05$.

Discussion

The responses from this survey reveal a lower quality of life (QoL) for participants with achondroplasia in comparison to participants without. The results of the study suggest that out of the areas included in the survey, achondroplasia most heavily impacts an individual's quality of life physically, their ability to find appropriate clothing, and their ability to have enough energy to fulfill daily tasks.

However, although the smallest difference in scores were found in the questions about confidence and appearance satisfaction (difference of 0.48, $p > 0.05$ for confidence; difference of 0.44, $p > 0.05$ for appearance satisfaction), this does not suggest that people with achondroplasia have high levels of confidence and appearance satisfaction. Instead, with mean scores of 3.33 ± 1.09 for the question about confidence and 3.08 ± 1.14 for the question about appearance satisfaction from participants with achondroplasia, the small difference in scores may be attributed to random variation, alongside the lower scores for these two questions from participants without achondroplasia as well (mean scores of 3.81 ± 0.81 for confidence and 3.52 ± 0.75 for appearance satisfaction). This suggests the possibility that both people with and without achondroplasia can have similar ratings of confidence and satisfaction with their appearance.

Some limitations of this study include that it had a small sample size and targeted recruitment to an achondroplasia support group and local community. By targeting a support group, the results could be skewed towards those more severely affected by the condition.

Additionally, the reference population was more geographically limited. Differences in age distribution (Fig 2.) could have skewed results as well, as different age groups will answer and interpret questions differently due to differences in their social roles, independence, and daily activities.

In addition to improving studies, current treatment options are still limited in their accessibility and success at increasing quality of life, which is why actionable changes are needed to improve the quality of life of people with achondroplasia while waiting for better treatment options. Some strategies include education surrounding achondroplasia, allowing for increased inclusivity in both schools and workplaces, confidence building, and body positivity. Additionally, providing accommodations for physical activities, particularly in school-aged children, and making the surrounding school and/or work environment more accessible could drastically improve individuals' quality of life. On a grander scale, there is a need for increased diversity in clothing sizes, allowing for more options when picking out clothes. Together, these efforts can bring about positive changes for those with achondroplasia and improve their quality of life.

Conclusion

The results of this study are consistent with other published quality of life studies (Witt et al.; Alves et al.; Llerena Jr. et al.). Together, these studies suggest that having achondroplasia can lead to a lower quality of life across different aspects (social, physical, emotional, future).

Moving forward, studies need to expand the participant pool and diversity of participants in order to better understand how an achondroplasia diagnosis impacts quality of life across larger regions and age groups, and in combination with other sociodemographic factors. Additionally, future studies could explore how novel or existing treatment options may improve a patient's quality of life, providing a better understanding of what the needs are for those with achondroplasia.

Conflict of Interest

The author declares that there are no conflicts of interest related to this work.

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A Methodological Reassessment of HK2, LDHA, and PKM Expression and Overall Survival in the CPTAC Glioblastoma Cohort By Heesoeng Kang

Abstract

Glioblastoma is an aggressive brain tumor with substantial metabolic reprogramming. This retrospective study tested whether expression of three glycolytic genes—HK2, LDHA, and PKM—was associated with overall survival in the independent CPTAC 2021 glioblastoma cohort. Expression data from 99 tumors and complete survival data from 97 patients were evaluated using correlation analysis, Mann-Whitney U tests, Kaplan-Meier curves, log-rank tests, and continuous Cox proportional hazards models. Age-adjusted p-values were corrected for three candidate genes. The transcripts were positively correlated, but median-split analyses identified no survival difference for any gene. Continuous HK2 expression was nominally associated with poorer survival before and after age adjustment (adjusted hazard ratio 1.327, 95% confidence interval 1.026–1.715, p = 0.031). The proportional hazards assumption was not violated, but the association did not remain significant after Bonferroni or false-discovery-rate correction (adjusted p = 0.093). LDHA and PKM showed no consistent association with survival; an isolated LDHA quartile result did not remain significant after correction across the three genes. Continuous modeling recovered a nominal signal that categorical thresholds obscured, while multiplicity correction showed that the evidence remained fragile. Larger cohorts with fuller clinical annotation and protein- or isoform-level validation are needed before HK2 can be considered an independent prognostic biomarker.

Keywords

Glioblastoma; aerobic glycolysis; HK2; LDHA; PKM; overall survival; Cox regression; CPTAC; transcriptomics; multiple testing; retrospective study.

1. Introduction

Glioblastoma (GBM) is an aggressive, rapidly progressing Grade 4 malignant brain tumor characterized by high lethality and poor clinical outcomes. Recently, molecular-based analysis and comprehensive understanding of this malignancy have advanced, driven by large-scale genomic profiling initiatives such as The Cancer Genome Atlas (TCGA) and the pioneering framework established by the TCGA Research Network including the foundational work of The Cancer Genome Atlas Research Network (1061). . These genomic efforts have shifted the paradigm of neuro-oncology from purely histological classifications to intricate molecular and transcriptomic characterizations.

Although standardized therapeutic interventions—including maximal safe surgical resection, radiotherapy, and temozolomide (TMZ) chemotherapy—are widely utilized as the standard of care, the clinical prognosis for GBM patients remains poor, with high mortality and rapid tumor recurrence. Consequently, metabolic reprogramming has been increasingly recognized as a hallmark of cancer and a potential avenue for novel therapeutic targeting, as it

may support and accompany the survival, proliferation, and adaptive growth of malignant glioma cells within the tumor microenvironment.

To facilitate rapid cellular duplication and biomass synthesis, glioblastoma cells undergo a metabolic shift known as the Warburg effect, in which glucose is preferentially metabolized through aerobic glycolysis even when oxygen is available. This pathway supports rapid ATP production and supplies intermediates for biosynthesis. Glycolytic enzymes including Hexokinase 2 (HK2), Lactate Dehydrogenase A (LDHA), and Pyruvate Kinase M (PKM) have been implicated in tumor growth, invasion, and treatment resistance (Valvona et al. ; Wolf et al.). Glycolytic alterations and their upstream regulators have also been studied across glioma grades (Liu et al. ; Xi et al.).

Previous retrospective work using the REMBRANDT dataset (GSE108476) linked higher HK2 and PKM2 expression with shorter overall survival (Stanke et al.). The same study separately used TCGA data to compare transcript expression between GBM and lower-grade glioma. Its survival analysis did not adjust for age, an established prognostic factor in glioblastoma, and it focused partly on the PKM2 isoform, whereas the present RNA-sequencing dataset measures total PKM transcript abundance. These differences leave open whether the reported survival associations are reproduced in another cohort after age adjustment.

To address these limitations, this study analyzes an independent cohort from the CPTAC 2021 Glioblastoma dataset. The primary objective was to test whether HK2, LDHA, and total PKM transcript expression were associated with overall survival after accounting for age, and to examine how the result changed across categorical and continuous models. All three genes were treated as parallel candidate tests, with correction for multiple comparisons.

2. Materials and Methods

2.1 Data Source and Preprocessing

Clinical and transcriptomic profiles were obtained from the CPTAC 2021 Glioblastoma cohort (Wang et al.), accessed through cBioPortal for Cancer Genomics (Cerami et al. ; Gao et al.). The original dataset comprised 99 patients.

The files (data_clinical_patient.txt, data_clinical_sample.txt, and data_mrna_seq_fpkm.txt) contained metadata lines preceded by a '#' character and were processed with an automated script that dynamically calculated the number of header rows rather than relying on hard-coded indices.

Patient-level clinical data and sample-level identifiers were linked through 'PATIENT_ID', assembling AGE, SEX, VITAL_STATUS, and SAMPLE_ID in a unified matrix. Among 44,963 quantified genes, HK2, LDHA, and PKM were selected by the HUGO symbol. The expression matrix was transposed from a gene-by-sample to a sample-by-gene structure and merged with the clinical dataset through the unique 'SAMPLE_ID' key.
Survival information was recorded in 'PATH_DIAG_TO_DEATH_DAYS' and 'PATH_DIAG_TO_LAST_CONTACT_DAYS'. These fields were recoded as 'TIME_DAYS'

for overall survival time and 'EVENT' for vital status. Two patients with missing values for both 'TIME_DAYS' and 'VITAL_STATUS' were excluded, leaving 97 patients in the survival cohort. Because upper-quartile-normalized FPKM (FPKM-UQ) expression values spanned several orders of magnitude, values were transformed as log2(value + 1). This monotonic transformation preserved rank order while stabilizing variance for statistical analysis.

2.2 Statistical Analysis

Pairwise Pearson correlation coefficients were calculated across all 99 tumors with expression data to evaluate co-expression among HK2, LDHA, and PKM. Survival analyses used the 97 patients with complete survival information. Patients were stratified as living or deceased, and log2-transformed FPKM-UQ values were compared with the non-parametric Mann-Whitney U test. This cross-sectional comparison does not account for differences in follow-up time and is descriptive rather than primary evidence of prognostic association. Candidate covariates were screened with an independent t-test for age and a chi-square test for sex, each assessed against vital status. Age, the only variable meeting the univariate screening threshold, was included in the multivariable model. This data-driven covariate-selection approach is a pragmatic limitation rather than a prespecified clinical justification because other established GBM prognostic factors, including IDH mutation status, MGMT promoter methylation, and extent of resection, were unavailable in the extracted clinical fields.

For an exploratory survival analysis, patients were dichotomized into high- and low-expression groups based on the median expression level of each gene, and differences in survival outcomes were evaluated using Kaplan-Meier survival curves and the log-rank test. As the primary analysis, gene expression levels were utilized as continuous variables to conduct univariable and age-adjusted multivariable analyses using Cox proportional hazards regression models, thereby mitigating the loss of statistical power associated with median-split dichotomization. Because three candidate genes were tested in the primary Cox models, Bonferroni and Benjamini-Hochberg false-discovery-rate (FDR) corrections were applied to the age-adjusted p-values to account for multiple comparisons. The proportional hazards assumption for each covariate was assessed using Schoenfeld residuals, testing for a significant correlation between scaled residuals and event time. Additionally, as a sensitivity analysis to assess differences in survival outcomes across extreme expression profiles, Kaplan-Meier curves and log-rank tests were applied to compare the upper and lower quartiles (top 25% vs. bottom 25%) of gene expression with respect to overall survival. All statistical tests were two-sided, and significance was defined as $p < 0.05$ prior to multiple-testing correction. Statistical computing was executed using Python packages, including pandas (version 2.2.2), SciPy (version 1.16.3), and lifelines (version 0.30.3).

2.3 AI Assistance Disclosure

Generative AI tools (Claude, Anthropic; Gemini, Google) were used to assist with drafting and troubleshooting data-processing and statistical analysis code and to provide editorial

feedback on manuscript prose. The author selected the research question, designed the analysis, reviewed the AI-assisted code, verified numerical outputs by re-deriving key statistics, and took full responsibility for the scientific interpretations and conclusions.

3. Results

3.1 Baseline Characteristics of the Survival Cohort

Of the 99 tumors in the CPTAC 2021 Glioblastoma cohort, 97 had complete survival information and were included in survival analyses. Among these patients, 62 were deceased and 35 were living at the last clinical contact. Mean age was 60.4 years in the deceased group and 53.1 years in the living group ($p = 0.0054$). Age was therefore included as an adjustment variable; however, selecting a covariate from its association with vital status was data-driven and remains a limitation. Sex was not associated with vital status in this cohort (chi-square $p = 1.0$) (Table 1).

Characteristic	Value	n
Survival analytic cohort	—	97
Vital status: Deceased	—	62
Vital status: Living	—	35
Mean age, deceased (years)	60.4	62
Mean age, living (years)	53.1	35
Sex: Male	—	54
Sex: Female	—	43
Follow-up time, median (IQR), days	390 (238–721)	97
Follow-up time, range, days	0–1107	97

Table 1. Baseline Characteristics of the CPTAC 2021 Glioblastoma Survival Cohort

3.2 Co-expression Analysis of Glycolytic Genes

Pairwise Pearson correlations were calculated across all 99 tumors with expression data to characterize co-expression among HK2, LDHA, and PKM (Figure 1). HK2 and LDHA were strongly correlated ($r = 0.742$, $p = 1.6 \times 1/10^{18}$), as were LDHA and PKM ($r = 0.671$, $p = 3.1 \times 1/10^{14}$); HK2 and PKM showed a moderate positive correlation ($r = 0.554$, $p = 2.7 \times 1/10^{9}$). These transcript-level correlations establish co-expression in this cohort, but they do not by themselves demonstrate coordinated metabolic activity.

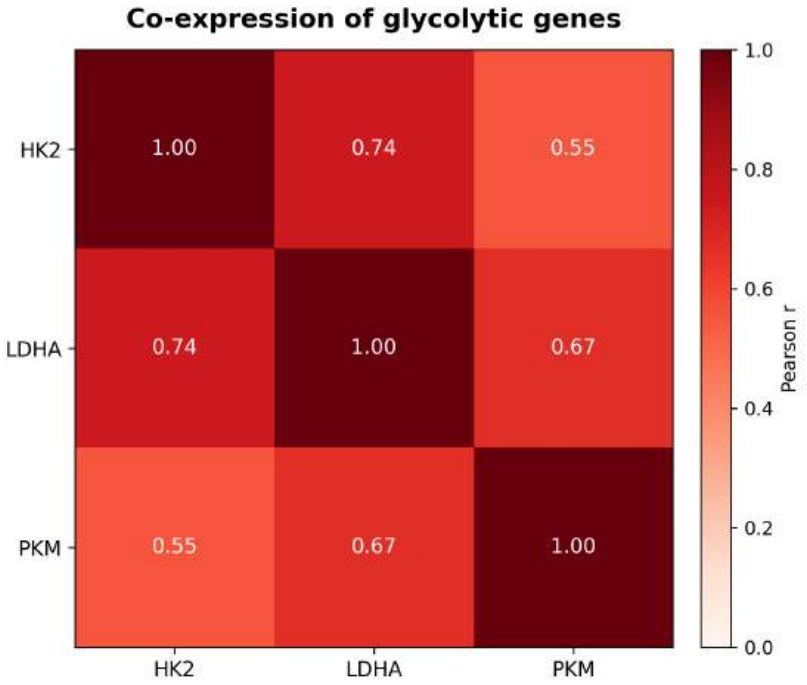

Figure 1. Pairwise Pearson correlation coefficients were calculated using $\log_2$(FPKM-UQ + 1)-transformed expression values from 99 tumor samples. The correlations were r = 0.742 for HK2–LDHA, r = 0.671 for LDHA–PKM, and r = 0.554 for HK2–PKM.

3.3 Differential Gene Expression Between Vital Status Subgroups

Expression levels were compared between living and deceased patients using Mann-Whitney U tests. Deceased patients had numerically higher expression of all three genes, with uncorrected p-values of 0.024 for PKM, 0.044 for LDHA, and 0.053 for HK2 (Figure 2). Because living patients were censored at last follow-up rather than confirmed as long-term survivors, this cross-sectional comparison does not account for follow-up time and is presented descriptively, not as primary evidence of prognostic association.

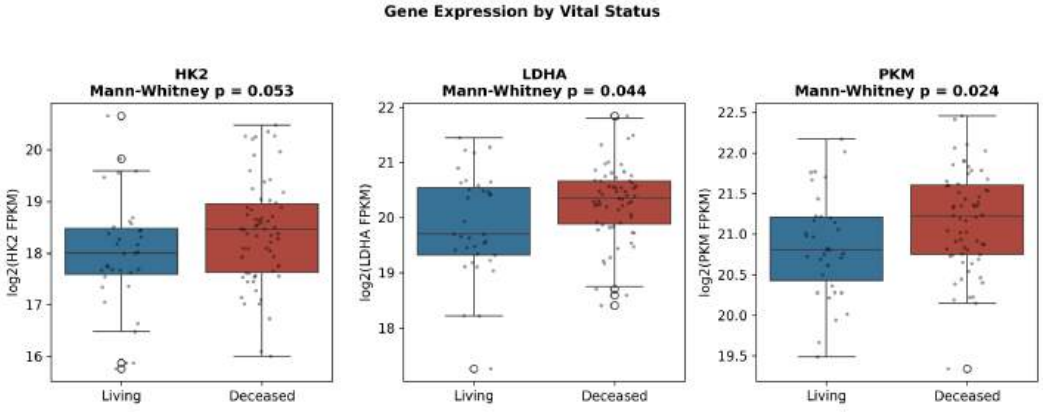

Figure 2. The analysis included 97 patients with complete survival data: 35 patients were alive at the last follow-up, and 62 were deceased. Gene-expression values were transformed using $\log_2$(FPKM-UQ + 1), and differences

between the two groups were evaluated using two-sided Mann–Whitney U tests. Each point represents the expression value of one patient. The center line represents the median, the box represents the interquartile range (25th–75th percentiles), and the whiskers represent values within 1.5 times the interquartile range. The deceased group showed numerically higher median expression of all three genes. The unadjusted p-values were 0.053 for HK2, 0.044 for LDHA, and 0.024 for PKM. Although the unadjusted p-values for LDHA and PKM were below 0.05, these findings should not be considered definitive evidence because three genes were evaluated in parallel and the comparison did not account for follow-up time or censoring.

3.4 Exploratory Survival Analysis via Kaplan-Meier Curves and Log-Rank Tests

To explore the prognostic significance of the target glycolytic genes using conventional clinical stratifications, patients were stratified into high- and low-expression subgroups based on the median expression level of each gene. Overall survival outcomes between these subgroups were subsequently evaluated using Kaplan-Meier survival curves and log-rank tests (Figure 3).

Under median-split dichotomization, none of the three genes was associated with overall survival by the log-rank test: HK2 p = 0.182, LDHA p = 0.792, and PKM p = 0.106. The result illustrates how a single threshold can discard information contained in continuous expression values; it does not establish the absence of a nonlinear or smaller association.

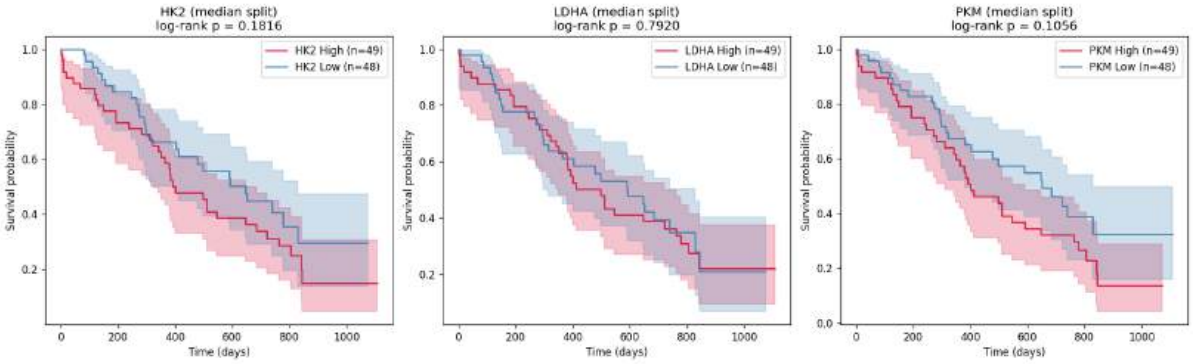

Figure 3. Kaplan–Meier curves compared overall survival between high- and low-expression groups defined by the median expression of each gene (high, n = 49; low, n = 48). No significant survival differences were observed for HK2 (p = 0.182), LDHA (p = 0.792), or PKM (p = 0.106) using log-rank tests. Shaded areas represent 95% confidence intervals.

3.5 Cox Proportional Hazards Regression Analysis

To reduce the information loss caused by median splitting, gene expression was modeled as a continuous variable in Cox proportional hazards regressions (Table 2). In the univariable model, HK2 was nominally associated with poorer overall survival (HR = 1.299, 95% CI: 1.019–1.655, p = 0.035). Each one-unit increase in log2-transformed HK2 expression corresponded to a 29.9% higher estimated hazard. LDHA (HR = 1.342, 95% CI: 0.960–1.877, p = 0.085) and PKM (HR = 1.331, 95% CI: 0.898–1.971, p = 0.154) were not statistically significant.

To assess whether the association of HK2 with survival persists after accounting for age, an age-adjusted multivariable Cox regression model was implemented. Within this multivariable framework, patient age was itself associated with a statistically significant increase in hazard,

with an HR of 1.029 (95% CI: 1.007–1.052, p = 0.0101) per year. After adjustment for age, HK2 expression remained nominally associated with adverse overall survival, with an HR of 1.327 (95% CI: 1.026–1.715, p = 0.031) (Figure 4). The age-adjusted HK2 association was nominally significant before multiple-testing correction (p = 0.031) but was not statistically significant after either Bonferroni correction (adjusted p = 0.093) or FDR correction (q = 0.093). The proportional hazards assumption was assessed via Schoenfeld residuals and was not violated for either the HK2 term (Spearman correlation with event time, p = 0.62) or the age term (p = 0.84) in this model, indicating that the Cox model's structural assumption was reasonably satisfied even though the corrected association fell short of statistical significance. Taken together, these findings indicate that HK2 shows a nominally significant, assumption-consistent association with overall survival after age adjustment, but this association should be interpreted as suggestive rather than confirmatory given the modest cohort size and the burden of multiple testing.

Gene	Univariable HR (95% CI)	Univariable p	Age-adjusted HR (95% CI)	Raw adjusted p	Bonferroni p	FDR q
HK2	1.299 (1.019–1.655)	0.035	1.327 (1.026–1.715)	0.031	0.093	0.093
LDHA	1.342 (0.960–1.877)	0.085	1.347 (0.954–1.903)	0.091	0.273	0.097
PKM	1.331 (0.898–1.971)	0.154	1.422 (0.939–2.154)	0.097	0.291	0.097

Table 2. Univariable and age-adjusted Cox proportional hazards models for glycolytic-gene expression and overall survival, with correction for three candidate genes

Note: HR = hazard ratio per one-unit increase in log2-transformed expression; CI = confidence interval. Raw age-adjusted p-values were corrected across the three candidate genes. No gene remained significant after Bonferroni or Benjamini-Hochberg false-discovery-rate correction.

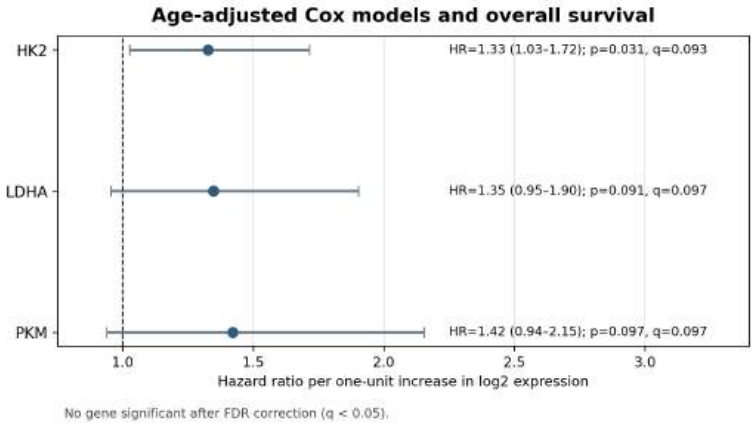

Figure 4. Forest plot of age-adjusted Cox proportional hazards models for continuous gene expression. Points represent hazard ratios per one-unit increase in $\log_2$ expression, horizontal lines represent 95% confidence intervals, and the dashed line indicates HR = 1. HK2 was nominally associated with increased mortality risk before correction (p = 0.031), but no gene remained significant after FDR correction.

3.6 Sensitivity Analysis via Quartile Stratification

To further investigate the impact of extreme expression profiles and assess the sensitivity of the dichotomized survival models, overall survival outcomes were re-evaluated by stratifying

patients into the upper quartile (top 25%, high expression) and the lower quartile (bottom 25%, low expression) for each gene (Figure 5).

In the extreme-quartile analysis, HK2 ($p = 0.192$) and PKM ($p = 0.349$) were not associated with overall survival. LDHA yielded a log-rank p-value of 0.025, with poorer survival in the highest quartile. Because this result arose from an exploratory threshold analysis and was not supported by the median-split or continuous models, it should be treated as hypothesis-generating. It did not remain statistically significant after Bonferroni correction across the three genes (adjusted $p = 0.076$).

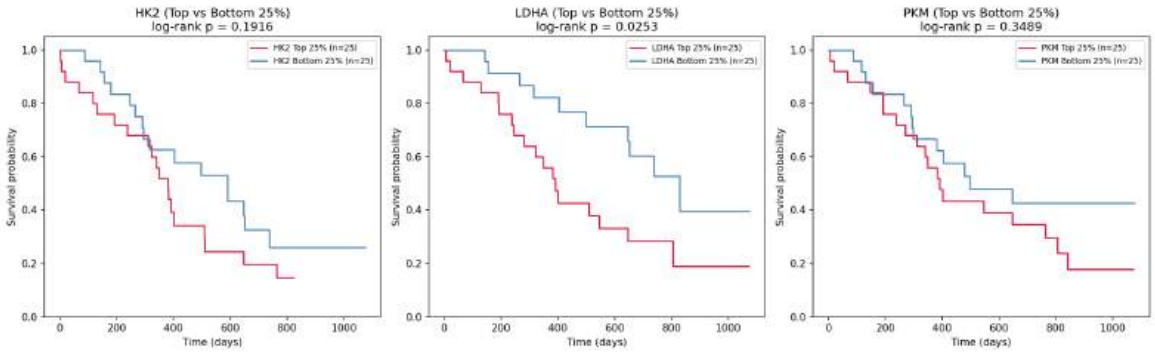

Figure 5. Kaplan–Meier curves compared overall survival between the upper and lower expression quartiles for each gene (n = 25 per group). LDHA showed an unadjusted log-rank p-value of 0.025, but this result was not significant after Bonferroni correction for three genes (adjusted $p = 0.076$). No significant differences were observed for HK2 or PKM.

4. Discussion

4.1 Summary of Findings and Methodological Implications

The apparent association between HK2 expression and overall survival changed with the analytic specification. Median and extreme-quartile splits did not identify an HK2 survival difference. Continuous Cox models did recover a nominal association before and after age adjustment ($p = 0.035$ and $p = 0.031$, respectively), and the proportional hazards assumption was not violated for the HK2 term. Correction for three candidate genes then changed the inferential status of the finding: both the Bonferroni-adjusted p-value and the FDR q-value were 0.093. The association is therefore suggestive, not confirmatory. The contrast between models makes a narrower methodological point. Dichotomization can obscure information, but continuous modeling does not eliminate the need for multiplicity control, adequate sample size, or replication.

4.2 Biochemical Plausibility of an HK2-Mediated Effect

The HK2 result is biologically plausible, but plausibility does not convert a nominal association into validation. HK2 catalyzes the first step of glucose metabolism by phosphorylating glucose to glucose-6-phosphate and has been linked experimentally to aerobic

glycolysis and tumor growth in glioblastoma (3). Its mitochondrial interactions may also connect metabolism with resistance to apoptosis. These mechanisms provide a rationale for further study; they do not show that transcript abundance is causal or clinically predictive in the present cohort.

4.3 Explaining the Lack of Prognostic Significance in LDHA and PKM and StratificationSensitivity

Neither LDHA nor PKM was significantly associated with survival in the continuous Cox models. Several explanations remain possible, including limited power, nonlinear effects, post-transcriptional regulation, and measurement of total rather than isoform-specific expression. The present data cannot distinguish among these explanations, and pathway position alone does not establish why one transcript did or did not show a prognostic association.

Bulk RNA sequencing measures transcript abundance rather than protein abundance or enzymatic activity. Total PKM expression also combines biologically distinct PKM isoforms, including PKM1 and PKM2 (Christofk et al. 230–33) . This analysis therefore cannot determine whether an isoform-specific or protein-level signal was masked by aggregation at the gene level.

The isolated LDHA quartile result should be read with the same restraint. It was not reproduced by the median-split or continuous analyses, and its dependence on one threshold makes it sensitive to sampling variation. It may reflect a nonlinear extreme-expression effect, threshold sensitivity, or one positive result among several exploratory comparisons. A Bonferroni correction across the three genes gave an adjusted p-value of 0.076, above the 0.05 threshold.

4.4 Relationship to Prior Literature

This study re-evaluates the retrospective framework reported by Stanke et al. (6), who associated glycolytic-gene expression with adverse outcomes in the REMBRANDT dataset (GSE108476). A directionally consistent, nominal HK2 association in the separate CPTAC cohort adds limited evidence of reproducibility, but it does not establish HK2 as a validated biomarker.

The principal contribution is methodological. Compared with the REMBRANDT survival analysis, this study models expression continuously, adjusts for age, tests the proportional hazards assumption, and corrects for three parallel candidate genes. Those decisions make the result less dramatic but more accurately bounded: the direction of the HK2 estimate was stable after age adjustment, while its statistical support was fragile after correction.

4.5 Study Limitations and Future Directions

Several limitations must be acknowledged. First, the age-adjusted HK2 association did not survive correction for three candidate genes and should be regarded as a nominal, hypothesis-generating signal rather than a confirmed independent biomarker. Second, the multivariable model adjusted only for age, which was selected through univariate screening rather than prespecified on clinical grounds. Established prognostic factors such as IDH mutation

status, MGMT promoter methylation, extent of surgical resection, and treatment regimen were unavailable, so residual confounding cannot be excluded. Third, the CPTAC 2021 cohort was modest in size (n = 97, 62 events), and the descriptive living-versus-deceased comparison did not account for differential follow-up. Fourth, the proportional hazards assumption was assessed by a residual-correlation approach rather than a full Grambsch-Therneau scaled-residual test, and linearity of the log-hazard relationship was not formally evaluated with methods such as restricted cubic splines. Finally, this retrospective analysis of public data lacked direct experimental and protein-level validation; mechanistic wet-lab experiments, including functional in vitro knockdown or in vivo pharmacological inhibition of HK2, could not be conducted. Larger multicenter cohorts with complete clinical annotation, formal diagnostic testing, and functional laboratory validation are needed before HK2 can be considered a clinically actionable prognostic marker.

Additionally, the current study evaluated three candidate transcripts separately rather than using a prespecified composite glycolysis-pathway score. Therefore, it may fail to capture pathway-level dysregulation distributed across several glycolytic genes and regulatory factors. Future studies should select the gene set, weighting method, and decision threshold before examining survival outcomes and validate the resulting score in an independent cohort. Moreover, this study was limited to RNA expression data. Transcript abundance does not necessarily reflect protein expression, post-translational regulation, enzymatic activity, or actual metabolic activity. The study also cannot distinguish PKM1 from PKM2 by measuring only total PKM expression. Therefore, matched proteomic, phosphoproteomic, isoform-specific expression, and metabolomic data should be integrated to determine whether the nominally observed HK2 signal can be reproduced at functionally meaningful molecular levels.

Lastly, the association observed in this study was not formally validated in an independent cohort. Comparison with the REMBRANDT study results cannot be considered external validation because the two studies used different expression measurement methods, covariate-adjustment strategies, and statistical models. To establish the generalizability and clinical utility of the HK2 finding, future studies should apply the same preprocessing procedures, continuous Cox model, covariates, and multiple-testing correction methods to an independent cohort and determine whether the result can be reproduced. Expression of HK2, LDHA, and PKM should not be interpreted as a direct measure of overall glycolytic activity because three transcript measurements cannot capture the coordinated enzyme activity or metabolic flux of the entire pathway.

5. Conclusion

The signal was consistent in direction, but it was not conclusive. In the CPTAC cohort, higher HK2 expression was nominally associated with poorer survival after age adjustment (HR =1.327, p = 0.031), yet the association did not survive correction for three candidate genes (adjusted p = 0.093). LDHA and PKM showed no consistent survival association, and the isolated LDHA quartile result remains exploratory. The main conclusion is methodological:

model choice altered the apparent result, and multiple-testing correction altered its inferential status. Larger cohorts with fuller clinical annotation, protein- or isoform-level measurements, and prespecified analyses are required before any claim of clinical utility is justified.

Data Availability

The clinical and transcriptomic data underlying this study are publicly available from cBioPortal for Cancer Genomics (https://www.cbioportal.org/), CPTAC 2021 Glioblastoma study. The processed, de-identified analytic dataset generated for this study is available from the author upon reasonable request.

Code Availability

The analysis code used for data preprocessing, statistical tests, and figure generation is available from the author upon reasonable request

Ethics Statement

This study used publicly available, de-identified patient data obtained from the CPTAC 2021 Glioblastoma cohort via cBioPortal. No new human subjects data were collected, and no identifiable patient information was accessed; institutional review board approval was therefore not required for this secondary analysis.

Acknowledgments

This study was conducted independently by the student author without a formal research mentor. No external funding was received. Generative AI tools were used only as disclosed in the Methods for code troubleshooting and editorial feedback; the author verified the analyses and accepted responsibility for the final work.

Conflict of Interest

The author declares no conflict of interest.

Author Contributions

The student author conceived the study, acquired the public data, reviewed and verified the analysis, interpreted the results, prepared the figures, and approved the final manuscript. mGenerative AI assistance is described in the Methods.

List of Abbreviations

ATP, adenosine triphosphate; CI, confidence interval; CPTAC, Clinical Proteomic Tumor Analysis Consortium; CSV, comma-separated values; FDR, false discovery rate; FPKM-UQ, upper-quartile-normalized fragments per kilobase of transcript per million mapped reads; GBM, glioblastoma; HK2, hexokinase 2; HR, hazard ratio; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; MGMT, O6-methylguanine-DNA methyltransferase; PKM, pyruvate kinase M; TCGA, The Cancer Genome Atlas; TMZ, temozolomide.

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Community-Informed Development of Nutrition Education for Older Adults in San Francisco's Chinatown Through a Community Needs Assessment

By William Choi

Abstract

Background Nutrition education programs are often developed without direct community input, limiting their cultural relevance and responsiveness to local priorities. Community-based participatory research (CBPR) emphasizes engaging communities early in program development, yet few published studies have examined how community needs assessments inform nutrition education for older adults in San Francisco's Chinatown.

Objective To conduct a community needs assessment among older adults connected to San Francisco Chinatown single-room occupancy (SRO) communities and use the findings to develop a culturally responsive nutrition education intervention.

Methods This pilot community-informed implementation study, guided by CBPR principles, engaged community organizations before curriculum development. A convenience sample of 18 older adults completed an investigator-developed exploratory needs assessment examining cooking habits, nutrition knowledge, and health concerns. Findings informed the development of bilingual educational materials and two nutrition workshops delivered in English, Mandarin, and Cantonese to approximately 40 older adults. Outcomes focused on feasibility and immediate participant perceptions.

Results The needs assessment challenged the initial assumption that limited cooking facilities were the primary barrier to healthy eating. Most participants reported preparing their own meals and identified Nutrition Facts label interpretation as their biggest educational challenge. The curriculum was designed to emphasize label literacy, including serving size, sodium, added sugars, and protein. Following the workshops, more than 75% of participants reported greater confidence interpreting Nutrition Facts labels, although sustained improvements in nutrition knowledge or behavior changes were not assessed.

Conclusions This pilot study demonstrates the feasibility of using a community needs assessment to guide development of a culturally responsive nutrition education program. Its primary contribution is illustrating how early community input can redirect educational priorities before implementation. Future studies should incorporate validated assessment instruments, systematically collect demographic information, and evaluate longer-term educational and behavioral outcomes.

Keywords community-based participatory research; community engagement; nutrition literacy; health literacy; older adults; Chinatown; Chinese American; nutrition education; implementation science; pilot study

Introduction

Improving nutrition education among older adults in San Francisco's Chinatown presents several challenges. Approximately 62% of Chinatown households are linguistically isolated, and

limited English proficiency has been associated with lower health literacy and reduced use of preventive health services (San Francisco Planning and Chinatown Community Development Center; Berkman et al.). Language barriers, cultural food preferences, access to health information, and trust in educational resources all influence how nutrition information is understood and applied.

Educational priorities identified by health care professionals and researchers do not always align with those identified by the communities they intend to serve. In Chinatown, the built environment further shapes assumptions about nutrition education. About half of neighborhood housing consists of single-room occupancy (SRO) hotels, many occupied by older adults living on fixed incomes (San Francisco Planning and Chinatown Community Development Center). Limited kitchen facilities may reasonably lead educators to prioritize healthy cooking or meal preparation when designing nutrition programs. Whether these assumptions reflect the priorities of older adults themselves remains largely unexplored.

Nutrition Facts labels provide information about serving size, sodium, added sugars, saturated fat, protein, and other nutrients that influence food choices (U.S. Food and Drug Administration). Studies have shown that older adults often experience greater difficulty interpreting these labels than younger adults, particularly when serving-size calculations and numerical comparisons are required (Macon et al.). Despite the assumed additional negative impact of the language barrier, evidence describing nutrition label literacy among Chinese American older adults remains limited.

Research involving Chinese American communities suggests that effective health education depends on more than translation alone. In a community-based participatory research study conducted in Chicago's Chinatown, older adults identified language, affordability, transportation, and access to culturally appropriate health care as important barriers to maintaining health (Dong et al.). Systematic reviews have found that educational interventions incorporating discussion, collaborative learning, and opportunities to apply information to participants' own experiences achieve greater engagement than passive information delivery (de Wit et al.). Together, these findings support early community involvement in the development of health education programs to ensure that educational priorities reflect local needs.

Despite the widespread use of CBPR in public health, limited published research has examined how a structured community needs assessment can guide the development of nutrition education for older adults in San Francisco's Chinatown.

This project originated through a Community Impact Grant awarded by Lick-Wilmerding High School to support development of a public health initiative serving the local community. Working in partnership with community organizations, the project began with a community needs assessment rather than a predetermined curriculum. The objective of this pilot community-informed implementation study was to determine community-identified nutrition education priorities and use those findings to develop a feasible, culturally responsive nutrition education intervention.

Methods

Study Design

This pilot community-informed implementation study was guided by principles of community-based participatory research (CBPR). A community needs assessment was conducted before curriculum development to identify participant priorities that would inform the design of a nutrition education intervention. The study evaluated the feasibility of using this approach to develop a community-informed educational program rather than the effectiveness of the intervention itself.

Community Partnerships

The project was conducted with input from and collaboration with organizations serving older adults in San Francisco's Chinatown, including the Chinatown Community Development Center (CCDC), the Chinese Presbyterian Church food pantry, and the San Francisco Department of Public Health. Community partners assisted with participant recruitment, provided workshop locations, and advised on language accessibility. They did not influence survey responses, data analysis, interpretation of findings, or curriculum development.

Community Needs Assessment

An investigator-developed survey was used to assess cooking habits, meal preparation, confidence interpreting Nutrition Facts labels, and self-reported chronic health conditions. The complete survey instrument is provided in Appendix A.

Surveys were administered in May 2026 in Mandarin and Cantonese. Translation assistance was provided when needed using an application called Translate Now AI Translator. Because no existing instrument adequately addressed the specific objectives of this exploratory project, the survey was developed for this study and was not adapted from a validated nutrition literacy instrument.

Survey participants were recruited through convenience sampling during community coffee hours at two CCDC single-room occupancy (SRO) buildings and in the line outside the Chinese Presbyterian Church food pantry. Eligible participants were older adults connected to San Francisco's Chinatown who were willing to complete a brief survey regarding cooking habits, nutrition knowledge, and health concerns.

Of 45 individuals approached, 18 completed the survey and were included in the analysis. Participant demographic characteristics, including age, primary language, and living situation, were requested but not uniformly reported.

Responses were summarized descriptively to identify educational priorities that would guide curriculum development. Given the exploratory design and small sample size, no formal statistical analyses were performed.

Intervention Development and Implementation

The curriculum was developed directly from priorities identified in the community needs assessment. Educational materials were created in English and Traditional Chinese and highlighted examples of foods commonly available in Chinatown grocery stores. A link to the educational materials is provided here: Community Nutrition Workshop Slide Deck.

Two multilingual nutrition education workshops were conducted at CCDC SRO locations, reaching approximately 40 older adults across both sessions. Workshops were delivered in English, Mandarin, and Cantonese and lasted approximately 50 minutes. Each session included a brief presentation, guided practice interpreting Nutrition Facts labels, participant discussion, and opportunities to ask questions.

Workshop Evaluation

Immediately following each workshop, participants completed a brief show-of-hands assessment of their confidence interpreting Nutrition Facts labels. Evaluation was limited to participants' immediate perceptions. A pre-workshop assessment and objective measures of nutrition knowledge or behavior were not collected, and the original survey evaluation instrument and response scale were not retained.

Ethical Considerations

Participation was voluntary, no financial incentives were provided, and personally identifiable information was not collected. The project was conducted as a student-led community education initiative in which the needs assessment was used to inform curriculum development. Evaluation of intervention effectiveness was not within the scope of the project. Future studies that collect research data should obtain appropriate ethics review and follow informed-consent procedures.

Results

Participant Characteristics

Eighteen community needs assessment surveys were completed and were included in the analysis. Participants were 65 years of age or older (range 65–80), and the primary language in 16 of 17 participants (94.1%) was Chinese (Mandarin, Cantonese, or another dialect). Of 17 respondents, 15 (88.2%) were SRO residents. Other demographic factors, including sex, educational attainment, country of origin, and years living in Chinatown, were not systematically collected and therefore could not be analyzed.

Community Needs Assessment

The community needs assessment challenged the initial assumption that access to cooking appliances and healthy cooking instruction should be the primary focus of the educational intervention. Of 14 participants, 13 (92.9%) reported never or rarely worrying about having enough to eat. Of 18 participants, 18 (100%) reported preparing their own meals, and 16

of 18 (88.9%) had access to a rice cooker and 17 of 18 (94.4%) had access to a refrigerator. Of 18 participants, 14 (77.8%) were at least somewhat familiar with the main food groups, whereas 4 of 18 participants (22.2%) were at least somewhat familiar with food nutrition labels. Of 14 participants, 11 (78.6%) had not been advised on their nutrition in the last year. When asked about chronic conditions, 8 of 13 (61.5%) reported diabetes, 6 of 13 (46.2%) reported hypertension, and 6 of 13 (46.2%) reported hyperlipidemia.

Because the survey was exploratory, percentages should be interpreted descriptively rather than as estimates of population prevalence.

Intervention Development and Workshop Delivery

The findings from the needs assessment informed development of a multilingual nutrition education curriculum emphasizing Nutrition Facts label interpretation. Two workshops were conducted at Chinatown Community Development Center single-room occupancy (SRO) locations, reaching approximately 40 older adults. Participants engaged in guided practice interpreting Nutrition Facts labels using examples of foods commonly available in Chinatown grocery stores and participated in group discussion and question-and-answer sessions.

Participant Evaluation

Immediately following the workshops, a brief show-of-hands assessment was used to evaluate participants' confidence interpreting Nutrition Facts labels. More than 75% reported greater confidence after participating in the workshops. Because the original evaluation instrument and response scale were not retained, additional statistical analysis was not possible. Objective measures of nutrition knowledge, dietary behavior, or health outcomes were not collected.

Table 1

Characteristics of the Pilot Community-Informed Implementation Study

Characteristic	Description
Study design	Pilot community-informed implementation study guided by CBPR principles
Study setting	Community organizations serving older adults living in single-room occupancy units in San Francisco's Chinatown
Community partners	Chinatown Community Development Center (CCDC), Chinese Presbyterian Church food pantry, and San Francisco Department of Public Health
Survey participant recruitment	Convenience sampling during community coffee hours at two CCDC SRO buildings and the Chinese Presbyterian Church food pantry line
Individuals approached	45
Surveys completed and included in analysis	18
Needs assessment topics	Cooking habits, meal preparation, confidence interpreting Nutrition Facts labels, and self-reported chronic health conditions

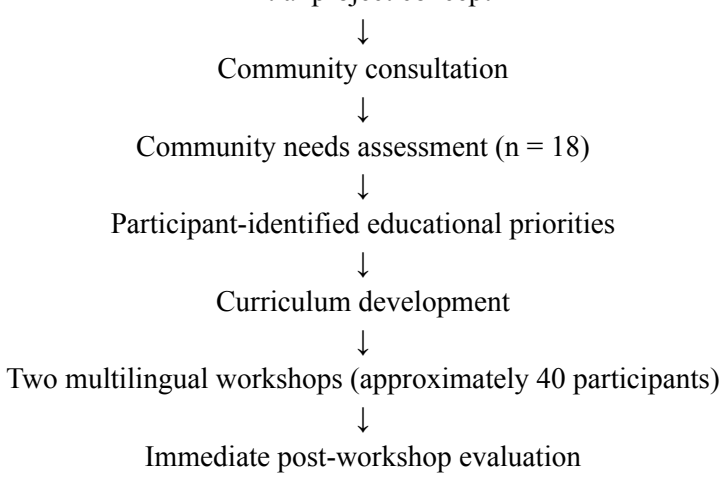
Curriculum development	Guided by findings from the community needs assessment
Educational intervention	Two nutrition education workshops delivered in English, Mandarin, and Cantonese
Workshop attendance	Approximately 40 older adults across two sessions
Primary educational focus	Nutrition Facts label literacy, including serving size, sodium, added sugars, and protein
Primary evaluation outcome	Participant confidence interpreting Nutrition Facts labels immediately after the workshops

Table 2

Changes to the Educational Intervention Following the Community Needs Assessment

Initial Project Assumption	Community Needs Assessment Finding	Resulting Curriculum Modification
Limited kitchen facilities would make healthy cooking strategies the highest educational priority.	Most participants reported preparing their own meals and having access to basic cooking facilities.	Supplying kitchen appliances was no longer the primary focus of the program.
Cooking skills would be the area of greatest educational need.	Participants identified Nutrition Facts label interpretation as a knowledge gap.	The curriculum was structured to emphasize Nutrition Facts label literacy.
General nutrition education would be sufficient.	Participants reported uncertainty interpreting information on packaged foods while managing chronic conditions such as diabetes, hypertension, and hyperlipidemia.	Educational materials focused on Nutrition Facts labels, with particular attention to serving size, sodium, added sugars, and protein.
Standard nutrition education materials would meet participant needs.	Participants represented a linguistically diverse community with limited English proficiency.	Educational materials and workshops were delivered in English, Mandarin, and Cantonese using examples of foods commonly available in Chinatown grocery stores.

Figure 1. Development of the Intervention



Discussion

The primary finding of this study was that the community needs assessment changed the educational intervention before it was implemented. The project began with the expectation that access to kitchen appliances and recipes would impact the nutrition and health of older adults living in San Francisco's Chinatown, particularly given the prevalence of single-room occupancy housing. Instead, participants identified Nutrition Facts label interpretation as the educational topic of greatest need. This shift highlights the value of obtaining community input before developing educational programs and demonstrates the pitfalls of relying on assumptions based on the local environment.

This approach aligns with the principles of community-based participatory research (CBPR), which emphasize involving community members throughout the development of public health interventions (Israel et al.). Previous CBPR studies have focused on partnership development, trust, and community engagement. In this study, the community needs assessment directly influenced the educational curriculum before implementation. The project provides an example of how community input can shape an intervention during the planning phase.

Published studies describing nutrition education among older Chinese American adults remain limited. Research in older adult populations has shown that interpreting Nutrition Facts labels can be challenging, particularly when serving size and nutrient information must be applied to everyday food choices (Miller et al.; Macon et al.). Participants in this population reported similar difficulties with understanding the nutritional value of what they buy and eat. They also reported a high rate of diabetes, hypertension, and hyperlipidemia. These findings support Nutrition Facts label literacy as an appropriate focus for future nutrition education programs in this population.

The project also highlighted the value of collaboration with existing community organizations serving older adults. These organizations provided access to participants and locations for workshop delivery. Presenting the workshops in English, Mandarin, and Cantonese and using examples of foods commonly available in Chinatown grocery stores helped tailor the curriculum to the community served. The relative importance of each of these elements to participant engagement was not evaluated independently.

The principal contribution of this study is methodological rather than clinical. It demonstrates that a structured community needs assessment can meaningfully influence intervention design and that such an approach is feasible within existing community organizations. Although modest in scale, the project provides a practical model for future student-led community health initiatives. The findings support the feasibility of a community-informed educational approach rather than intervention effectiveness.

Limitations

Several limitations should be considered. First, the study included a small convenience sample recruited through community organizations in a single neighborhood, limiting generalizability beyond the study population. Second, participant demographic characteristics

were not collected systematically, preventing analysis of whether educational priorities differed across participant groups. Third, the needs assessment survey was developed specifically for this project and was not based on a validated nutrition literacy instrument. Evaluation of workshop efficacy was limited to participants' confidence immediately after the workshops. Because no pre-intervention assessment or objective measures of nutrition knowledge or behavior were collected, the study cannot determine whether the intervention changed nutrition knowledge, food purchasing decisions, dietary intake, or health outcomes.

Future Directions

Future studies should incorporate validated nutrition literacy instruments, collect participant demographic information systematically, and include both pre- and post-intervention assessments. Longer-term follow-up of the study and workshop participants would allow evaluation of whether improvements in Nutrition Facts label literacy are associated with changes in food choices or health behaviors. Although the educational priorities identified in this study may differ across communities, the process of conducting a community needs assessment before developing an intervention can be applied to other community-based nutrition education programs serving linguistically diverse older adult populations.

Conclusion

This pilot study demonstrates that a community needs assessment can redirect a nutrition education intervention toward priorities identified by the people it is intended to serve. Among older adults connected to San Francisco Chinatown SRO communities, the assessment shifted the program's focus from assumed cooking-resource barriers toward Nutrition Facts label literacy. The project does not establish intervention effectiveness, but it demonstrates a feasible process for community-informed program development and provides a foundation for more rigorous future evaluation.

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The Brain's Lie: Neurobiological Self-Defense in Self-Deceit, Schizophrenia, and DID
By Jiayi Zheng

Abstract

Self-deceit is a paradox for any living organisms on Earth, that how can one be both the deceiver and the deceived. This essay explores self-deception from the neurobiological and psychiatric perspectives, analyzing brain structures such as amygdala's function in self-deception and mental disorders such as Schizophrenia as a representation of self-deception. Evidence shows that self-deception can function as a part of unconscious self-defense against emotional threats, and it could cause delusions or cognitive disorders when exaggerated in extreme conditions such as DID. These findings offer a neurobiological insight to understand self-deceit as a defensive response rather than an intentional choice.
Keywords: self-deceit, prefrontal cortex, amygdala, schizophrenia, dissociative identity disorder

Introduction

We all lie, at times. The behavior of deceit indicates that people are causing others to accept as true or valid what is false or invalid (Merriam-Webster, n.d.). Human beings often deceive one another to avoid punishment, embarrassment or take on unwanted responsibilities. Moreover, individuals can also deceive themselves through self-deceit. However self-deceit is not simply lying behavior. There appears to be a contradiction: although lying is an internally driven behavior, from a logical perspective, one cannot always force themself to forget the "true and valid" and believe in what is "false or invalid" (Merriam-Webster,n.d.) . Given this line of thought on self-deceit, the following question is raised: is self-deceit a purposeful action that people utilize to achieve certain goals such as satisfaction or well-being? This paper intends to answer that basic question through reviewing and analyzing the areas of psychiatry and neuropsychiatry that discuss the neural bases of self-deceit. It is essential to review the brain's contribution to the behavior of self-deception because your neurons coordinate all behavior. Delving into brain systems involved in our tendency to deceive ourselves combined with an analysis of psychiatric disorders, where self-deceit is a part of the symptomology will provide an insightful and comprehensive analysis of the subject from a brain perspective. If we consider that our minds are a reflection of our brain activity, then we recognize the pivotal role that brain research is playing in our overall understanding of our psyche.

Brain Systems Involved in Self-deception

We can begin this discussion with the review of brain systems that have been identified in the processing of feelings such as deceit and self-deceit. The human brain is a highly complex system in which not a single part of the brain works solitarily. However specific parts are the primary coordinator of functions. To answer the question of whether self-deceit is a behavior caused by neurobiological actions, researchers have investigated different brain areas and identified regions that are involved in emotional expressions and conflict processing.

The prefrontal cortex (PFC) orchestrates lying behavior. The PFC is located in the front part of the human brain, and is capable of receiving input from multiple cortical regions to process "in the moment" information (Hathaway & Newton,2023). This systematic process was discovered by a group of researchers who compared people's brain activity while they lie and tell the truth. They used functional magnetic resonance imaging (fMRI), and they found that the prefrontal cortex has increased activity when people lie (Allison Curley, n.d.). Additionally, the researchers reported that the PFC processes higher-order executive functions and thus helps adapt the human body to the environment (Hathaway & Newton, 2023); when people are going through situations that make them uncomfortable, the PFC enhances its activity to inhibit awareness of emotionally distressing events, thereby promoting psychological stability.

Another important brain region involved in telling untruths is called the amygdala, a small almond-shaped structure located deep within the neurons and is pivotal in carrying out emotions, controlling behavior, and detecting danger automatically (Cleveland Clinic, 2025). Additionally, the amygdala processes emotions and also forms memories about these feelings (Cleveland Clinic, 2023). When it detects negative feelings such as fear, the human brain may suppress the perception of this danger to protect itself from leaving trauma. In such situations, the amygdala may alter or reconstruct the memory — deceiving the brain into believing that nothing harmful occurred — or it may lead to the development of disruptive symptoms (Cleveland Clinic, 2023), such as hallucinations, delusion and disorganized thinking (Mayo Clinic, 2024). These pathological symptoms that present in behaviors such as dissociation will be discussed later in this essay.

There are many more regions in human brain, such as the hippocampus which is closely associated with the amygdala (Fogwe et al., 2023), the thalamocortical connections, amygdalo-striate pathways, cortico-striatal-limbic hubs, default mode network, salience networks (Dabiri et al., 2022), even the grey matter (Howes et al., 2022) in the spinal cord (Cleveland Clinic, 2023) plays a role in your emotion. These brain structures are crucial for emotional response including self-deceit as they together form the neurobiological framework of lying, including self-deceit (Dabiri et al., 2022; Howes et al., 2022; Fogwe et al., 2023). This indicates that self-deceit is not a purposeful decision made by us, instead, it's a protective strategy of the brain to live through extreme fear and other severe negative emotions, to maintain psychological equilibrium.

From a Psychiatry Perspective

Psychiatric diseases have been associated with higher levels of self-deceit. Given our discussion that self-deceit is an unconscious brain protective strategy, my interest was peaked regarding self-deceit in psychopathology symptoms associated with schizophrenia (SCZ) and Dissociative Identity Disorder (DID). Could the brain's attempt to avoid fear drive it to engage in self-deceit, resulting in the hyperactivation of certain regions and the emergence of disruptive emotional and psychological symptoms?

Take schizophrenia, for example, research is just starting to understand its dynamic neural underpinnings. Schizophrenia is a psychiatric condition where people's thoughts and memories are interfered with and become chaotic (Cleveland Clinic, 2024). Schizophrenia is associated with a mix of genetics, brain chemistry and environmental variables (Mayo Clinic, 2024). Self-deceit is not the cause of SCZ, instead, it's a protection or delusion mechanism of the human brain that diminishes the feeling of reality — the origin of fear and anger — as the affected individuals accept "false and invalid" and but not the "true and valid".. Researchers have shown that some delusional subjects are, indeed, self-deceived (Gibson, 2017). As mentioned before, the behavior of deceiving is often produced automatically by brain regions such as PFC and amygdala when confronted with extreme, chaotic feelings. Therefore, generally and in schizophrenics, self-deceit is usually unaware, and people seek hope in subconsciously created lies. Researchers have demonstrated that patients who have schizophrenia show amygdala dysfunctions, and they are not able to effectively process feelings such as fear and anger (Rasetti et al., 2008). These dysfunctions are often linked to life trauma that the brain detects but struggles to regulate or integrate through processing.

Additionally, hyperactivity of the lateral frontal cortex and in the right TPJ (Fuentes-Claramonte et al., 2020) were detected among SCZ patients, supporting the claim that these psychotic symptoms are the reflection of protection, which includes self-deceit.

Self-deceit is also often associated with Dissociative Identity Disorder (DID), and has notable differences with its relationship to schizophrenia. DID is a psychiatric condition that involves people experiencing a loss of connection between thoughts, memories, feelings, surroundings, behavior and identity (Mayo Clinic, 2023). In other words, it is when one being has more than one "personality" that lives in it. In DID, self-deceit is the essential mechanism that drives the dissociation. Researchers have found that among those people who had DID, their amygdala is often overactive, particularly in response to trauma-related triggers (Shimiaie, 2025). This evidence suggests that the human brain will actively hide or delete the past memories that cause extreme fear or distress, and this is, indeed, associated with self untruths.

In this moment, self-deceit reaches an extraordinarily complex and sophisticated level that it fragments the individual's sense of self into distinct pieces, or personalities. Each of these identities only have to face a certain negative context; only processing some of the painful emotional burden. This dynamic partitioning creates the illusion for each identity that their suffering remains compartmentalized and endurable, preventing any single personality from succumbing to emotional collapse. Yet neurobiologically, no true division occurs—the same individual bears the undiminished totality of the trauma. This form of self-deceit is not a simple act of denial, but a deeply embedded psychological defense—a made up reality created by the mind. It is a lie inwardly to protect the self from emotional collapse. Paradoxically, while this kind of self-deceit seems to be a fake world that can protect the person's emotion temporarily, it functions as a life-preserving mechanism, shielding the person from the devastating reality that may lead to self-destruction such as suicide (Hamlyn, 1985).

Conclusion: Integration of Neural Systems and Psychiatric Evidence

The neurobiology and psychiatric evidence demonstrates that self-deceit is, indeed, often without the conscious and mostly towards the goal of protection or promoting well-being, even when the emotional state is somewhat fabricated.

What appears as irrational denial or distorted belief may, in fact, be the brain's last line of defense—an involuntary act of self-deceit designed to guard the mind against psychological disintegration and pathology. Notice that in both psychiatric diseases we discussed above, whether self-deceit is a crucial reason that drives the symptoms or not, the goal of the brain system deluding itself is to protect from danger, distress, or fear. Moreover, we see from the neurological evidence that even in psychiatric diseases, self-deceit was carried out by brain systems automatically. This explains the paradox of "how can people actively forget something" that we mentioned in the introduction of this essay. Ultimately, self-deceit is often not a conscious choice, but a subconscious mechanism. Moreover, it is an involuntary, emotionally driven process by which the brain sustains false beliefs — despite evidence to the contrary — due to the mind's desperate need to feel some safety, even if that safety is against the truth (Stanford Encyclopedia of Philosophy, 2006).

Limitations

Neuroimaging researchers have found that different brain regions are activated under certain conditions, but do not always capture the entire brain system that is involved in producing the investigated behavior. This is a limitation because they fail to find the actual brain system that is working for the behavior. Secondly, there is no one to one correspondence between the increased brain activity and behaviors. Additionally, the brain activity increases should not be considered as promoting the behaviors, rather, they may happen simultaneously with the behaviors–their temporal occurrence is not definite.

Future Considerations

Though this essay tends to discuss the appearance of unconscious self-deceit, there does exist self-deceit that can be realized clearly. Additionally, some cognitive biases — unconscious and systematic errors in people's thinking that distort an individual's perception of its surroundings (Silva et al., 2023); an example could be self-serving bias where people tend to use information that advance their self-interest (The University of Texas at Austin, n.d.) Further, this self-serving bias can function like self-deceit and may be misrecognized as decisive behaviors. Thus, it is very important for future researchers to distinguish between those variables especially in clinical practices.

Furthermore , while both self-deceit and lying on purpose are very important mechanisms that could be used for defensive reasons, researchers should continue to explore the neurobiological and methodological differences between them going forward. This will not only help improve clinical context such as the psychiatric symptoms, but also the dynamically organized model of brain behavior.

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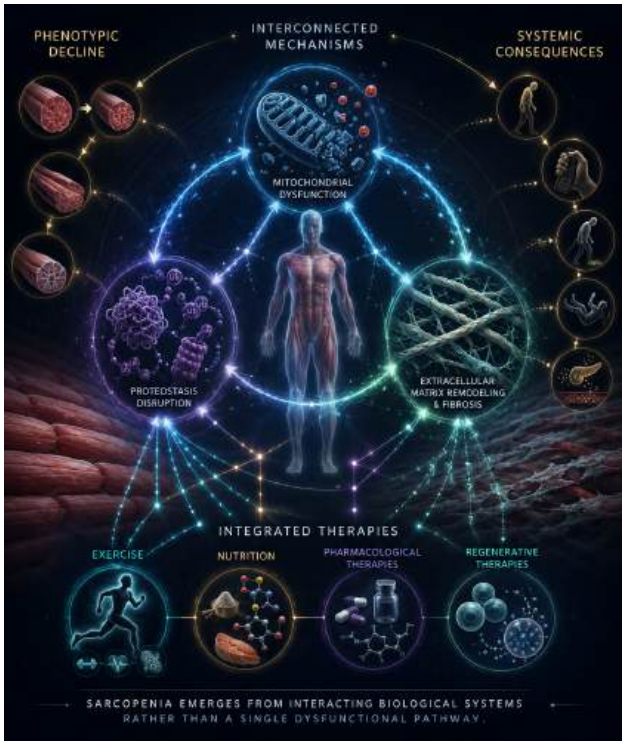
A Review of Phenotypic Hallmarks, Biological Mechanisms, and Evidence-Based Interventions in Skeletal Muscle Ageing By Alessia Arisi



ABSTRACT

Skeletal muscle is essential for longevity and overall systemic health, as it plays a critical role in movement, metabolic regulation, and physiological homeostasis. Maintaining skeletal muscle throughout life is therefore essential, yet with advancing age it undergoes progressive structural and functional decline, contributing to the development of sarcopenia.

This review synthesizes current literature on the phenotypic and functional hallmarks of skeletal muscle ageing, the biological mechanisms underlying these changes, and the effectiveness of current therapeutic strategies. By examining how these processes interact, it provides a more integrated understanding of skeletal muscle ageing, highlights the limitations of viewing sarcopenia through isolated biological pathways, and identifies directions for future therapeutic development. The literature suggests that muscle ageing arises from the dysregulation of interconnected biological systems rather than a single pathological process. Mitochondrial dysfunction, impaired proteostasis, and extracellular matrix remodeling collectively reduce muscle quality, regenerative capacity, and physiological resilience, driving the progressive declines in muscle mass, strength, and physical performance that characterize sarcopenia. Among current interventions, exercise remains the most consistently effective strategy because it simultaneously targets multiple pathways involved in muscular ageing, whereas nutritional, pharmacological, and regenerative therapies have shown more limited or inconsistent long-term functional benefits.

Overall, understanding the complexity and interconnected nature of skeletal muscle ageing is essential for developing more effective, multimodal interventions capable of preserving

muscle function, promoting healthy longevity, and improving quality of life in ageing populations.

Keywords

Sarcopenia; Skeletal muscle aging; Mitochondrial dysfunction; Proteostasis; Extracellular matrix remodeling; Exercise interventions; Gut–muscle axis; Healthy aging.

SECTION 1- Introduction

1.1 The Role of Skeletal Muscle in Human Health and Physiological Homeostasis

Skeletal muscle is a fundamental tissue that underpins a wide range of physiological functions extending far beyond voluntary movement. While it is most commonly associated with actions such as walking, lifting, and maintaining posture, skeletal muscle also plays essential roles in metabolic regulation, thermogenesis, and whole-body homeostasis. It contributes significantly to glucose regulation and serves as a major reservoir of protein, accounting for approximately 40% of total body mass and nearly half of total protein content[1] Importantly, skeletal muscle is not limited to large locomotor groups, but also includes specialized structures such as the muscles of the vocal cords, highlighting the breadth of its functional diversity. Because of this wide distribution and specialization, both the manifestation of muscle decline and the response to treatment can vary considerably across muscle types. At a physiological level, skeletal muscle is maintained through tightly regulated processes such as muscle protein synthesis (MPS) and muscle protein breakdown (MPB), which exist in a dynamic equilibrium influenced by factors including nutrition and physical activity[2]. Disruption of this balance, whether due to aging, disease, or disuse, can result in muscle atrophy, reduced force production, and diminished functional capacity[3].

1.2 Clinical Significance and Lifespan Progression of Sarcopenia

The progressive decline of skeletal muscle function is a major contributor to reduced mobility, loss of independence, and decreased quality of life, particularly in aging populations. This decline is most described as sarcopenia; a condition originally defined as the age-related loss of skeletal muscle mass and later expanded to include reductions in strength and functional performance[4]. Clinically, sarcopenia is assessed through measures of muscle mass, strength, and physical function, while biologically it reflects complex alterations at molecular, cellular, and tissue levels. Importantly, sarcopenia is not solely a consequence of chronological aging but represents a pathological state that can be influenced by multiple factors, including chronic disease and lifestyle. Its relevance extends beyond musculoskeletal health, as low muscle mass has been associated with cognitive decline and increased mortality risk, suggesting broader systemic implications[5].

Contrary to traditional assumptions, skeletal muscle decline does not begin exclusively in old age but emerges earlier in the lifespan and progresses gradually over time[6]. Longitudinal

evidence demonstrates that muscle strength, endurance, and aerobic capacity typically peak before the age of 40 and subsequently decline, with the rate of decline accelerating in later decades[7]. This trajectory indicates that the foundations of sarcopenia are established well before clinical symptoms become apparent. Furthermore, the progression of muscle aging is not uniform across populations. For example, sex-specific differences have been observed, with hormonal changes such as declining estrogen levels potentially contributing to distinct patterns of muscle deterioration in females[8]. Despite increasing research in this area, there remains a relative lack of comprehensive human studies, particularly those focusing on underrepresented groups, which limits a full understanding of variability in muscle aging.

Table 1. Age-related trajectory of skeletal muscle decline across adulthood

Summary of the age-related trajectory of skeletal muscle decline synthesized from representative studies. Age thresholds and rates of decline vary according to population characteristics and measurement techniques. ASM = appendicular skeletal muscle.

Age	Trajectory of Skeletal Muscle Aging
20–39 years	Skeletal muscle reaches peak mass during early adulthood. Appendicular skeletal muscle (ASM) generally peaks in the 20s–30s, while total muscle mass may peak later (around the fourth decade) depending on sex and measurement method[9]. Following peak muscle mass, a gradual decline of approximately **3–8% per decade begins**[10].
40–59 years	Progressive declines in total muscle mass and ASM become increasingly evident. Although the rate of loss remains relatively gradual, measurable reductions in muscle quantity and strength are observed[9].
60–74 years	The rate of muscle loss accelerates compared with earlier adulthood. In women from the Brazilian cohort, muscle mass declined by an average of **5.7% per decade** from the sixth decade onward[9,10]. Functional impairments and detectable deterioration in muscle tissue become increasingly apparent[11].
75–82 years	This period is associated with the most rapid decline in skeletal muscle mass, accompanied by substantial losses in muscle strength and physical performance[11].
≥83 years	The rate of muscle mass loss begins to plateau; however, muscle quality, strength, and physical function continue to deteriorate, reflecting ongoing neuromuscular and structural changes[11].

1.3 Biological Mechanisms Underlying Skeletal Muscle Aging

At a mechanistic level, skeletal muscle aging cannot be attributed simply to cumulative "wear and tear," but instead reflects the dysregulation of interconnected biological systems. Among the most critical of these are mitochondrial function, proteostasis, and extracellular matrix (ECM) integrity. Mitochondria play a central role in ATP production and metabolic regulation, making them essential for muscle contraction and endurance. Proteostasis, or the balance between protein synthesis and degradation, is equally essential for maintaining muscle mass and function. The extracellular matrix provides structural support and facilitates communication between muscle cells and their environment. Importantly, these systems do not operate independently. Mitochondrial dysfunction, loss of proteostasis, and ECM remodeling

form an interconnected network in which disruption in one component can propagate to others, supporting the view of muscle aging as a network-level process rather than a result of isolated pathways[12].

1.4 Review Scope and Integrated Perspective on Skeletal Muscle Aging

The consequences of skeletal muscle aging extend beyond localized tissue changes to impact whole-body health. Declines in muscle mass and strength are associated with increased risk of falls, frailty, and loss of independence, as well as broader metabolic disturbances such as insulin resistance and increased susceptibility to chronic disease. Sarcopenia has also been linked to higher rates of mortality and poorer clinical outcomes across a range of conditions [13]. Despite the growing body of research, much of the existing literature focuses on individual mechanisms in isolation, leaving gaps in understanding how these processes interact and which interventions are most effective.

This review aims to synthesize current research on skeletal muscle aging by identifying key phenotypic and functional hallmarks, examining the underlying molecular and cellular mechanisms, and evaluating evidence-based intervention strategies. In doing so, it seeks to provide an integrated perspective that connects processes across biological scales, from molecular dysfunction to whole-body outcomes. Additionally, this review highlights gaps in current research, including limited representation of certain populations and the need for alternative therapeutic approaches for individuals unable to engage in conventional interventions such as exercise or nutritional supplementation. Emerging perspectives, such as the potential role of the gut–muscle axis, further emphasize the importance of exploring new frameworks for understanding and treating sarcopenia[14].

SECTION 2 – Skeletal Muscle Structure and Function

Skeletal muscle is organized into highly specialized structures that enable efficient force production and coordinated whole-body function. During development, muscle precursor cells fuse to form elongated, multinucleated muscle fibers that can extend several centimeters in length. These fibers are composed of repeating contractile units known as sarcomeres, which function as the fundamental basis of muscle contraction. Within each sarcomere, thin actin filaments and thick myosin filaments are arranged in a precise overlapping pattern that enables coordinated force generation through cyclical cross-bridge interactions. Structural proteins such as titin, together with anchoring regions including the Z-disc and M-band, provide stability, maintain sarcomere alignment during contraction, and contribute to intracellular mechanical signaling[15].

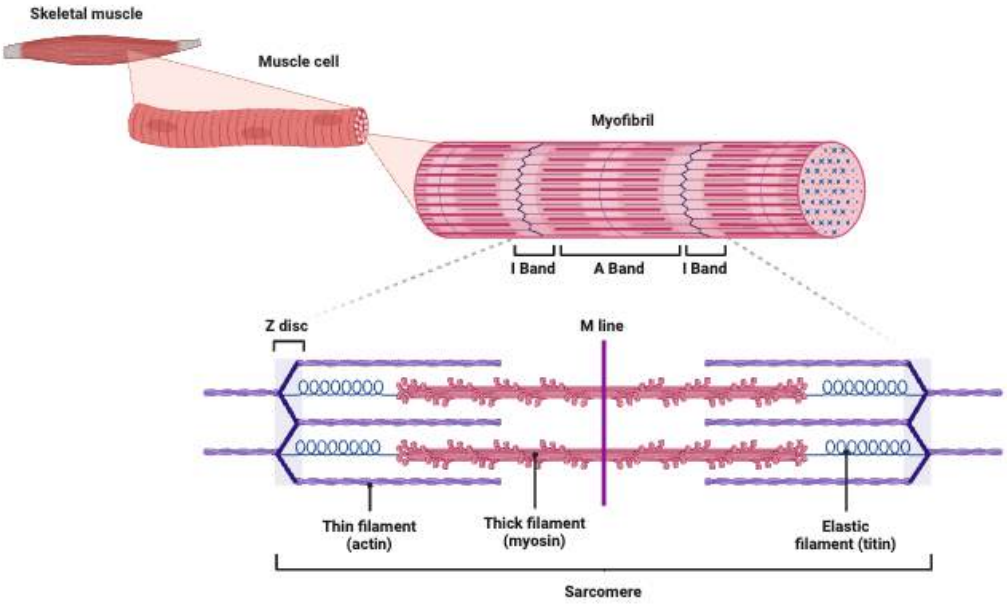
Skeletal muscle fibers can be broadly categorized into type I and type II fibers, each possessing distinct structural and metabolic characteristics. Type I fibers (also known as slow-twitch fibers) rely primarily on aerobic respiration and have high fatigue resistance. While type II fibers (fast-twitch fibers) are specialized for short explosive movement, relying primarily on anaerobic pathways, causing easier fatigue. Type II muscle fibers contain a more developed

sarcoplasmic reticulum and greater myofibrillar content, allowing for faster and more forceful contractions compared to type I fibers[3]. These differences in fiber composition contribute to variations in strength, endurance, fatigue resistance, and metabolic activity across skeletal muscle groups.

This highly ordered architecture allows skeletal muscle to efficiently convert chemical energy into mechanical work. Beyond its role in locomotion and voluntary movement, skeletal muscle also functions as a major regulator of metabolic homeostasis, contributing to glucose regulation, energy balance, thermogenesis, and systemic physiological stability[15]. Furthermore, skeletal muscle demonstrates considerable adaptability in response to exercise, injury, and physiological stress through continuous remodeling and repair processes that help maintain muscular integrity and function across the lifespan[16].

However, despite this adaptive capacity, skeletal muscle becomes increasingly vulnerable to structural and functional decline with advancing age. Progressive impairments in muscle mass, strength, regenerative potential, and physical performance contribute to the development of sarcopenia, a complex age-associated condition with significant physiological and clinical consequences. The following section will therefore examine the major phenotypic and functional hallmarks that characterize sarcopenic skeletal muscle ageing.

Figure 1. *Hierarchical organization of skeletal muscle structure, illustrating the anatomical progression from whole skeletal muscle to the sarcomere, the fundamental contractile unit responsible for force generation.* ***Created with BioRender.com.***



SECTION 3 – Phenotypic and Functional Hallmarks of Skeletal Muscle Ageing

3.1 Sarcopenia as a Clinical Indicator of Skeletal Muscle Ageing

Sarcopenia is characterized by progressive age-related declines in skeletal muscle mass, strength, and physical performance, contributing significantly to frailty and functional impairment in older populations[17]. As aging populations continue to expand globally, sarcopenia and its associated fall risk are increasingly recognized as major public health concerns. Falls are strongly associated with reduced independence, chronic disability, hospitalization, morbidity, and mortality in older adults[18], while secondary injuries such as fractures generate substantial healthcare and economic burdens[19].

Recent research has challenged the use of chronological age alone as a predictor of physiological decline, instead emphasizing biological age as a more accurate reflection of accumulated systemic damage and reduced functional reserve. In this context, sarcopenia has been proposed as a potential clinical indicator of biological ageing due to its association with phenotypic, genetic, and metabolic deterioration[20]. The muscle loss observed in sarcopenia is therefore not simply a reflection of reduced muscular regenerative capacity, but rather an indicator of broader impairments in tissue repair, metabolic regulation, and physiological resilience. Because skeletal muscle plays a central role in mobility, stability, and voluntary movement, its decline directly affects independence and the ability to respond to physical stressors[21]. Furthermore, skeletal muscle contributes significantly to insulin regulation, metabolic homeostasis, and whole-body energy balance, meaning muscular deterioration may contribute to wider systemic dysfunction[22]. Which is why understanding the mechanisms underlying sarcopenia is clinically important, as the interconnected nature of muscular ageing suggests that isolated treatments targeting a single pathway may be insufficient to address the broader biological decline associated with ageing.

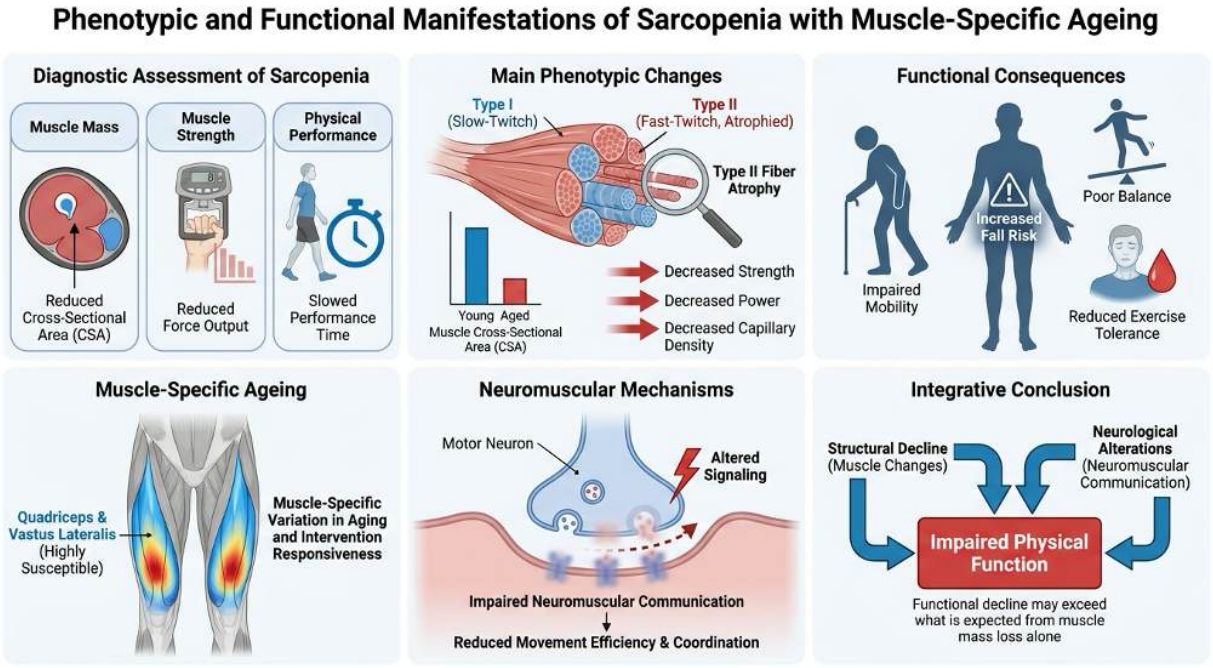
3.2 Phenotypic Manifestations of Sarcopenia

The diagnosis of sarcopenia involves the combined assessment of muscle mass, muscle strength, and physical performance[23]. These declines are reflected through measurable phenotypic changes including reductions in muscle cross-sectional area (CSA), muscular strength, power generation, and capillary density[23]. Functionally, these structural alterations contribute to impaired mobility, reduced exercise tolerance, diminished balance, and increased fall risk. In particular, the preferential atrophy of type II muscle fibers may contribute disproportionately to losses in strength and motor control[24], further compromising physical performance in older adults.

Age-related muscular decline is not always uniform across the body. Much of the current literature has focused primarily on the quadriceps and vastus lateralis muscles due to the accessibility of imaging, biopsy collection, and exercise intervention studies involving these muscle groups. However, emerging evidence suggests that skeletal muscle ageing may occur in a muscle-specific manner, with different muscle groups demonstrating varying susceptibility to atrophy and responsiveness to exercise interventions[25]. This variability complicates the generalized understanding and treatment of sarcopenia, as muscular decline cannot be

approached as a completely uniform process across all individuals or muscle groups. Additionally, functional decline may not solely reflect muscular atrophy itself, but also alterations in neurological signaling pathways and neuromuscular communication that impair movement efficiency and coordination[26]. Consequently, reductions in physical function may exceed what would be expected from muscle mass loss alone.

Figure 2. Phenotypic manifestations of sarcopenia, illustrating the progressive structural and functional changes associated with skeletal muscle ageing, including reductions in muscle cross-sectional area (CSA), preferential atrophy of type II muscle fibers, decreased strength and power generation, diminished capillary density, and the resulting impairments in mobility, balance, and physical performance. The figure also highlights the heterogeneous and muscle-specific nature of sarcopenic decline and its contribution to increased frailty and fall risk.



3.3 Clinical Assessment and Heterogeneity of Sarcopenia

Several tools are currently used to assess sarcopenia in clinical practice, with grip strength and gait speed tests remaining among the simplest and most accessible methods for evaluating muscle function and physical performance. Muscle mass may also be estimated using radiological imaging techniques and bioelectrical impedance analysis (BIA), which remains widely available in clinical settings. However, BIA presents several limitations, particularly in individuals with altered hydration or electrolyte balance, reducing the consistency and accuracy of measurements across populations[27].

Furthermore, while BIA can estimate fat-free mass and total body water, it provides limited insight into muscle structure or functional quality. Alternative diagnostic tools such as muscle ultrasound have recently emerged as promising methods for evaluating muscle

composition and architecture[28]. Nevertheless, diagnosing sarcopenia remains challenging because the condition is not defined by a single measurable alteration, but rather by a combination of molecular, structural, and functional decline. The heterogeneity of skeletal muscle ageing across individuals further complicates diagnosis, often requiring highly patient-specific assessment strategies that may not always be practical or accessible in clinical settings. In addition, functional decline is not solely quantitative but also qualitative; muscle tissue may retain apparent mass while experiencing substantial reductions in contractile efficiency, coordination, or functional capacity[29]. Consequently, measurements of muscle quantity alone may fail to fully capture the physiological impact of sarcopenia on quality of life and daily functioning.

Sarcopenic decline is not limited to appendicular skeletal muscle and may also affect respiratory musculature, a condition referred to as respiratory sarcopenia. Respiratory sarcopenia is characterized by reduced respiratory muscle strength and reduced respiratory muscle mass. Decreases in respiratory muscle function have been associated with poorer physical performance, impaired nutritional status, and adverse clinical outcomes in older adults[30]. However, the diagnosis of respiratory sarcopenia remains challenging due to the difficulty of accurately measuring respiratory muscle mass and establishing standardized diagnostic criteria[30].

This presence of sarcopenia across diverse muscle groups reinforces the systemic nature of muscular ageing and demonstrates that sarcopenic decline cannot be understood solely through measurements of isolated muscle groups such as the quadriceps. Instead, the widespread involvement of skeletal and respiratory musculature suggests that muscle ageing reflects broader disruptions in metabolic regulation, tissue maintenance, and intercellular communication. Consequently, the observable hallmarks of sarcopenia may represent surface-level manifestations of deeper biological mechanisms driving whole-body ageing processes.

The widespread phenotypic and functional manifestations of sarcopenia suggest that skeletal muscle ageing is driven by deeper biological processes occurring at molecular and cellular levels[31]. Ageing has been conceptualized through several interconnected hallmarks involving progressive dysregulation of genetic, metabolic, and intercellular pathways[17]. Among these hallmarks, mitochondrial dysfunction, impaired proteostasis, chronic inflammation, and altered intercellular communication appear particularly relevant to skeletal muscle ageing and may collectively contribute to the progressive decline in muscular structure and function observed with advancing age[32]. The following sections will therefore explore the interconnected molecular and cellular mechanisms underlying sarcopenia and how their interaction contributes to the progression of age-related muscular decline.

SECTION 4 – Biological Mechanisms Underlying Skeletal Muscle Ageing

4.1 Mitochondrial Dysfunction

Mitochondria play a central role in ATP production and metabolic regulation, making them essential for muscle contraction and endurance. In healthy skeletal muscle, mitochondrial

quality is maintained through tightly regulated processes including fusion, fission, and mitophagy, which collectively preserve mitochondrial integrity and energy efficiency[33]. However, with aging, mitochondrial dysfunction leads to reduced oxidative capacity, impaired ATP production, increased reactive oxygen species (ROS) generation, and activation of catabolic signaling pathways that contribute to muscle atrophy and weakness[34].

Mitochondrial dysfunction and impaired mitophagy have therefore emerged as major drivers of skeletal muscle deterioration[35]. As critical regulators of energy metabolism, redox balance, and intracellular signaling, mitochondria are essential for maintaining muscle fiber integrity and plasticity. Aging is associated with structural and functional mitochondrial impairments including diminished mitochondrial biogenesis, accumulation of damaged mitochondria, reduced respiratory efficiency, and mitochondrial DNA (mtDNA) damage. These alterations compromise cellular energy metabolism while simultaneously increasing oxidative stress within muscle tissue[36].

The excessive production of ROS further disrupts intracellular homeostasis by damaging proteins, lipids, and nucleic acids, contributing to impaired cellular repair and regenerative capacity[37]. As mitochondrial quality declines, muscle fibers become progressively less capable of sustaining efficient contraction and metabolic adaptation, accelerating the progression of sarcopenia[36]. Importantly, this oxidative and energetic stress places additional strain on proteostatic systems responsible for maintaining protein integrity[38], further linking mitochondrial dysfunction to broader mechanisms of skeletal muscle ageing.

4.2 Proteostasis Disruption

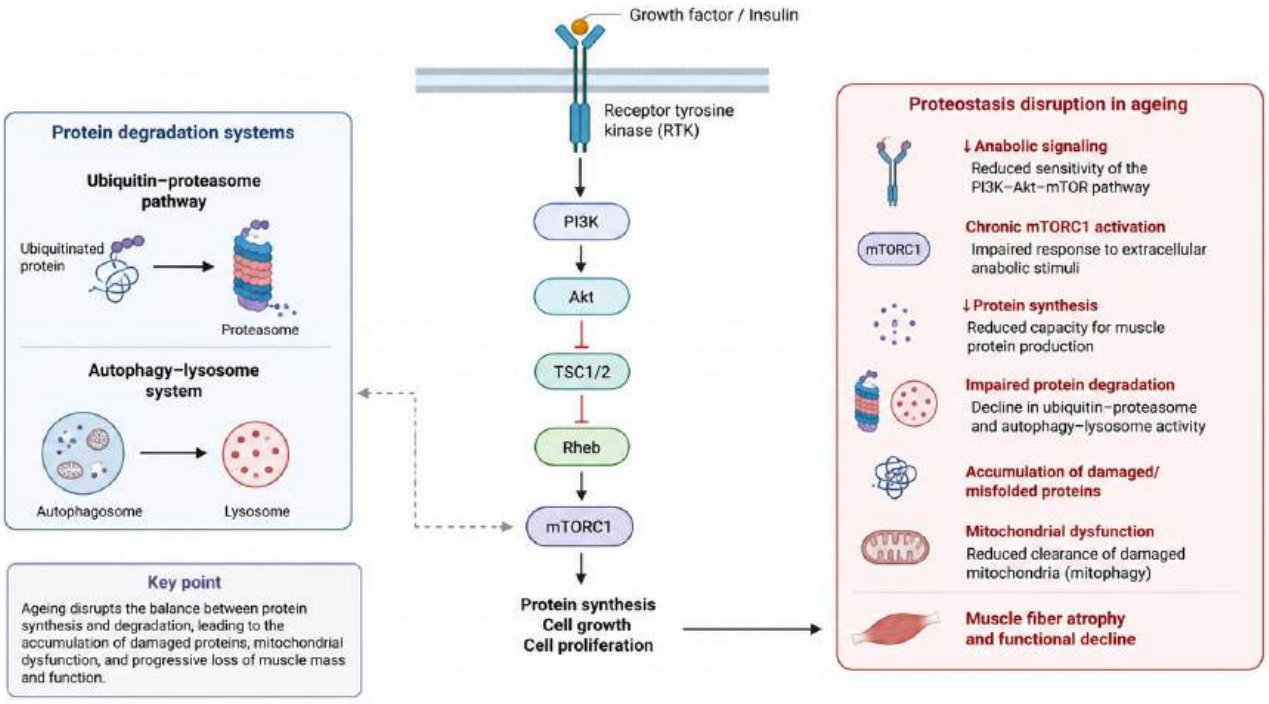
Proteostasis, or the balance between protein synthesis and degradation, is equally essential for maintaining skeletal muscle mass and function. This balance is regulated by interconnected systems including the ubiquitin–proteasome pathway, autophagy–lysosome system, and signaling networks such as AKT–mTOR[39]. Under healthy physiological conditions, these pathways ensure continuous protein turnover, removal of damaged proteins, and regeneration of muscle tissue. With aging, however, proteostatic mechanisms become progressively dysregulated, resulting in reduced protein synthesis capacity, accumulation of damaged or misfolded proteins, and impaired regeneration of muscle fibers.

Protein homeostasis has consequently been proposed as one of the central mechanisms underlying the development of sarcopenia. As a major regulator of protein synthesis and muscle hypertrophy, the mammalian target of rapamycin (mTOR) pathway plays a critical role in maintaining muscular integrity[39]. However, sustained activation of mTOR complex 1 (mTORC1) in ageing skeletal muscle has paradoxically been associated with reduced responsiveness to extracellular anabolic signaling despite its known role in promoting muscle growth[39]. This dysregulation suggests that chronic alterations in anabolic signaling may impair the muscle's ability to appropriately respond to stress, repair demands, and nutrient availability over time.

As damaged proteins accumulate and protein turnover efficiency declines, muscle fibers progressively lose structural and functional integrity[40]. The inability to effectively maintain

proteostasis also exacerbates mitochondrial dysfunction, as defective proteins involved in oxidative phosphorylation and mitochondrial maintenance accumulate within ageing cells[41]. Simultaneously, impaired autophagy limits the clearance of dysfunctional mitochondria, reinforcing oxidative stress and energetic decline. Consequently, disruptions in proteostasis and mitochondrial quality control become increasingly interconnected during skeletal muscle ageing.

Figure 3. Regulation of proteostasis in healthy skeletal muscle and its disruption during ageing through alterations in the PI3K–Akt–mTOR signaling pathway, ubiquitin–proteasome degradation, and autophagy–lysosome function. **Created with BioRender.com.**



4.3 ECM Remodeling and Fibrosis

The extracellular matrix (ECM) provides structural support and facilitates communication between muscle cells and their surrounding environment. In healthy skeletal muscle, the ECM plays an important role in force transmission, tissue organization, mechanical stability, and regenerative signaling. Under normal physiological conditions, ECM remodeling remains tightly regulated in order to maintain tissue integrity and adaptability. However, during aging and disease states, excessive collagen deposition and fibrosis may occur, resulting in increased tissue stiffness, altered cellular signaling, and impaired regenerative capacity[42].

Fibroblasts, once considered passive structural cells responsible primarily for ECM production, are now increasingly recognized as dynamic regulators of tissue homeostasis, inflammation, and immune signaling[43],[44]. In multiple fibrotic diseases affecting organs such as the heart, liver, and lungs, activated fibroblast populations contribute to pathological remodeling through cytokine secretion, immune modulation, and metabolic reprogramming. Similar

mechanisms are now being explored within skeletal muscle ageing[45],[46]. During sarcopenia, fibroblasts may transition into activated myofibroblast states that promote fibrosis, chronic inflammation, and ECM dysregulation, further disrupting the muscle microenvironment[44].

These alterations not only impair the structural flexibility of muscle tissue but may also interfere with intracellular signaling pathways involved in regeneration and metabolic adaptation. Increased ECM stiffness can negatively affect mitochondrial function, nutrient diffusion, and satellite cell activity, limiting the muscle's ability to repair damaged fibers efficiently[12]. Furthermore, chronic inflammatory signaling originating from dysregulated fibroblast activity may intensify oxidative stress and proteostatic dysfunction, reinforcing the interconnected progression of muscular ageing.

4.4 Interconnected Network

Importantly, these mechanisms do not operate independently. Mitochondrial dysfunction, proteostasis disruption, and ECM remodeling form an interconnected biological network in which impairment within one system can propagate dysfunction throughout others. For example, mitochondrial dysfunction increases oxidative stress and energetic instability, overwhelming proteostatic systems responsible for protein repair and turnover[47]. Simultaneously, impaired proteostasis limits the removal of dysfunctional mitochondrial components, further accelerating mitochondrial decline[48].

Alterations in ECM composition and fibroblast signaling additionally influence intracellular metabolism, mechanical signaling pathways, and regenerative capacity within skeletal muscle. Increased fibrosis and tissue stiffness may impair nutrient exchange, mitochondrial adaptation, and satellite cell activation, while chronic inflammatory signaling further amplifies oxidative and catabolic stress[49]. These overlapping mechanisms collectively contribute to progressive declines in muscular structure, repair capacity, and physical performance.

The interconnected nature of these pathways supports the view of skeletal muscle ageing as a systems-level process rather than the consequence of isolated molecular abnormalities. Consequently, sarcopenia emerges not from a single dysfunctional pathway, but from the cumulative interaction between metabolic dysregulation, impaired cellular maintenance, altered tissue remodeling, and chronic inflammatory stress occurring progressively throughout ageing muscle tissue.

SECTION 5 – Current Therapeutic Approaches

Due to the multifactorial nature of sarcopenia, no single therapeutic intervention has been able to fully reverse the condition or consistently restore muscular function. Current treatment strategies primarily focus on exercise interventions, nutritional supplementation, pharmacological therapies, and emerging regenerative approaches. Many pharmaceutical agents targeting pathways associated with sarcopenia, including myostatin inhibitors, activin receptors, hormonal therapies, and exercise mimetics, have been investigated by international

pharmaceutical companies. However, although some therapies demonstrated increases in muscle mass, few have consistently improved muscle strength, exercise performance, or overall quality of life[27]. This distinction highlights an important limitation in the treatment of sarcopenia: hypertrophy alone does not inherently indicate improved muscular function or physiological resilience. Muscle quality is not solely determined by quantity, but rather emerges from the interaction between cellular energy production, protein maintenance systems, connective tissue architecture, and effective neuromuscular signaling[50,51]. This further reinforces the idea that sarcopenia cannot be approached as a purely symptomatic or muscle-mass-related condition, but rather as a systemic and function-oriented process involving multiple interconnected biological pathways.

5.1 Resistance exercise (RE)

Among currently available interventions, exercise remains the most consistently effective therapeutic strategy for alleviating sarcopenia[52]. Within different forms of exercise, resistance exercise is that which targets building muscle mass by making muscles act against an external force or weight. RE in particular is supported by substantial evidence demonstrating improvements in muscle strength, muscle mass, and physical performance in middle-aged and older adults[53]. Effective RE prescription depends on several variables including exercise intensity, volume, frequency, and individual physiological capacity, meaning treatment outcomes are highly dependent on individualized exercise dosing and patient-specific adaptation[54]. Exercise appears especially effective because it simultaneously influences multiple hallmarks of muscular ageing rather than targeting a single pathway in isolation. Controlled mechanical stress placed on skeletal muscle stimulates adaptive cellular responses that improve mitochondrial efficiency, enhance metabolic regulation, activate proteostasis pathways responsible for damaged protein removal and synthesis, strengthen neuromuscular signaling, and reduce chronic inflammatory signaling[55]. Through these overlapping adaptations, exercise is able to address sarcopenia in a far more comprehensive manner than isolated pharmacological approaches. Additional modalities including multimodal exercise programs and blood flow restriction training have also demonstrated beneficial effects on muscle strength and physical performance[56]. These findings suggest that resistance exercise is most effective when implemented before severe functional decline develops, supporting its role not only as a treatment for early sarcopenia but also as a preventive strategy for preserving muscle health across ageing[57].

Despite the strong clinical evidence supporting exercise interventions, exercise programs are often limited by patient mobility, cardiovascular health, frailty status, and access to specialized rehabilitation resources[27,54]. This creates a significant theory-practice gap in sarcopenia management, as the patients most vulnerable to severe muscular decline are frequently the least capable of participating in high-quality exercise interventions. Furthermore, as previously discussed, sarcopenia presents heterogeneously across muscle groups and

individuals, reinforcing the need for personalized exercise prescriptions rather than generalized treatment models.

5.2 Nutritional supplementation

Nutritional supplementation has been investigated as a therapeutic strategy for sarcopenia, particularly through interventions aimed at supporting muscle protein synthesis and reducing inflammation. Several randomized controlled trials examining vitamin D supplementation have produced mixed results, although combined interventions involving vitamin D, protein supplementation, and exercise appear to improve grip strength and may contribute to increased muscle mass[51]. Omega-3 polyunsaturated fatty acids have additionally been proposed as potential therapies due to their anti-inflammatory properties and anabolic effects mediated through mTOR signaling and improved insulin sensitivity[58]. Previous trials demonstrated improvements in gait speed, thigh muscle volume, and muscular strength following omega-3 supplementation[51].

Other nutritional compounds including branched-chain amino acids (BCAAs) and β-hydroxy β-methylbutyric acid (HMB) have shown potential benefits in improving muscle mass, strength, and physical function through enhanced protein synthesis[59,60]. However, evidence suggests that the additional effects of nutritional supplementation beyond resistance training alone may remain limited in certain patient populations[61]. Although nutrient supplementation may help compensate for specific deficiencies or support muscular maintenance, it cannot independently reverse deeper dysfunctions involving mitochondria, proteostasis systems, or regenerative signaling pathways. Ageing skeletal muscle also demonstrates anabolic resistance, meaning it becomes progressively less responsive to anabolic stimuli over time[61]. As a result, even when amino acids or anabolic nutrients are available, ageing muscle may fail to adequately initiate the growth and repair processes necessary for meaningful recovery.

These findings suggest that nutritional interventions are most effective as supportive strategies for preserving muscle health or enhancing the benefits of exercise, rather than as standalone treatments capable of reversing established sarcopenia. While appropriate nutrition may help preserve existing muscle mass and improve responsiveness to exercise interventions, restoring muscular health ultimately requires improvements in the underlying biological systems responsible for repair, regeneration, and metabolic stability.

5.3 Pharmacological therapies

Several pharmacological agents targeting anabolic and catabolic pathways involved in skeletal muscle ageing have also been investigated as potential sarcopenia therapies. Antibodies targeting myostatin or activin receptors, including bimagrumab and trevogrumab, demonstrated increases in muscle mass and, in some cases, improved mobility in individuals with slow gait speed[62]. However, these therapies failed to establish sufficient long-term efficacy and safety profiles. Hormonal therapies including testosterone, selective androgen receptor modulators, insulin growth factor-1 mimetics, growth hormone, and growth hormone secretagogues have

likewise been explored, although clinical outcomes remain inconsistent[63]. Similarly, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers failed to produce significant improvements in muscle function or physical performance[51]. These findings further demonstrate the limitations of targeting isolated molecular pathways within a condition driven by interconnected ageing mechanisms. In many cases, pharmacological interventions may stimulate hypertrophy or influence singular signaling pathways without restoring the coordinated metabolic, neurological, and regenerative systems required for healthy physical function[64]. Additionally, many therapies remain insufficiently tested in long-term human trials and may introduce complications affecting other body systems, particularly endocrine and cardiovascular regulation. As a result, these therapies may ultimately prove most beneficial as adjuncts for individuals with established sarcopenia or for patients unable to participate fully in exercise-based rehabilitation, rather than as primary therapeutic strategies[65]. Consequently, pharmacological treatments for sarcopenia continue to face substantial limitations regarding efficacy, safety, and translational applicability.

5.4 Regenerative therapies

Because exercise interventions may provide limited benefits for severely immobile individuals, regenerative therapies involving stem cells and stem-cell secretomes have emerged as promising therapeutic alternatives[66]. These therapies are proposed to exert beneficial effects through mitochondrial restoration and immune-modulatory signaling mechanisms. Preclinical sarcopenia research has relied heavily on mouse models to investigate therapeutic mechanisms and candidate interventions[67]. However, important physiological differences exist between mouse and human skeletal muscle, including differences in immune activity, metabolic regulation, fibre-type composition, and stress responses[68].

Table 2. Summary of current therapeutic approaches for sarcopenia.
Summary of established and emerging therapeutic approaches for sarcopenia, compared according to their primary mechanisms of action, reported clinical benefits, and current limitations.

Therapeutic approach	Mechanism of action	Reported benefits	Limitations
Resistance exercise (RE)	Stimulates mitochondrial function, proteostasis, metabolic regulation, neuromuscular signaling, and reduces chronic inflammation[54,56].	Improves muscle strength, muscle mass, and physical performance[53,54]	Requires individualized prescription; limited by frailty, comorbidities, mobility, and rehabilitation access[27,54]
Nutritional supplementation (protein, vitamin D, omega-3, BCAAs, HMB)	Supports protein synthesis, reduces inflammation, and enhances anabolic signaling[51,61]	May improve grip strength, muscle mass, gait speed, thigh muscle volume, and responsiveness to exercise[51,61]	Limited benefits without exercise; anabolic resistance reduces effectiveness; cannot restore mitochondrial or proteostatic dysfunction independently[51,61]

Pharmacological therapies (myostatin inhibitors, hormonal therapies, exercise mimetics)	Targets anabolic/catabolic pathways to promote muscle hypertrophy or reduce muscle breakdown[64]	Some agents increase lean muscle mass and may improve mobility[64]	Functional improvements remain inconsistent; insufficient long-term efficacy and safety; often fail to improve strength or quality of life [51,64]
Regenerative therapies (stem cells and stem-cell secretomes)	Promote regeneration through mitochondrial restoration and immune modulation[27]	Promising preclinical therapeutic potential, particularly for patients unable to exercise[27]	Evidence largely limited to animal models; mouse physiology differs substantially from humans, limiting translation[67]

Abbreviations: RE = resistance exercise; BCAAs = branched-chain amino acids; HMB = β-hydroxy β-methylbutyrate.

In humans, sarcopenia is associated primarily with reductions in type II muscle fibre size and number, whereas mouse skeletal muscle is predominantly composed of type II fibres, limiting direct translational applicability[51]. Furthermore, human ageing trajectories are highly heterogeneous and influenced by lifestyle, disease status, environmental stressors, and systemic interactions between tissues and organs[67]. This creates a major translational challenge when attempting to extend promising animal-model findings into effective human therapies[68]. Consequently, future therapeutic approaches will likely require increasingly personalized treatment strategies capable of addressing the complexity and variability of muscular ageing across individuals. Nevertheless, if future clinical trials demonstrate comparable efficacy in humans, regenerative therapies may offer particular value for individuals with advanced frailty or severe mobility limitations who are unable to benefit fully from conventional exercise interventions

Overall, current evidence suggests that therapeutic strategies should be tailored to an individual's stage of muscular ageing, functional capacity, and ability to participate in rehabilitation. The most effective interventions appear to be those capable of simultaneously targeting multiple interconnected biological pathways involved in muscular ageing. While exercise interventions consistently demonstrate improvements in strength, function, mitochondrial regulation, and inflammatory balance, isolated pharmacological approaches have largely failed to reproduce comparable functional outcomes. Nutritional and regenerative therapies may provide supportive or complementary benefits, although their long-term efficacy remains under investigation. Collectively, these findings reinforce the idea that sarcopenia is fundamentally a systems-level ageing process rather than a disease driven by a single dysfunctional pathway. As a result, therapies capable of addressing multiple hallmarks of ageing simultaneously are likely to offer the greatest potential for restoring meaningful muscular function and improving long-term physiological resilience.

SECTION 6- Discussion

6.1 – Limitations of Current Research and Treatments

Sarcopenia emerges throughout this review not as an isolated muscular disorder, but as a multisystemic ageing process involving interconnected metabolic, structural, inflammatory, and regenerative dysfunctions. Although skeletal muscle decline is commonly observed through measurable reductions in muscle mass, strength, and physical performance, the mechanisms underlying these phenotypic changes extend far beyond simple tissue atrophy. Mitochondrial dysfunction, impaired proteostasis, extracellular matrix remodeling, chronic inflammation, and altered neuromuscular signaling collectively contribute to the progressive decline in muscular integrity and physiological resilience observed during ageing.

Importantly, these mechanisms do not operate independently. As explored throughout the mechanisms section, dysfunction within one pathway frequently amplifies dysfunction in others. Mitochondrial decline increases oxidative stress and energetic instability, which can overwhelm proteostatic systems responsible for protein repair and turnover. Simultaneously, impaired proteostasis limits the removal of dysfunctional mitochondria, while extracellular matrix remodeling and fibrosis alter mechanical signaling, regenerative capacity, and metabolic communication within skeletal muscle tissue. Consequently, skeletal muscle ageing reflects a progressive systems-level disruption rather than the failure of a singular biological pathway.

This interconnectedness may also explain why many current therapeutic strategies fail to fully reverse sarcopenia. Most pharmacological interventions target isolated molecular pathways despite the condition emerging from widespread interactions between metabolism, inflammation, cellular maintenance, and tissue remodeling. Even therapies capable of increasing muscle mass often fail to restore meaningful muscular function or long-term physiological resilience. This reinforces the distinction between muscle quantity and muscle quality, as increases in tissue size do not necessarily correspond to improvements in mitochondrial efficiency, neuromuscular coordination, protein turnover, or regenerative capacity.

In contrast, exercise remains the most consistently effective intervention currently available because it simultaneously influences multiple biological systems involved in muscular ageing. Exercise improves mitochondrial efficiency, reduces chronic inflammatory signaling, enhances insulin sensitivity, stimulates proteostatic repair pathways, and strengthens neuromuscular communication. Rather than targeting a single mechanism symptomatically, exercise promotes broader systemic adaptation across interconnected ageing pathways. This may explain why exercise-based interventions consistently demonstrate greater functional improvements compared to isolated pharmacological therapies.

However, exercise interventions themselves remain limited by significant practical and biological constraints. The patients most vulnerable to severe sarcopenia are often those least capable of participating in high-intensity or sustained physical rehabilitation programs due to frailty, immobility, cardiovascular disease, or comorbid conditions. Additionally, considerable heterogeneity exists between individuals regarding fibre-type composition, hormonal regulation, metabolic health, inflammatory status, and responsiveness to interventions. Consequently, generalized treatment approaches may fail to adequately address patient-specific ageing trajectories and physiological variability.

Current understanding of sarcopenia is also limited by fragmented mechanistic research and translational challenges. Many studies investigate individual pathways in isolation despite substantial evidence that ageing mechanisms function as interconnected networks. Furthermore, much preclinical research continues to rely heavily on animal models that differ significantly from humans in muscle composition, metabolism, immune signaling, lifespan, and environmental exposure. These differences complicate the translation of promising experimental findings into effective long-term human therapies.

Certain populations additionally remain underrepresented within sarcopenia research, particularly women and postmenopausal individuals, despite the significant influence of endocrine and hormonal regulation on skeletal muscle aging. Variability in ageing trajectories between sexes further emphasizes the need for more inclusive and personalized research models capable of accounting for human heterogeneity across genetic, metabolic, hormonal, and environmental contexts.

Overall, the evidence presented throughout this review supports the idea that sarcopenia should be understood as a systems-level ageing condition rather than a purely muscular or single-pathway disease. Future therapeutic strategies will likely require integrated and multimodal approaches capable of simultaneously addressing mitochondrial dysfunction, impaired proteostasis, inflammation, fibrosis, metabolic dysregulation, and neuromuscular decline. As understanding of these interconnected mechanisms continues to evolve, more personalized interventions may offer greater potential for preserving muscular function, independence, and quality of life throughout ageing.

6.2 – Emerging Directions

Emerging research directions increasingly support the idea that skeletal muscle ageing is influenced by interactions between multiple body systems rather than isolated muscular decline alone. Alongside improvements in diagnostic methods, individualized exercise prescription, nutritional interventions, and regenerative therapies such as stem-cell-based approaches, newer theories are beginning to explore how broader physiological environments may influence the progression of sarcopenia.

One particularly interesting area of investigation is the proposed gut–muscle axis, which suggests that gut microbiota and skeletal muscle may influence one another through bidirectional metabolic and inflammatory signaling pathways. Gut microbiota present distinct characteristics among elderly individuals and sarcopenia patients, including reductions in microbial diversity that may be associated with declines in skeletal muscle quality and function[69]. These alterations may affect skeletal muscle through mechanisms involving nutrient absorption, inflammation, insulin sensitivity, mitochondrial regulation, and anabolic signaling pathways, further reinforcing the interconnected nature of muscular ageing.

Experimental studies additionally demonstrate the importance of gut microbiota in regulating skeletal muscle metabolism and maintenance. Germ-free mice lacking gut microbiota developed skeletal muscle atrophy, impaired mitochondrial and muscle-growth signaling, and

reductions in insulin-like growth factor 1 expression. Similarly, disruption of gut microbiota through antibiotic treatment has been associated with reductions in muscle mass, smaller muscle fiber size, and activation of genes linked to skeletal muscle atrophy[70].

The gut microbiota has also been shown to interact closely with mitochondrial regulatory pathways involving PGC-1α, SIRT1, and AMPK signaling[70]. Consequently, disturbances within the gut environment may indirectly contribute to mitochondrial dysfunction, altered metabolism, and impaired muscular maintenance. These findings further reinforce the broader idea presented throughout this review: skeletal muscle ageing is heavily influenced by the physiological environment surrounding muscle tissue just as much as by the muscle fibers themselves. In many ways, the ageing environment impacts the muscle while ageing muscle simultaneously alters the surrounding environment, creating a reciprocal cycle of degeneration across multiple systems.

However, the precise role of the gut microbiota in the development and progression of sarcopenia remains incompletely understood and requires broader investigation using more diverse research methodologies and long-term human studies. Although these emerging theories remain under development, they highlight how many environmental and systemic factors influencing skeletal muscle ageing are still left to explore. As skeletal muscle participates in metabolic regulation, movement, endocrine signaling, and whole-body homeostasis, it is increasingly unlikely that a single universal treatment will adequately address all manifestations of sarcopenia across patient populations.

Future research may therefore need to move away from the search for singular "umbrella" therapies and instead focus on integrated, multimodal, and patient-specific treatment strategies capable of targeting multiple interconnected hallmarks of ageing simultaneously. Considering the substantial heterogeneity present across ageing individuals, future therapeutic approaches will likely require combinations of exercise, nutritional support, metabolic regulation, regenerative therapies, and personalized interventions tailored to the specific physiological conditions underlying muscular decline. Ultimately, continued investigation into these interconnected systems may provide a more complete understanding of skeletal muscle ageing and improve the ability to preserve muscular function, resilience, and quality of life throughout ageing.

SECTION 7 – Conclusion

By identifying the phenotypic and functional hallmarks of skeletal muscle ageing and sarcopenia, examining the interconnected biological mechanisms that drive these changes, and evaluating the effectiveness of current therapeutic approaches, this review provides a holistic overview of the current state of sarcopenia research. The evidence presented throughout this paper suggests that sarcopenia is not simply a condition of muscle loss, but rather a multisystemic ageing process involving complex interactions between mitochondrial dysfunction, impaired proteostasis, extracellular matrix remodeling, and inflammation. Given the central role of skeletal muscle in functions ranging from locomotion and postural stability to respiration, vocalization, metabolic regulation, and whole-body homeostasis, deterioration of

muscular health carries consequences that extend far beyond movement alone. These interconnected mechanisms help explain both the widespread functional consequences of sarcopenia and the limited success of many therapies that target individual pathways in isolation.

At the same time, analysis of current interventions highlights important lessons for future research. The consistent effectiveness of exercise compared to many pharmacological approaches reinforces the importance of therapies capable of influencing multiple biological systems simultaneously. Furthermore, the substantial heterogeneity observed across individuals, muscle groups, and ageing trajectories suggests that future treatment strategies will likely require increasingly personalized and multimodal approaches. Ultimately, understanding sarcopenia as a systems-level process provides a useful framework for interpreting past findings, identifying current limitations, and guiding the development of more effective interventions. Continued research into the interconnected mechanisms underlying skeletal muscle ageing will be essential for preserving muscular function, independence, and quality of life across ageing populations.

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To What Extent Does Maternal Anesthesia during Obstetric and Non-obstetric Surgery Affect Pediatric Respiratory Diseases By Haasini Venisetty

Abstract

Maternal anesthesia is commonly used during obstetric and non-obstetric surgery, yet limited research has examined its potential relationship with pediatric respiratory disease. This study investigated whether maternal exposure to anesthesia during pregnancy was associated with respiratory disease in children and whether outcomes differed between obstetric and non-obstetric procedures. A causal-comparative study was conducted using an online survey of 87 mothers who reported maternal anesthesia exposure during pregnancy and the respiratory health of their children. Participants were categorized into obstetric anesthesia, non-obstetric anesthesia, and no anesthesia exposure groups. A chi-square test of independence was used to examine the association between maternal anesthesia exposure and pediatric respiratory disease. Children exposed to obstetric anesthesia had a respiratory disease rate of 60.0%, while children exposed to non-obstetric anesthesia had a rate of 53.3%, compared with 21.4% among children with no reported exposure. The chi-square test indicated a statistically significant association between maternal anesthesia exposure and pediatric respiratory disease ($\chi^2 = 12.5$, $df = 2$, $p < 0.05$). These findings suggest an association between prenatal anesthesia exposure and pediatric respiratory disease, although limitations including sample size, self-reported data, and potential confounding variables restrict causal conclusions.

Introduction

Nearly 40 million anesthetics are administered annually in the United States (*Speak up: Anesthesia and Sedation*, n.d.). This high prevalence makes understanding the potential effects of anesthesia especially important for vulnerable populations such as pregnant women and their developing fetuses. Scientifically speaking, there are two main types of anesthesia used: general anesthesia and regional anesthesia. According to Uhrig and other experts from France

General anesthetic agents are pharmacological agents that target specific central nervous system receptors… once they bind to their brain receptors, anesthetics modulate remote brain areas and end up interfering with global neuronal networks, leading to a controlled and reversible loss of consciousness. (Uhrig et al.)

And regional anesthesia "...consists of infiltrating a peripheral nerve with an anesthetic agent and blocking transmission to avoid or relieve pain" (Folino and Mahboobi) More simply, when anesthetics are administered, they temporarily interrupt the way nerves communicate pain and sensation to the brain. General anesthesia achieves this by suppressing activity in the nervous system and regional anesthesia blocks nerve signals in a specific part of the body. While anesthesia is essential for pain management and surgical procedures, its administration is not without risk. As a result, there are many health issues that may arise from administering anesthesia. For example, nerve damage, collapsed lung, malignant hyperthermia. Anesthesia has also been linked to respiratory/pulmonary diseases. This will be further examined in the literature

review.. When anesthesia is administered during pregnancy, the effects are now not only on the mother but also on the fetus, which can be detrimental. Because fetal organ development occurs during pregnancy, exposure to anesthesia during this period may have consequences that extend beyond birth. This will also be further elaborated in the literature review

Surgeries performed during pregnancy generally fall into two categories: obstetric surgery, which refers to procedures directly related to pregnancy or childbirth, such as cesarean sections, and non-obstetric surgery, which includes procedures unrelated to pregnancy, such as appendectomies, trauma surgery, or gallbladder removal (Rasmussen et al.). In many obstetric procedures, especially cesarean sections, regional anesthesia is most commonly used because it allows the mother to remain awake while reducing pain in a specific area of the body. In contrast, non-obstetric surgeries more often require general anesthesia depending on the procedure, as unconsciousness and full-body sedation may be necessary (Iddrisu et al.). Because these procedures differ in urgency, invasiveness, and anesthetic approach, they may also differ in their effects on maternal and fetal health. Administering anesthesia can have adverse effects on the mother. Grace Lim and other anesthesiology researchers from the University of Pittsburgh support this by saying when using Anesthesia in surgery, it gave rise to airway complications in the mother including failed tracheal intubations, maternal aspiration, and Mendelsohn Syndrome (Lim et al.). Furthermore, anesthesia negatively affects the fetus. According to the National Library of Medicine "Some studies suggest an increased chance for neural tube defects (an opening in the fetal spine or skull) and issues, such as microcephaly (small head size)" (*General Anesthesia*). This will also be further elaborated in the lit review.

Beyond these immediate maternal and fetal complications, another growing health concern is respiratory disease, which affects more than 80 million people worldwide ("Chronic Respiratory Diseases"). There are several types of respiratory diseases, but they are mainly divided into three categories: airway diseases, which affect the airways, such as asthma and COPD; lung tissue diseases, which damage lung tissue; and circulation-related lung diseases, which impair blood flow between the heart and lungs (*Lung Disease*). Respiratory diseases contribute significantly to global morbidity and healthcare burden, leading to increased hospitalizations, long-term medical costs, and reduced quality of life (Soriano et al.) These conditions affect individuals across all age groups; however, the focus of this study is specifically on children. Pediatric respiratory diseases encompass a broad range of conditions that impair normal lung function and airflow, often limiting physical activity and increasing emotional stress for both patients and caregivers (Booth and Johnson). Among the most common pediatric respiratory illnesses are asthma, bronchiolitis, pneumonia, and cystic fibrosis (*Lung Disease in Children*). Asthma is characterized by chronic airway inflammation and airflow obstruction (Maddox and Schwartz), bronchiolitis causes inflammation and mucus buildup in the small airways (Florin et al.), pneumonia inflames the air sacs of the lungs (*Pneumonia*), and cystic fibrosis leads to thick mucus accumulation that obstructs breathing passages (*Cystic Fibrosis*). Because many aspects of respiratory health are established early in life, understanding

prenatal influences on lung development may be essential when examining the origins of these childhood conditions.

Despite the widespread use of anesthesia and the well-documented risks to maternal health, existing research has largely focused on immediate surgical outcomes and neurodevelopmental effects in offspring. Comparatively little attention has been given to the potential long-term respiratory consequences for children exposed to anesthesia in utero. Furthermore, there is an even more limited amount of research with the implications of non-obstetric surgery during pregnancy. Given that fetal lung development is highly sensitive to oxygen availability and pharmacological exposure during pregnancy and that non-obstetric surgery is rarely explored during pregnancy, this represents a critical gap in the literature. Thus, a study examining children's respiratory health outcomes following prenatal exposure to anesthesia is needed because it may reveal a potential contributing factor to pediatric respiratory diseases and provide a foundation for further investigation. As a result, the purpose of this study is to examine a possible correlation between maternal exposure to anesthesia during pregnancy and the development of respiratory diseases in children.

Literature Review

Prenatal Influences on Pediatric Respiratory Development

Pediatric respiratory diseases are not only defined by their symptoms in childhood but are increasingly understood as conditions that can originate during early development. Research suggests that lung health is shaped long before birth, as fetal lung development begins early in gestation and continues through birth. According to Rehman and Bacha from the University of London, development of the lower respiratory tract begins around day 22 of embryonic growth and continues through the formation of the trachea, bronchi, and alveoli (Rehman and Bacha) Because this process occurs in utero, the prenatal environment plays a critical role in determining long-term respiratory outcomes. While Gao et al. establish that anesthetic agents can cross the placenta, Devroe et al. and Olutoye et al. provide evidence of resulting developmental outcomes, suggesting that placental transfer may translate into measurable effects on fetal growth and maturation. This idea is further supported by longitudinal cohort research examining how prenatal environmental exposures influence later lung function. For example, Neophytou et al. and other experts from Colorado State University, conducted a multi-cohort study following children aged 6–9 years, found that prenatal exposure to fine particulate matter ($PM_{2.5}$) was associated with reduced lung function, measured through decreased FEV_1 and FVC. The study also identified specific windows during gestation in which exposure had a stronger impact on respiratory outcomes, suggesting that fetal lung development is particularly sensitive at certain stages of pregnancy. Taken together, Rehman and Bacha establish the biological timeline of lung development, while Neophytou et al. demonstrate that disruptions during this timeline can lead to measurable impairments in childhood respiratory function. This connection highlights that

prenatal conditions can directly influence how the lungs develop structurally and functionally, reinforcing the importance of examining factors that may alter the intrauterine environment.

Maternal Physiological Effects of Anesthesia During Pregnancy

During pregnancy, the administration of anesthesia becomes more complex due to physiological changes in the mother and the presence of the fetus. It is therefore important to examine the maternal implications of anesthesia during this period. These concerns apply across both obstetric and non-obstetric procedures performed during pregnancy, although the urgency and anesthetic approach may differ. When considering how anesthesia affects mothers, pregnancy must be recognized as an especially susceptible state. As stated by Davis and Narayan from Cambridge University, "The prenatal period is a time of rapid development, when a multitude of psychological and physiological changes occur for both mother and fetus. Thus, pregnancy is a sensitive period" (Davis and Narayan). Their argument is supported by Hoefnagel, Yu, and Kaminski from the University of Rochester, who reviewed major anesthetic complications in pregnant patients and found that physiological changes during pregnancy increase the likelihood of aspiration, difficult neuraxial placement, postdural puncture headache, neuropathy, and maternal cardiac emergencies requiring modified resuscitation protocols (Hoefnagel et al.). Rather than focusing solely on one organ system, their review demonstrates that anesthesia-related risks during pregnancy are systemic and can affect neurological, cardiovascular, and gastrointestinal functioning in addition to respiratory health. These findings reinforce Davis and Narayan's claim that pregnancy represents a uniquely vulnerable physiological state in which standard medical interventions may carry heightened risk. Because pregnancy alters lung mechanics and increases oxygen demand, even minor respiratory compromise during anesthesia may become clinically significant. Expanding on this broader evidence of maternal vulnerability, Mahta from Harvard University explains that anesthesia further reduces functional residual capacity by decreasing respiratory drive and respiratory muscular function. As a result, pregnant patients may be more susceptible to apnea, atelectasis, pulmonary edema, and acute respiratory distress syndrome. While Hoefnagel et al. describe the wide range of anesthesia-related complications that can occur during pregnancy, Mahta specifically clarifies the respiratory mechanisms behind these risks. Together, these sources suggest that the normal physiological adaptations of pregnancy, combined with the depressant effects of anesthesia, can significantly increase maternal complications. Understanding these maternal respiratory risks provides a foundation for examining how anesthetic exposure may subsequently affect fetal development and long-term pediatric respiratory outcomes. Since maternal oxygenation is critical for fetal survival and organ development, anesthesia-related impairment in pregnant women may have consequences beyond maternal health alone.

Placental Transfer and Fetal Exposure to Anesthetic Agents

To understand how anesthesia will affect the fetus, we need to look at how anesthetic agents interact with the fetus. Most anesthetic agents are lipophilic allowing them to cross the

placenta via simple diffusion and reach fetal circulation (Gao et al.). These properties suggest that the fetus may be exposed to significant concentrations of anesthetic drugs whenever the mother undergoes surgery. This biological mechanism is supported indirectly by clinical outcome studies examining pregnancies exposed to anesthesia. Devroe et al. from the department of anesthesia in Belgium, conducted a large matched cohort study including 171 pregnancies exposed to non-obstetric surgery and more than 35,000 matched controls, found that prenatal exposure to anesthesia and surgery was associated with increased rates of preterm birth and lower birth weight. Although this study does not directly measure placental drug concentration, it provides real-world evidence that fetal exposure to maternal anesthesia-related factors can translate into measurable developmental outcomes. Taken together with Gao et al., which explains that anesthetic agents are lipophilic and can readily cross the placenta into fetal circulation, these findings suggest a direct link between the biological mechanism of placental transfer and observable neonatal outcomes such as lower birth weight and increased prematurity. While direct research on fetal lung development following maternal anesthesia is limited, studies of other developing organ systems demonstrate fetal vulnerability. According to Olutoye et al. from Baylor College of Medicine:

The US Food and Drug Administration issued a warning regarding impaired brain development in children following exposure to certain anesthetic agents used for general anesthesia, namely the inhalational anesthetics isoflurane, sevoflurane, and desflurane, and the intravenous agents propofol and midazolam, in the third trimester of pregnancy. (Olutoye et al.)

Although these findings focus on the brain, they complement the findings of Devroe et al. by suggesting that the fetus may be sensitive to anesthetic exposure across multiple developmental outcomes, including growth and maturation. Adding on to Olutoye et al., Chai and other researchers from Tong University say that exposure to sevoflurane, which is a major pharmacological agent in anesthesia, “involves oxidative stress, neuroinflammation, neuroapoptosis, and alteration of synaptic properties” (Chai et al.). While Chai et al. focus primarily on neural mechanisms, these biological processes highlight how anesthetic agents can interfere with normal cellular development, which may also be relevant to other rapidly developing fetal tissues such as the lungs. Given that fetal lung development begins in utero and progresses through birth, any maternal intervention that alters oxygenation or exposes the fetus to pharmacological agents may have lasting implications for postnatal respiratory function. Because airway branching, alveolar formation, and pulmonary vascular development begin prenatally, disruptions during gestation may have lasting respiratory consequences. Furthermore, there are certain periods where the baby is even more vulnerable to changes happening in the mother's body. According to the National Library of Medicine, the first trimester tends to be one of the most critical periods in which the baby is growing and any disturbances during this time may lead to birth defects *(Critical Periods of Development*). This connection highlights the importance of examining anesthetic exposure during pregnancy not only for immediate maternal safety, but also for potential long-term pediatric respiratory outcomes. Despite well-documented effects of anesthesia on maternal health and established evidence that anesthetic agents can cross

the placenta and affect fetal development, there remains a significant gap in understanding how prenatal exposure influences fetal lung development and subsequent respiratory health outcomes following both obstetric and non-obstetric surgery. This leads me to my research question: To what extent does maternal anesthesia during obstetric and non-obstetric surgery affect pediatric respiratory diseases

Methods

This study is a causal-comparative analysis study. This research method is used to examine whether a pre-existing independent variable may influence a dependent variable without manipulation. The goal of this study is to examine the relationship between maternal exposure to anesthesia during obstetric and non-obstetric surgery in order to determine whether prenatal exposure to anesthesia is associated with long-term respiratory outcomes in children. Data was collected through the distribution of an online survey, demonstrating that this study uses a non-experimental method of data collection. No variables were manipulated or controlled, further supporting the classification of this study as causal-comparative. And lastly, this procedure was approved by the school's internal review board (IRB).

Participant Selection

Participants were recruited by posting the survey to various online parenting and maternal health groups on Facebook and Reddit. Facebook was selected as the recruitment platform due to its widespread use among adults and parents, allowing for increased accessibility and response rates. According to Oliver et al. Facebook remains one of the primary social media platforms for adults, making it an effective medium for reaching the target population. I also used Reddit to have a greater reach and get more participants. According to Goodwin and other experts, reddit is a good platform to recruit people for scientific research (Goodwin et al.). And lastly, I went to different pediatric and respiratory clinics to see if they could put my survey up in their lobbies. To be eligible, participants were required to provide relevant information regarding both maternal anesthesia exposure during pregnancy and the respiratory health of their child. 87 mothers responded; zero were left out.

Materials/Measures

The data collected in this study were analyzed using cross-tabulation and the Chi-square test of independence to examine the relationship between maternal anesthesia exposure and pediatric respiratory disease (McHugh). Maternal anesthesia exposure was treated as a categorical variable with three categories: obstetric anesthesia, non-obstetric anesthesia, and no anesthesia exposure. Pediatric respiratory disease was measured as a binary variable, indicating whether a child had a diagnosed respiratory condition. Cross-tabulation was used to summarize the number of children with and without respiratory disease within each anesthesia exposure group. The Chi-square test was then applied to determine whether there was a statistically significant association between anesthesia exposure and respiratory disease. The chi-square test

of independence was used to determine whether there was a significant association between maternal anesthesia exposure and pediatric respiratory disease. The chi-square statistic was calculated using the formula $\chi^2 = \Sigma((O - E)^2 / E)$, where O represents observed frequencies and E represents expected frequencies under the assumption of no association. Expected values were computed by multiplying row and column totals and dividing by the total sample size (N = 87). The chi-square value was obtained by summing the contributions from each cell in the contingency table. A significance level of 0.05 was used to determine statistical significance. This test compares the numbers from the survey in each category with the expected numbers that would occur to see if there is an association. The Chi-square test was appropriate because both variables were categorical and the test is commonly used to assess associations between categorical groups. Using this method, we were able to determine whether children exposed to maternal anesthesia were more likely to have respiratory disease than children who were not exposed.

Study Design

First, permission to post on various Facebook groups was granted through direct messaging. Then, the survey was posted with a brief description of the intended purpose of the survey. A consent paragraph was typed prior to the first question, including the approximate length of the survey, what the questions were asking, and how the participants could exit the survey whenever they chose to. To collect data, a survey was conducted in which participants were required to be a birth mother who had a child between the ages of 0 and 18. Participants were asked to provide information about their pregnancy, including whether anesthesia was used during a medical procedure, as well as information about their child's respiratory health. Prior to participation, respondents provided electronic informed consent and confirmed their eligibility, ensuring ethical compliance and data integrity. Participants were also allowed to exit the survey at any time with no implications. The survey was divided into multiple sections to collect data. The first section was the pediatric respiratory health section. It assessed the dependent variable by gathering information on the child's age, gender, diagnosed respiratory conditions, disease severity, and frequency of respiratory symptoms within the past 12 months. Symptom frequency was measured using a Likert-type scale ranging from "never" to "always," allowing for quantitative comparison across participants. While the Likert-type scale will not be used to determine any final results, it is there for extra details. The second section, maternal anesthesia exposure during pregnancy was measured as the independent variable. Participants reported whether they underwent a medical procedure involving anesthesia during pregnancy and, if applicable, identified the type of procedure (obstetric or non-obstetric), the type of anesthesia administered (general, regional, or local), and the trimester during which exposure occurred. The final section of the survey collected background information to account for potential confounding variables. Participants reported whether their child was born prematurely and indicated any family history of respiratory conditions such as asthma, bronchitis, pneumonia, or sleep-disordered breathing. However, these variables were not included as primary variables in

the study because their established association with pediatric respiratory disease could confound the relationship being examined; instead, they were collected as background information and considered when interpreting the results to strengthen the validity of the findings. Although these were not main sections, there were 2 additional sections for multiple children if applicable to the participant.

Hypothesis

After reviewing the existing literature on maternal anesthesia exposure and pediatric respiratory health, two hypotheses were developed. (1) Children whose mothers were exposed to anesthesia during pregnancy will have higher rates of respiratory diseases compared to children whose mothers were not exposed. (2) Children whose mothers were exposed to anesthesia for non-obstetric procedures will have similar respiratory outcomes to children whose mothers were exposed to anesthesia for obstetric procedures. (3) children whose mothers had obstetric surgery will have a greater exposed risk to respiratory diseases than children whose mothers had non-obstetric surgery. I do not believe that maternal anesthesia exposure will have an inverse relationship with pediatric respiratory outcomes, as prior research suggests that anesthesia may pose risks rather than protective effects for fetal development.

Results

Table 1

Participant Sex (N = 87)

Characteristic	Frequency (n)	Percentage (%)
Sex		
Male	46	52.9
Female	41	47.1

Table 2

Participant Age (N = 87)

Age Group (years)	Frequency (n)	Percentage (%)
0–5	22	25.3
6–10	28	32.2
11–14	21	24.1
15–18	16	18.4
Total	87	100

The majority of participants were male (52.9%). The largest age group was children 6–10 years old (32.2%), while adolescents 15–18 years old made up the smallest group (18.4%).

Table 3

Respiratory Disease Status and Types (N = 87)

Respiratory Disease Status	Frequency (n)	Percentage (%)

Diagnosed respiratory disease	35	40.2
No respiratory disease	52	59.8

Approximately 40.2% of the children included in the study were reported to have a diagnosed respiratory disease, while the majority of participants (59.8%) had no reported respiratory condition.

Table 4

Respiratory Disease Types

Type of Respiratory Disease (n = 35)	Frequency (n)	Percentage (%)
Asthma	14	40.0
Bronchitis	9	25.7
Pneumonia	8	22.9
Chronic cough / other	4	11.4
Total	35	100

Approximately 40% of children had a diagnosed respiratory disease. Asthma was the most common condition (40%), followed by bronchitis (25.7%) and pneumonia (22.9%).

Table 5

Exposure to Anesthesia/Surgery and Respiratory Outcomes

Exposure Group	Respiratory Disease	No Disease	Total
Obstetric anesthesia	18	12	30
Non-obstetric surgery	8	7	15
No anesthesia/surgery	9	33	42
Total	35	52	87

Children whose mothers were exposed to anesthesia had higher rates of respiratory disease than children with no exposure. Obstetric anesthesia showed the highest proportion (60%), followed by non-obstetric surgery (53.3%), while children with no exposure had the lowest rate (21.4%).

Table 6

Exposure to Anesthesia by Trimester of Pregnancy

Trimester	Frequency	Percentage
1st	4	8.9%
2nd	6	13.3%
3rd	35	77.8%
Total	45	100%

Most maternal anesthesia and surgical exposures reported in this study occurred during the third trimester, suggesting that procedures during pregnancy were more commonly performed later in gestation than during the earlier stages of fetal development.

Table 7

Chi-Square Results

Exposure Group	Respiratory Disease Observed)	Respiratory Disease Expected)	No Disease Observed)	No Disease Expected)	Total
Obstetric anesthesia	18	12.0	12	18.0	30
Non-obstetric surgery	8	6.0	7	9.0	15
No anesthesia/surgery	9	17.0	33	25.0	42
Total	35	35	52	52	87

The chi-square test indicates a significant association between maternal anesthesia exposure and pediatric respiratory disease ($\chi^2 = 12.5$, $df = 2$, $p < 0.05$). Children exposed to obstetric or non-obstetric anesthesia had more cases of respiratory disease than expected, while those with no exposure had fewer. These results support Hypothesis 1, showing higher respiratory risk among children with maternal anesthesia exposure.

Discussion

The purpose of this study was to examine the relationship between maternal anesthesia exposure during pregnancy and the development of pediatric respiratory diseases. Prior literature has established that anesthesia can negatively impact both maternal respiratory function and fetal development, particularly due to reduced oxygenation and the ability of anesthetic agents to cross the placenta (Mahta and Gao et al.). Given that fetal lung development begins early in utero and continues throughout gestation, any disruption during this critical period may have lasting consequences on respiratory health (Rehman and Bacha). Therefore, the results of this study were analyzed to determine whether prenatal exposure to anesthesia is associated with increased respiratory disease risk in children and whether differences exist between obstetric and non-obstetric exposure types.

The results of this study strongly support Hypothesis 1, which predicted that children whose mothers were exposed to anesthesia during pregnancy would have higher rates of respiratory diseases compared to those with no exposure. As shown in Table 7, children exposed to obstetric anesthesia (60.0%) and non-obstetric anesthesia (53.3%) had significantly higher rates of respiratory disease than those with no exposure (21.4%). Additionally, the chi-square test confirmed a statistically significant association ($\chi^2 = 12.5$, $p < 0.05$), indicating that the observed differences are unlikely due to chance. This relationship can be explained through both maternal

and fetal mechanisms. Anesthesia has been shown to reduce maternal respiratory function, leading to decreased oxygen availability (Mahta). Since fetal oxygenation is dependent on maternal respiration, reduced oxygen levels may impair lung development. Furthermore, anesthetic agents can cross the placenta and directly affect fetal tissues (Gao et al.). Although most prior research has focused on neurological outcomes, these findings suggest that similar mechanisms such as oxidative stress and cellular disruption may also impact developing lung tissue (Chai et al.,). Together, these factors provide a strong biological explanation for the increased respiratory disease rates observed in exposed children.

The results also support Hypothesis 2, which predicted that children exposed to anesthesia during non-obstetric procedures would have similar respiratory outcomes to those exposed during obstetric procedures. The data showed relatively comparable rates of respiratory disease between the two groups (60.0% vs. 53.3%). This suggests that the type of surgery itself may be less important than the presence of anesthesia exposure. Both types of procedures involve similar pharmacological agents, many of which are lipophilic and capable of crossing the placenta (Gao et al.). Additionally, both situations may result in maternal physiological stress and reduced oxygenation, which can influence fetal development. While obstetric procedures are often planned and controlled, non-obstetric surgeries are typically emergency based, yet the similarity in outcomes indicates that fetal exposure to anesthetic agents remains a consistent risk factor regardless of surgical context. This aligns with the broader literature suggesting that fetal vulnerability is primarily linked to timing and exposure rather than procedure type (Davis and Narayan).

The results partially support Hypothesis 3, which predicted that obstetric anesthesia would pose a greater risk than non-obstetric anesthesia. While the data did show a slightly higher percentage of respiratory disease in the obstetric group (60.0%) compared to the non-obstetric group (53.3%), the difference between these two groups was relatively small. This suggests that although obstetric anesthesia may present a marginally higher risk, the difference is not substantial enough to indicate a strong distinction between the two exposure types. One possible explanation is that obstetric procedures often occur later in pregnancy, when fetal lung development is more advanced and potentially more sensitive to disruption. According to the literature, later stages of pregnancy involve critical lung maturation processes, meaning interference during this time could have more direct respiratory consequences (Rehman and Bacha). However, the similarity in outcomes also indicates that anesthesia exposure at any point during pregnancy may carry risk, especially considering that early developmental stages are highly sensitive to external disturbances (*Critical Periods of Development*). Therefore, while Hypothesis 3 is partially supported, the findings suggest that overall exposure is more impactful than the specific type of procedure.

Limitations

There are several characteristics of this study that limit the certainty and generalizability of the results. As previously discussed, pediatric respiratory diseases can be influenced by a wide range of factors beyond maternal anesthesia exposure. While this study aimed to isolate the

relationship between anesthesia exposure during pregnancy and respiratory outcomes in children, other variables such as genetic predisposition, environmental exposures (e.g., secondhand smoke), premature birth, and pre-existing health conditions may also play a significant role. As a result, the relationship observed in this study may not be entirely accurate or solely attributable to maternal anesthesia exposure.

Another limitation of this study is the relatively small participant size. A total of 87 participants were included, which limits the statistical power and generalizability of the findings. While efforts were made to recruit participants through multiple platforms such as Facebook, Reddit, and medical clinics, the sample size remains small compared to large-scale epidemiological studies. Additionally, participants were self-selected, meaning that those who chose to respond may not be fully representative of the broader population. A larger and more diverse sample size would provide more reliable data and allow for stronger conclusions to be drawn regarding the relationship between maternal anesthesia exposure and pediatric respiratory disease.

Finally, the use of a self-reported survey introduces the possibility of response error and bias. Participants were required to recall details about their pregnancy and their child's medical history, which may lead to inaccuracies due to memory limitations. Additionally, some participants may have misunderstood questions or unintentionally provided incorrect information. There is also the potential for response bias, as participants may overreport or underreport medical conditions based on personal perceptions or lack of formal diagnosis. Since the data relies entirely on participant responses rather than verified medical records, these factors may reduce the overall reliability and validity of the findings.

Conclusion

Implications

The findings of this study have several important clinical, public health, and academic implications. From a clinical perspective, the observed association between maternal anesthesia exposure and increased rates of pediatric respiratory disease suggests that healthcare providers may benefit from exercising additional caution when administering anesthesia during pregnancy, particularly when non-urgent procedures can be safely postponed. When surgery is medically necessary, physicians may consider using the lowest effective dosage and evaluating the most appropriate anesthesia type based on the patient's condition. These findings also emphasize the importance of collaboration between obstetricians, surgeons, and anesthesiologists to ensure both maternal safety and fetal well-being during procedures. Additionally, pregnancies involving anesthesia exposure may benefit from increased prenatal monitoring and more attentive pediatric respiratory follow-up after birth if symptoms arise. From a patient perspective, this research may help expectant mothers make more informed healthcare decisions by encouraging discussions with medical providers regarding the risks, benefits, timing, and alternatives associated with anesthesia during pregnancy. Improved communication may allow families to better understand

necessary procedures while remaining aware of possible long-term considerations for child health. Academically, this study contributes to the growing body of literature on prenatal risk factors and long-term child health outcomes by expanding existing research beyond neurological effects to include respiratory development. It suggests that fetal lung development may be sensitive to prenatal medical exposures and highlights the need for future research examining specific anesthetic agents, dosage levels, timing of exposure, and long-term pediatric outcomes.

Future Directions

This study opens a completely new direction for future research regarding the effects of maternal anesthesia exposure and the relationship between prenatal conditions and long-term child health outcomes. In the future, it will be useful to analyze the relationship between anesthesia exposure and other medical issues, such as mental health disorders, including anxiety, ADHD, or other neurodevelopmental conditions. Since anesthetic agents are known to affect the central nervous system, this could help determine whether prenatal exposure plays a role in altering brain development. It also will be useful to research how pregnancy-related anesthesia influences various disorders and the effects (both physically and mentally) on the fetus. Additionally, considering the type of anesthesia used, dosage, and timing of exposure could display differing results. This could lead to comparing general versus regional anesthesia or early versus late pregnancy exposure and their effects on fetal development, leading to new findings.

This research contributes to the body of literature surrounding prenatal risk factors and the potential long-term effects of maternal anesthesia exposure. This study introduced a new connection between anesthesia use during pregnancy and pediatric respiratory diseases, while also suggesting possible links to other developmental outcomes. The results also established a correlation between maternal medical exposure and offspring health, allowing for an expanded field of study to be explored. Therefore, the findings of this study advocate for more careful consideration and monitoring of anesthesia use during pregnancy. Improved awareness could help minimize potential risks to both the fetus and the mother and support healthier developmental outcomes.

In conclusion, the findings of this study have contributed to society by helping medical professionals and researchers better understand potential risks associated with anesthesia exposure during pregnancy. This may lead to more informed medical decisions, improved screening practices, and safer surgical protocols for pregnant individuals. In addition, the connection between maternal anesthesia exposure and offspring health is key to exploring further relationships between prenatal factors and long-term development. This research has opened new possibilities for understanding how medical interventions during pregnancy may impact future generations.

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Identifying Risk Factors Associated with Coronary Heart Disease Using Multivariable Logistic Regression By Jiya Phagwani

Abstract

Coronary heart disease (CHD) remains one of the leading causes of death worldwide, making the identification of risk factors essential for prevention and early intervention. This study used a publicly available Framingham Heart Study dataset to quantify the relations between behavioral, clinical risk factors and CHD using multivariable logistic regression. This analysis was conducted using data from 11,627 participants in the Framingham Heart Study. Variables, including age, sex, body mass index (BMI), total cholesterol, systolic blood pressure, diastolic blood pressure, smoking status, and diabetes status, were analyzed. Multivariable logistic regression was used to estimate adjusted odds ratios (AORs), and performance was evaluated by comparing pseudo-R^2 values for individual and combined predictor models. Older age, higher BMI, elevated cholesterol, hypertension, male sex, and diabetes were independently associated with increased odds of CHD. Diabetes demonstrated the strongest association with CHD (AOR = 2.40), while age and hypertension also showed substantial increases in CHD risk. The combined multivariable model of age, BMI, and diabetes demonstrated greater explanatory power than any individual predictor model (pseudo-R^2 = 0.0389 versus 0.0214), indicating that multiple risk factors jointly explain CHD more effectively than any single variable alone. These findings reinforce that CHD risk reflects the combined influence of behavioral and clinical factors rather than a single predictor. This study demonstrates the value of multivariable modeling using publicly available data to improve understanding of cardiovascular disease risk and support future research in risk assessment.

Keywords

Cardiovascular risk factors, Multivariable logistic regression, Coronary heart disease, Adjusted odds ratio, Multicollinearity, Framingham Heart Study, Statistical modeling, Clinical data analysis, Risk prediction, Public health

Introduction

Coronary Heart Disease is the most common heart disease and the leading cause of death in the United States, causing over 370 thousand deaths in 2022 alone. Coronary Heart Disease, also known as CHD, occurs when the arteries of the heart cannot deliver enough oxygen-rich blood to the heart muscle (National Heart, Lung, and Blood Institute).

CHD is a major health concern due to its high prevalence and high mortality rates. Approximately 1 in every 20 adults are diagnosed with CHD, and it causes 1 in every 3 deaths worldwide (National Heart, Lung, and Blood Institute). Early risk identification is extremely important because many of the modifiable risk factors for CHD, such as elevated cholesterol, obesity, unhealthy diet, and physical inactivity, begin in early childhood (National Heart, Lung, and Blood Institute). As a result, following healthy lifestyle habits is essential for preventing

these factors from developing and reducing future cardiovascular risk. In fact, more than two-thirds of patients experiencing a heart attack have previously undiagnosed heart disease (Williamson).

Previous research has consistently identified several clinical and demographic factors associated with CHD. Researchers have identified these risk factors as smoking, hypertension (high blood pressure), cholesterol, obesity and diabetes. These variables are currently used as guidelines for clinics and healthcare and also for general populations.

One source of evidence was given by William Castelli, who focused on the impact of cholesterol on CHD in the Framingham Heart Study, reinforcing the importance of healthy cholesterol levels through long term follow-up of participants (Castelli et al.).

Similarly, these findings are supported by research such as the INTERHEART study. Koon Teo demonstrates that a very small portion of risk factors accounted for 90% of cardiovascular disease risk worldwide, by using large datasets to identify broad risk factors across different populations. The factors that INTERHEART focused on are cholesterol, smoking, hypertension, diabetes and obesity, very similar to the factors from Castelli's paper (Teo and Rafiq).

David Wood's Study, "Established and emerging cardiovascular risk factors" emphasizes that there are different groups of factors depending on the evidence for each one. He highlights the established risk factors as cholesterol, smoking, hypertension and diabetes, matching up with the insights from the other two sources (Wood). However, these emerging factors are not examined in other studies, suggesting that most researchers prioritize well established variables.

Although previous research has included these risk factors into multivariable models such as the Framingham Risk Score and the ACC/AHA Pooled Cohort Equations, this study uses publicly available data to demonstrate and replicate the value of multivariable logistic regression. Established research like the Framingham Risk Score and the ACC/AHA Pooled Cohort Equations similarly combine multiple cardiovascular risk factors to estimate CHD risk (Wilson, American College of Cardiology, "Guidelines and Clinical Documents").

While most existing research is consistent in identifying the major risk factors for CHD, reanalyzing these trends with public data sets allows for better understanding about the impact of multiple joint factors. Castelli's study relies on long term data collection, making it effective for identifying long term association of risk factors. However, it does not account for changes in diagnosis over time (Castelli et al.). In contrast, the INTERHEART study uses large international datasets, increasing the generalizability of its findings but providing less insight into how the factors interact over time (Teo and Rafiq).

However, there is some limitation in focusing on the same small group of variables across different studies. While these factors are important, they are analyzed independently and also minimize the exploration of other factors and their interactions.

This study aims to answer whether publicly available anonymous patient data can be used to identify and quantify associations between demographic, behavioral and clinical factors and Coronary Heart Disease (CHD) using multivariable logistic regression. In addition to identifying

the strongest independent risk factors, this research also examines the correlation among predictors.

By applying multivariable logistic regression to publicly available data, we can understand which factors play the biggest role in a patient developing CHD and how these factors interact with each other. These findings can then be translated into actionable recommendations, such as identifying high-risk individuals for earlier screening, prioritizing lifestyle interventions (smoking, improved diet, increased physical activity) and guiding preventative treatments to reduce future cardiovascular risk. The American Heart Association emphasizes that multivariable risk prediction models can be used to match the intensity of preventative care to an individual's cardiovascular risk (Khan et al.).

This research demonstrates accessibility of public data to understand CHD by exploring not only the main factors of CHD, but also how they influence CHD risk when evaluated together rather than independently. This research applies a multivariable logistic regression to understand how combined risk factors influence CHD likelihood in order to better inform targeted interventions.

Materials and Methods

We conducted an observational study to identify the most significant risk factors of Coronary Heart Disease. The data for this research came from a publicly available dataset with anonymous patient data. No direct patient interaction occurred and no personally identifiable information is included in the dataset.

The dataset used in this analysis is a derived and pre-processed version of the Framingham Heart Study data, consisting of 11,627 observations (Framingham Heart Study Dataset). The original Framingham Heart Study is a long term cardiovascular study conducted in Framingham, Massachusetts. However, publicly available and instructional versions of this dataset often differ in size and structure due to cleaning, feature selection, and cohort merging. The version used in this analysis includes demographic, behavioral, and clinical variables such as age, sex, BMI, cholesterol levels, blood pressure, smoking status, diabetes status, and coronary heart disease (CHD) outcome. A total of 3,158 individuals in this dataset were recorded with CHD.

The variables selected for this research were the patient’s sex, age, BMI, cholesterol level, diastolic blood pressure level, systolic blood pressure level, current smoking status, current diabetic status and whether or not they were diagnosed with CHD. These variables were selected because of their medical correlation and relevance to heart disease.

CHD status was treated as the binary dependent value, while Age, Sex, BMI, total cholesterol, systolic blood pressure, diastolic blood pressure, smoking status and diabetic status were treated as independent variables.

For all the numeric data (age, BMI, cholesterol and blood pressure), median values were imputed for the missing data. Median values allowed us to handle the patient’s data without getting rid of the complete rows. However, it may also make the data less variable than it

actually is, and may introduce some biases. Sex, current smoking status, current diabetic status and CHD diagnosis were dichotomized into binary classes. Continuous variables including age, BMI, cholesterol, and blood pressure were categorized into clinically relevant groups to improve the interpretability of the regression results and to make the comparison between each group easier to understand. Any missing binary values were excluded from the modeling, but the rest of the patient's data was still used.

Table 1. Missing Data Summary of Selected Variables

Variable	Missing (Count)	Missing (Percentage)
CHD (yes/no)	0	0.00%
AGE	0	0.00%
SEX	0	0.00%
TOTAL CHOLESTEROL	409	3.52%
SYSTOLIC BLOOD PRESSURE	0	0.00%
DIASTOLIC BLOOD PRESSURE	0	0.00%
BMI	52	0.45%
SMOKING STATUS	0	0.00%
DIABETES STATUS	18	0.15%

Continuous variables were converted into clinical categories, which were based on medical guidelines. The outcome variable in this study was ANYCHD, an indicator of whether or not the participant had Coronary Heart Disease.

BMI categories were used because they come from CDC adult BMI classification guidelines used in public health and clinical screening in the United States. These are standard national-level guidelines used for population health studies (Centers for Disease Control and Prevention, "Adult BMI Categories"). Cholesterol categories were used because they come from Cleveland Clinic clinical information and standard medical cholesterol risk guidelines used in healthcare practice in the United States. These are commonly used clinical thresholds for classifying cardiovascular risk (Cleveland Clinic, "Understanding Your Cholesterol Numbers"). Systolic blood pressure categories were based on standardized blood pressure classification systems used in major U.S. cardiovascular health organizations and clinical references, including guidance summarized by Cleveland Clinic and the American Heart Association (Cleveland Clinic, "What Is Blood Pressure?"). Diastolic blood pressure categories were also based on the same standardized clinical blood pressure classification systems used in U.S. cardiovascular health guidelines from organizations such as Cleveland Clinic and the American Heart Association (American Heart Association, "Understanding Blood Pressure Readings").

Table 2. Clinical categorization of study variables

Variable	Range	Converted Label
AGE	30-39	30s
	40-49	40s
	50-59	50s
	60-69	60s
	70+	70+
BMI	<18.5	Underweight
	18.5 - 24.9	Normal
	25 - 29.9	Overweight
	>30	Obese
CHOLESTEROL	<200	Desirable
	200-239	Borderline High
	>240	High
SYSTOLIC BLOOD PRESSURE	<120	Normal
	120-129	Elevated
	130-139	Stage 1 Hypertension
	140 - 179	Stage 2 Hypertension
	>180	Emergency
DIASTOLIC BLOOD PRESSURE	<80	Normal
	80-89	Elevated
	>90	High
SMOKING STATUS	0	Non-smoker
	1	Current Smoker
DIABETIC STATUS	0	Non-diabetic
	1	Diabetic

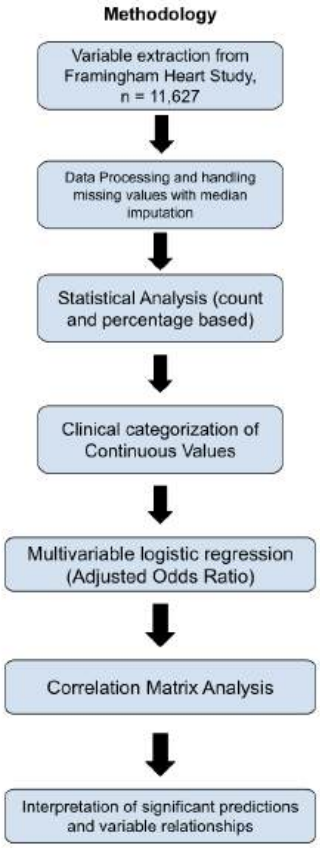

Fig 1: Workflow diagram of research methodology

All analyses were done using Python 3.13.19. Key libraries included pandas (2.3.3), numpy (2.3.5), and statsmodels (0.14.5), which were used for data preprocessing, statistical analysis, and multivariable logistic regression modeling.

Results

Table 3. Descriptive Statistics

Variable	Category	# of people	% of people
AGE	**30s**	388	3.3
	40s	2908	25.0
	50s	4193	36.1
	60s	3125	26.9
	70+	1013	8.7
SEX	**Male**	5022	43.2

	Female	6605	56.8
BMI	**Underweight**	166	1.4
	Normal	5033	43.5
	Overweight	4808	41.5
	Obese	1568	13.5
	****Missing**	52	0.45
Total Cholesterol	**Desirable**	1931	17.2
	Borderline High	3786	33.7
	High	5501	49.0
	****Missing**	409	3.5
Systolic Blood Pressure	**Normal**	2746	23.6
	Elevated	2351	20.2
	Stage 1 Hypertension	2066	17.8
	Stage 2 Hypertension	3876	33.3
	Emergency	588	5.1
Diastolic Blood Pressure	**Normal**	4367	37.6
	Elevated	4180	36.0
	High	3080	26.5
Smoking Status	**Current Smoker**	5029	43.3
	Non-smoker	6598	56.7
Diabetes Status	**Diabetic**	530	4.6
	Non-diabetic	11079	95.4
	****Missing**	18	0.2

Table 3 summarizes the distribution of demographic and clinical characteristics among the 11,627 participants included in the study. The table reports the number and percentage of individuals within each category for age, BMI, total cholesterol, systolic and diastolic blood pressure, smoking status, and diabetes status. This overview provides context for the study population and establishes the prevalence of each potential risk factor before examining its relationship with coronary heart disease.

Table 4. CHD across groups

Variable	Category	CHD Yes (%)	CHD No (%)
AGE	**30s**	15.5	84.5
	40s	18.9	81.1
	50s	25.7	74.3
	60s	34.0	66.0
	70+	40.3	59.7
SEX	**Male**	35.2	64.8
	Female	21.1	78.9
BMI	**Underweight**	18.1	81.9
	Normal	21.8	78.2
	Overweight	30.3	69.7
	Obese	35.6	64.4
Total Cholesterol	**Desirable**	20.1	79.9
	Borderline High	25.7	74.3
	High	30.6	69.4
Systolic Blood Pressure	**Normal**	17.0	83.0
	Elevated	22.2	77.8
	Stage 1 Hypertension	27.2	72.8
	Stage 2 Hypertension	33.9	66.1
	Emergency	49.5	50.5
Diastolic Blood Pressure	**Normal**	22.3	77.7
	Elevated	25.4	74.6
	High	36.3	63.7
Smoking Status	**Current Smoker**	27.1	72.9
	Non-smoker	27.2	72.8
Diabetes Status	**Diabetic**	52.8	47.2
	Non-diabetic	25.9	74.1

Table 4 presents the percentage of participants with and without coronary heart disease (CHD) across each category of the examined risk factors. Overall, the prevalence of CHD generally increased with higher age, BMI, cholesterol levels, blood pressure, and the presence of diabetes. These descriptive results do not account for the influence of other variables.

Fig 2: Correlation matrix of CHD Risk Factors

Table 5. Adjusted Odds Ratio

Category	Adjusted Odds Ratio	Confidence Interval (Lower)	Confidence Interval (Upper)	p-value
Age				
30-39	REF			
40-49	1.061	0.826	1.362	0.644
50-59	1.338	1.047	1.712	0.0202
60+	2.022	1.576	2.594	<0.001
BMI				
Underweight	0.850	0.561	1.288	0.4443
Normal	REF			

Overweight	1.228	1.114	1.353	<0.001
Obese	1.556	1.366	1.774	<0.001
Cholesterol				
Desirable	REF			
Borderline High	1.354	1.177	1.557	<0.001
High	1.715	1.502	1.959	<0.001
Systolic Blood Pressure				
Normal	REF			
Elevated	1.240	1.074	1.431	0.0034
Stage 1 Hypertension	1.434	1.240	1.659	<0.001
Stage 2 Hypertension	1.966	1.728	2.235	<0.001
Sex				
Female	REF			
Male	2.210	2.020	2.419	<0.001
Smoking				
Non - smoker	REF			
Current Smoker	1.174	1.071	1.287	<0.001
Diabetes				
Non - diabetic	REF			
Diabetic	2.439	2.030	2.930	<0.001

Diastolic Blood Pressure was excluded from the final multivariable regression model due to strong multicollinearity with Systolic Blood Pressure (r = 0.71) to preserve model stability.

Table 5 displays the adjusted odds ratios (AORs) for developing CHD, with each category compared to its reference group while controlling the other variables included in the model. An odds ratio greater than 1 indicates a higher likelihood of developing CHD relative to the reference category. These results identify the independent contribution of each risk factor, highlighting those most strongly associated with CHD risk.

Table 6. Pseudo R^2 Comparison

Model	**R^2 value (nearest thousand)**

Age Only	0.0214
BMI Only	0.0117
Diabetes Only	0.0121
Combined Model (Age + BMI + Diabetes)	0.0389

Table 6 compares R^2 of regression models using age, BMI, and diabetes individually and in combination. The combined model produced the highest R^2 value, indicating that incorporating multiple significant predictors explains more variation in CHD outcomes than any single variable alone. This finding demonstrates the importance of considering multiple risk factors together when modeling coronary heart disease risk.

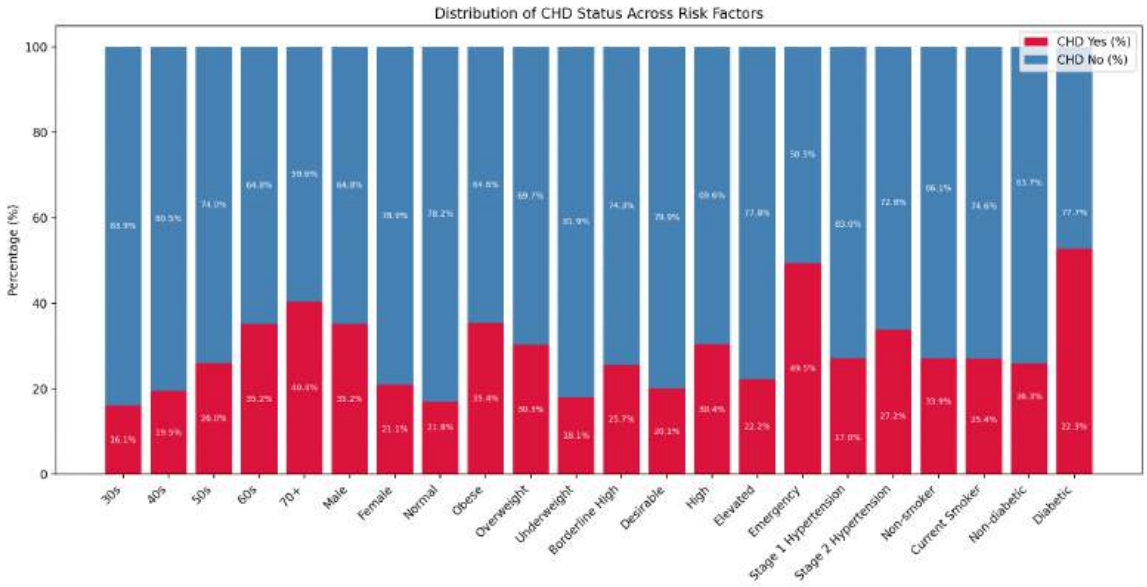

Fig 3: Distribution of Coronary Heart Disease (CHD) prevalence

The adjusted odds ratios were derived from a logistic regression model to investigate how strongly each risk factor is associated with CHD. The predictors of the model were age, BMI, cholesterol, systolic and diastolic blood pressure, smoking status and diabetes status. These predictors were chosen because they are already proven to have some correlation to coronary heart disease (Chow et al.). Adjusted Odds Ratio values greater than 1 indicate increased odds of having CHD, and values less than 1 indicate decreased odds. Additionally, we created a correlation matrix to indicate the strength of relations between the variables themselves. The heat map color scheme visually displayed whether there was collinearity between CHD risk factors. Collinearity indicates that two (or more) independent variables are highly correlated with each other in a regression model.

The correlation matrix showed a strong positive correlation between systolic and diastolic blood pressure ($r = 0.71$), suggesting multicollinearity. Moderate positive correlations were

observed between age and systolic blood pressure (r = 0.39) suggesting blood pressure tends to increase with age. Moderate positive correlation was also found between BMI and diastolic blood pressure (r = 0.33), suggesting association between higher body mass index and diastolic blood pressure.

Discussion

Throughout this research, publicly available anonymous patient data was used to identify significant Coronary Heart Disease risk factors. Descriptive analysis showed that CHD prevalence generally increased with higher age, BMI, cholesterol levels, blood pressure, and diabetes status. Logistic regression through adjusted odds ratios was used to distinguish the most significant risk predictors. Because this study was observational and based on existing patient data, the findings identify associations between risk factors and CHD but cannot establish causal relationships.

Increasing age was associated with progressively greater odds of CHD. Compared to individuals in their 30s, participants in their 60s or 70s have approximately twice the odds of having CHD.

Higher BMI was also associated with increased CHD risk. Compared to individuals with a normal BMI, overweight participants had 21% higher odds and obese participants had 63% higher odds of developing CHD. Previous studies have shown that obesity is associated with an increased risk of coronary heart disease by promoting systemic inflammation, insulin resistance and elevated blood pressure (CDC, "Consequences of Obesity").

Similarly cholesterol levels were strongly associated with CHD risk. Individuals with borderline high cholesterol had 35% higher odds and those with high cholesterol had 75% higher odds compared to participants with desirable cholesterol levels. High cholesterol has been associated with coronary heart disease and is thought to promote the buildup of fatty deposits (plaque) in the arteries. This can lead to the narrowing of blood vessels and eventually cardiovascular issues (American Heart Association, "Cholesterol").

Blood pressure was also a significant predictor of higher odds of CHD. Participants with Stage 1 or 2 hypertension had nearly twice the odds compared to individuals with normal blood pressure. High blood pressure increases the force exerted on artery walls, causing the heart to work harder and damage blood vessels (CDC, "High Blood Pressure Facts", American Heart Association, "High Blood Pressure").

Diabetes demonstrated the strongest association with CHD among the variables examined. Compared with non-diabetic participants, diabetic individuals had more than double odds of developing CHD. Diabetes has been associated with increased cardiovascular risk by accelerating atherosclerosis through chronically elevated blood glucose levels, which damages blood vessels over time (Centers for Disease Control and Prevention, "Your Heart and Diabetes").

Smoking status was associated with moderate increase in CHD. Although the descriptive analysis showed similar CHD prevalence between smokers and non-smokers, the multivariable

model suggested differently. After adjusting for age and other cardiovascular risk factors in the multivariable logistic regression model, smoking emerged as an independent predictor of CHD with an adjusted odds ratio greater than 1. This finding is consistent with previous research demonstrating that smoking damages blood vessels, reduces oxygen delivery, and accelerates plaque formation, which has been associated with a higher risk of coronary heart disease (CDC, "Smoking and Heart Disease and Stroke")).

The findings of this study are consistent with prior cardiovascular research. Similar to INTERHEART, it identifies age, hypertension, cholesterol levels, obesity and diabetes as major risk factors for coronary heart disease. Although the extent of the associations differ due to different populations and methods, the results were still consistent overall. Established clinical studies have consistently reported that these factors are associated with an increased risk of CHD and have been emphasized in risk prediction models (Castelli et al.). Prior research has also shown that a small amount of risk factors is responsible for the majority of CHD, emphasizing the importance of these variables in disease prevention (Teo and Rafiq).

Unlike many studies that examine each risk factor individually, this research evaluates multiple variables simultaneously using multivariable logistic regression. This provides a better understanding of each factor's independent association with CHD while also accounting for the influence of others. The correlation matrix highlighted relationships among several predictors, demonstrating that risk factors are interconnected.

The implication of this research can reach groups such as clinicians, public health, and other researchers. For clinicians, this reinforces the importance of prevention while also emphasizing what specific risk factors are important to check. In terms of public health, this research highlights the importance of healthy lifestyle choices, by highlighting the association between behaviors such as smoking and unhealthy eating habits and CHD risk. It also shows the importance of controlling blood pressure and cholesterol levels. Lastly, this research also demonstrates that publicly available datasets can generate meaningful insights.

One limitation of this study is that the analysis was based completely on the Framingham Heart study dataset. Although this dataset is widely used in cardiovascular research, it may not fully represent all populations because of demographic and geographic limitations.

Additionally, the dataset does not include potentially important variables, such as dietary habits, physical activity, family history, stress levels, or medication use, all of which may influence CHD risk. Finally, several continuous variables were categorized into clinical groups to improve interpretability. While this simplified analysis and made the findings more meaningful, it may have reduced precision by losing some information within the original data. Additionally, moderate multicollinearity between systolic and diastolic blood pressure ($r = 0.71$) may impact the regression coefficients, meaning their odds ratios may be less reliable.

Conclusion

This study investigated whether coronary heart disease risk can be understood more effectively by studying multiple factors simultaneously. Using data from the Framingham Heart

Study, a multivariable logistic regression was applied to evaluate how factors such as cholesterol, blood pressure, sex, diabetes, smoking status, BMI and age collectively contribute to CHD risk. The results indicate that increasing age, BMI, cholesterol, blood pressure and diabetes status were associated with the highest odds of developing CHD, although the strength of these associations varied. The correlation analysis further revealed positive relationships between systolic and diastolic blood pressure along with age, blood pressure, and BMI. Additionally, comparison of regression models showed that combining age, BMI and diabetes produced a higher R^2 value than models using each predictor independently (0.0389 vs 0.0214, 0.0117 and 0.0121).

These findings are important because they emphasize that Coronary heart disease is driven by a combination of factors rather than individual causes. Understanding the relationship between these factors is important for improving early detection and prevention. Identifying strong predictors, also established in previous research, such as hypertension, obesity and cholesterol levels has direct implications for risk assessment at clinical levels.

Future research should address the limitations of this study, starting with expanding the dataset to more diverse populations. In addition, incorporating more long term data to see the development of CHD over time. Future applications could also include models that are predictive of the amount of time until a patient develops CHD in relation to the factors they have. These applications could be integrated into clinical systems, to help healthcare with easier decision making. Overall, this research demonstrates that coronary heart disease risk is best understood through multiple joint factors rather than isolated factors. Multivariable analysis gives a more realistic understanding of CHD risk and highlights the relationship between variables. Development of similar models can help to improve early detection, build preventive care, and strengthen risk assessment.

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Ethical Considerations

This study used publicly available, anonymized data from the Framingham Heart Study dataset. No personally identifiable information was accessed, and no direct interaction with human participants occurred. Therefore, informed consent and institutional review board approval were not required for this secondary data analysis.

Conflict of Interest

The author declares no conflicts of interest.

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A Systematic Review: Role of Microplastics in ASD Development By Yeawon Ahn

Abstract

Overwhelming evidence suggests that humans ingest large quantities of small amounts of plastic through contaminated food, water, and air, although reported quantities vary across studies. Recent research has begun to link this microplastic exposure to disrupted neurodevelopment, including the increasing prevalence of autism spectrum disorders.

A systematic literature review of PubMed and Google Scholar was conducted to identify studies examining associations between plastic-related exposures, including microplastic particles and plastic-associated chemicals such as BPA and phthalates, and ASD or related neurodevelopmental outcomes. Using predefined search terms and inclusion criteria, we screened English-language, peer-reviewed primary studies published from 2016 to 2026 and excluded abstracts, commentaries, editorials, and non-full-text articles. After title, abstract, and full-text screening, 23 studies met all eligibility criteria and were included in the final analysis. Data extracted from included studies covered study design, sample characteristics, exposure type and timing, outcome measures, major findings, and reported limitations.

This review identifies a biologically plausible but still inconclusive link between plastic-related exposures—including microplastic particles and plastic-associated chemicals—and ASD-relevant neurodevelopmental outcomes. Across the reviewed studies, the most consistent signals appeared in early-life exposure windows, particularly in experimental models where prenatal or neonatal exposure was linked to ASD-like behaviors. Evidence also suggested that microplastic-related effects vary by exposure type, with animal studies pointing to neuroinflammation, gut-brain disruption, and vascular dysfunction as recurring mechanisms, while human studies produced more mixed and method-dependent findings.

Given the exponential increases in microplastic production and rates of autism, the body of evidence linking microplastic use with ASD has urgent implications for environmental health policy and could reshape our approach to preventing neurological disorders. Future studies are warranted to examine whether these associations vary across sex, region, developmental stage, and other relevant population subgroups.

Keywords Microplastics, autism, epidemiology, environmental health

Introduction

I. Background

Over the last several decades, industrialized societies have produced over 9 billion tons of plastic, creating a synthetic material so ubiquitous that it now cycles through Earth's atmosphere, oceans, and food webs (Barnes et al., 2009). While plastics have enabled major advancements in food processing, medicine, manufacturing, and packaging, the widespread environmental presence of microplastics has raised concerns regarding potential adverse health outcomes such

as heart disease, diabetes, and a spectrum of neurological disorders such as autism (Prüst et al., 2020).

II. Microplastics

Microplastics are a group of small (usually less than 5mm) particles of plastic breakdown (Prata et al., 2020).They can be present in the environment as primary microplastics (manufactured) or secondary microplastics (result of the continuous degradation of larger plastic pieces into smaller fragments due to environmental, physicochemical and biotic factors (Peñalver et al., 2020). Due to their size and ability to bind to a variety of surfaces, microplastics have become environmentally ubiquitous and are found in soil, air, and water (Kadac-Czapska et al., 2023). Additionally, microplastics can enter the human body through the respiratory system via inhalation, the digestive tract via consumption of contaminated food and water, or penetration through the skin via cosmetics and clothes contact (Winiarska et al., 2024). Because of their widespread distribution and ability to enter the human body, increasing attention has focused on whether microplastics may contribute to adverse biological effects, including disruptions in neurodevelopment.

III. Autism Spectrum Disorder

Autism prevalence has increased over time, although much of this rise has been attributed to changes in diagnostic criteria, awareness, and case identification rather than a simple true increase in incidence **(**Wing et al., 2002). The rise in autism diagnoses has also increased interest in whether environmental exposures, including pesticides, air pollution, and microplastics, may contribute alongside diagnostic and awareness-related changes (Dutheil et al., 2021). This rise has prompted researchers to examine how environmental toxins during pregnancy and early childhood may contribute to disruptions in neurodevelopmental pathways that can lead to autism spectrum disorders. Specifically, microplastic exposure has recently emerged as a novel focus of study due to its demonstrated neurotoxic properties (Xian et al., 2024). The etiology of autism spectrum disorder is multifactorial and incompletely understood, but proposed risk factors include genetic predisposition and chromosomal abnormalities, prenatal and perinatal infections, nutrient deficiencies, and/or exposure to environmental pollutants (Sauer et al., 2021).

IV. Microplastics and Autism Spectrum Disorder

While a growing number of studies have investigated plastic-related environmental exposures and autism, research directly examining microplastic particles remains limited. Consequently, much of the available human literature focuses on plastic-associated chemicals such as BPA and phthalates, whereas experimental studies more commonly investigate microplastic particles themselves (Zaheer et al., 2022). Importantly, microplastic particles and plastic-associated chemicals are related but distinct exposure categories. Microplastic particles may exert biological effects through tissue accumulation, inflammation, or barrier disruption (Amran et al., 2022; J. Xie et al., 2024), whereas additives such as BPA and phthalates are

thought to act primarily through endocrine and developmental signaling pathways (Yesildemir et al., 2024). Because direct human studies of microplastic particles remain relatively scarce, much of the available epidemiologic evidence instead focuses on these plastic-associated chemicals. Throughout this review, these exposure categories are considered complementary but are interpreted separately where appropriate.

Several studies have reported positive associations between plastic-related exposures and autism risk. For example, a 2024 study found that boys with higher prenatal BPA exposure had 3.5-fold greater odds of autism diagnosis (Symeonides et al., 2024.), while experimental work by Zaheer et al. demonstrated that prenatal polyethylene exposure produced ASD-like behavioral traits in mice (Zaheer et al., 2022.). Conversely, other investigations have failed to establish clear connections. For example, Hansen et al.'s study found no significant association between prenatal BPA exposure and ADHD symptoms at age of 5 years (Hansen et al., 2021.). This systematic review aims to evaluate evidence linking microplastic particles to ASD while also examining findings from studies of plastic-associated chemicals where direct human microplastic evidence remains limited. Particular attention is given to how these distinct exposure categories contribute to current understanding of potential biological mechanisms and neurodevelopmental risk.

Methods

This systematic literature review was conducted using searches of electronic databases, including PubMed and Google Scholar, to identify relevant studies examining associations between microplastic exposure and autism spectrum disorder (ASD) or related neurodevelopmental outcomes. The search strategy combined terms related to microplastics and neurodevelopment, using the following keywords and Boolean operators: ("microplastic" OR "micro plastic" OR "nanoplastic" OR "plastic exposure") AND ("autism" OR "autism spectrum disorder" OR "ASD" OR "neurodevelopment" OR "neurodevelopmental disorder"). The initial search yielded 263 records, which were then screened further using limits for studies published from the year 2016 to 2026, as research directly examining microplastic exposure and neurodevelopmental outcomes has substantially expanded only in recent years. and written in English, narrowing the total to 196 records.

Studies were eligible for inclusion if they met all of the following criteria: (1) examined the relationship between microplastic exposure and ASD, autistic traits, or broader neurodevelopmental outcomes; (2) were published in peer-reviewed journals; (3) used human participants or animal models; (4) were based on original primary data; and (5) were available as complete, full-text articles. Studies were excluded if they met any of the following criteria: (1) conference abstracts or proceedings from scientific conventions; (2) commentaries, editorials, letters, or other non-original publications; or (3) articles unavailable in full-text form.

After title and abstract screening, 144 records were excluded for not meeting the inclusion criteria. The most common reasons for exclusion were lack of relevance to microplastic exposure, lack of ASD or neurodevelopmental outcomes, review-only format, and duplicate

records. Full-text screening was then conducted on the remaining 52 studies, after which an additional 29 articles were excluded for the following reasons: (1) did not directly assess microplastic exposure or neurodevelopmental outcomes (2) were not accessible in full text, or (3) focused on unrelated outcomes. Following full-text review, 23 studies met all inclusion criteria and were retained for the review. These sources informed both the analytical synthesis presented in the body of the review and the contextual background presented in the introduction. Data extracted from included studies included study design, sample characteristics, exposure type, exposure timing, outcome measures, major findings, and reported limitations.

Biological Plausibility

Because autism spectrum disorder (ASD) emerges from disruptions of prenatal and early postnatal brain organization, exposure timing becomes particularly important: what may be a non-clinically significant contamination in adulthood could pose greater risk when exposure occurs while neural circuits and protective barriers are still maturing (Amran et al., 2022). The causal question—whether microplastics can significantly interact with the developing nervous system—requires evaluating not just environmental presence but biological accessibility during critical neurodevelopmental windows. This developmental framing, highlighted in Amran et al.'s synthesis of early-life exposure studies, reveals that much of the microplastic literature has yet to fully resolve how particle size, polymer chemistry, and exposure window interact in determining whether external contamination translates into internal biological effects and, ultimately, phenotypic outcomes.

This complexity becomes clearer when blood-brain barrier disruption studies are considered alongside evidence of human central nervous system accumulation. J. Xie et al. reports that microplastics and nanoplastics were detected in cerebrospinal fluid, including polyethylene (PE), polyvinyl chloride (PVC), polypropylene (PP), and polystyrene (PS), and that higher PP and PE concentrations were positively correlated with the cerebrospinal fluid albumin index, a marker of blood-brain barrier impairment (J. Xie et al., 2024). In patients with central nervous system infections, IL-6 and IL-8 (inflammatory cytokines) were also elevated, suggesting that plastic accumulation and inflammatory barrier dysfunction act synergistically rather than as separate processes. Ma et al. provides further evidence that barrier passage is not limited to disease states or animal models (Ma et al., 2025). In healthy humans, PE and PVC were among the most prevalent microplastics detected in plasma and the central nervous system, while the reported blood-brain barrier transmission efficiencies for PS, PVC, and PE were approximately 84.08 percent, 100.94 percent, and 62.86 percent, respectively. Taken together, J. Xie et al. and Ma et al. suggest a mechanistic continuum in which compromised barrier integrity may increase particle entry or accumulation in the central nervous system, while measurable transmission may also occur in apparently healthy individuals. Early-life exposure may be especially consequential because developmental barriers are still immature and therefore potentially more permissive than those in adulthood.

Taken together, this evidence provides a rationale for further mechanistic investigation. Amran et al., J. Xie et al., and Ma et al. together demonstrate that early-life microplastic exposure may represent a biologically plausible risk factor for ASD. This foundation provides direction for the animal behavioral studies that assess microplastic exposure and the ASD phenotype.

Experimental Evidence from Animal Models

Numerous studies have investigated whether microplastic exposure is associated with ASD-relevant outcomes in animal models. In a study by Zaheer et al., authors examined the accumulation of polyethylene (PE) in the brains of mice after exposure and then assessed subsequent behavioral effects using mouse models at different life stages, namely prenatal, post-weaning, puberty, and adult models (Zaheer et al., 2022). In the prenatal model, parent mice were fed polyethylene for two weeks, and the brains of offspring were later processed for direct quantification of polyethylene. In addition to measuring brain polyethylene, the authors used post-weaning mice to evaluate changes in brain gene expression, glucose metabolism, dopamine transporter activity, and gut microbiota in the context of polyethylene exposure. The authors found that polyethylene exposure significantly reduced dopamine transporter (DAT) binding and activity in the striatum, suggesting impaired dopaminergic signaling that could contribute to social behavior deficits characteristic of ASD. Validated behavioral tests such as the three-chamber test, Y-maze spontaneous alternation, nestlet shredding, marble burying, open field test, and adhesive removal test were used to assess traits in mice associated with ASD. Ultimately, they found that pre-/postnatal polyethylene exposure in mice produces ASD-like behaviors, which were accompanied by meaningful changes in gut microbial composition and diversity, including the loss of Lactobacillus reuteri and large increases in taxa such as Alistipes putredinis and Barnesiella intestini. Pathway analysis further showed decreased microbial carbohydrate and lipid metabolism and increased nucleotide and amino acid metabolism after polyethylene exposure, suggesting that reprogrammed gut metabolic activity may contribute to the observed brain changes. One major strength of this study is the direct quantification of polyethylene in brain tissue, which reduces reliance on indirect exposure biomarkers. However, the use of murine behavioral assays and a controlled dosing paradigm limits how directly the findings can be translated to the heterogeneous exposure patterns and behavioral complexity of human ASD.

Building on the Zaheer et al. study, Kopatz et al. and R. Xie et al. both showed that changes in polymer type, life stage, and exposure route can still produce ASD-relevant behavioral and molecular effects. Kopatz et al. exposed neonatal mice to polystyrene nanoplastics and found persistent increases in anxiety-like behavior and altered exploration, accompanied by oxidative stress and microglial activation in relevant brain regions (Kopatz et al., 2023). Specifically, they observed reduced time spent in the center of the open field test and impaired novel object recognition, alongside elevated malondialdehyde levels and increased Iba1+ microglial staining in the hippocampus and prefrontal cortex. Their work shows that a

different polymer and a narrower early-life window still produce durable behavioral and inflammatory changes, supporting the idea that microplastic exposure may prime the developing brain toward a pro-inflammatory, ASD-relevant state. At the same time, neonatal bolus exposure may not fully reflect the chronic, mixed exposures humans experience in everyday environments. R. Xie et al. focused on long-term polystyrene microplastic exposure and examined seizure symptoms, proposing that inflammation and ferroptosis may mediate the association between microplastic exposure and seizure exacerbation (R. Xie et al., 2025). This work adds seizure exacerbation, inflammation, and ferroptosis as additional pathways through which microplastic exposure may affect the nervous system, complementing the broader neurotoxic mechanisms described by Zaheer et al. and Kopatz et al. This finding strengthens the mechanistic case, although questions remain about how closely a vagotomy-based model captures human exposure physiology. Taken together, these studies suggest that different microplastic types and exposure patterns may affect the nervous system through distinct mechanisms, including neuroinflammation, oxidative stress, and barrier disruption.

In contrast, Huang et al. emphasize a vascular route by which microplastics can impair brain function without extensive parenchymal penetration (Huang et al., 2025). Using *in vivo* two-photon imaging, they showed that microplastics are phagocytosed by immune cells, predominantly neutrophils and macrophages marked by F4/80+ and Ly6G+, which then become lodged in small cortical vessels, particularly at capillary branch points. These blockages created long-lasting microthrombi that persisted up to 7 days and reduced local blood flow by up to 18% in affected regions. These vascular obstructions led to neurobehavioral abnormalities, including impaired motor coordination and cognitive deficits in maze tasks. This work adds cerebral hypoperfusion and microvascular obstruction as an additional mechanism of microplastic-induced neurotoxicity, complementing the neuroinflammatory and gut-brain pathways described by Zaheer et al., Kopatz et al., and R. Xie et al. Even so, the study examines acute vascular responses under a controlled exposure paradigm, leaving open whether similar cerebrovascular effects occur under environmentally relevant chronic exposure levels. Still, the combined evidence reinforces the idea that multiple converging biological routes can lead from microplastic exposure to ASD-relevant behavioral change.

Epidemiological Findings

Although emerging evidence suggests that environmental exposures may contribute to ASD-related outcomes, the human literature remains mixed, with some studies detecting associations at specific developmental windows or in specific subgroups and others finding no overall relationship. This pattern suggests that disagreement may reflect differences in exposure metrics, timing windows, and analytic choices.

Hansen et al. is one of the clearest examples of a study reporting a positive association rather than a null result. In that prospective cohort, prenatal BPA exposure was assessed in gestational week 28, and elevated ASD scores were observed at age 5 years, with the strongest association reported in girls rather than boys. The study is notable because it reports a

sex-specific association between prenatal BPA exposure and ASD symptoms, which is consistent with the possibility that endocrine-disrupting chemicals may affect neurodevelopment differently across sexes. Hansen et al. provides human cohort evidence that is directionally consistent with the mechanistic concerns raised by the animal literature on top of resolving the broader question on its own.

Mustieles et al. extends this pattern by focusing on a mixture of phenols and phthalates rather than a single chemical. The key finding was not simply that exposure mattered, but that the relationship was strongest for early-life exposure and for specific social-behavior domains (Mustieles et al., 2023). This detail aligns with the developmental emphasis of the rest of the review: if the earliest window is the most sensitive, then a study centered on infancy may detect effects that a broader prenatal-only analysis would miss. The phthalate findings therefore suggest that exposure timing may partly explain why some studies observe effects while others do not.

Cunha et al. and Uldbjerg et al. point in the opposite direction and are methodologically important because they help define the limits of the human evidence. Cunha et al., the systematic review, did not identify a consistent link between prenatal endocrine-disrupting chemical exposure and ASD later in life (Cunha et al., 2023). That conclusion weakens the casual relationship between BPA, phthalates, and ASD, but it also requires a careful evaluation of the positive studies: if a large review cannot find convergence across the literature, then the apparent signal in individual cohorts may depend heavily on who was studied, how exposure was measured, and when outcomes were assessed. Because systematic reviews synthesize evidence across multiple cohorts, their conclusions generally carry greater evidentiary weight than any single observational study, although they are still constrained by heterogeneity in study design, exposure assessment, and outcome definition. Uldbjerg et al. illustrates that issue by testing phthalates and BPA across maternal and paternal preconception as well as maternal pregnancy windows and still finding no association with autistic behaviors. Because this study examines multiple windows rather than relying on one narrow exposure period, it complicates the assumption that the problem is simply the wrong timing in null studies. At the same time, its negative result does not necessarily invalidate Hansen et al. and Cunha et al.; instead, it suggests that human findings may depend on exposure range, regional patterns of plastic chemical use, assay sensitivity, and outcome definition. In that sense, Uldbjerg et al. reinforces the point that the human literature is sensitive to design choices in ways that animal models are not.

Yesildemir et al. helps contextualize why human data are so difficult to interpret. By treating plastic-related chemicals as part of a broader endocrine-disrupting chemical (EDC) landscape, the review makes clear that human exposure is almost always mixed rather than isolated (Yesildemir et al., 2024). BPA and phthalates rarely act in a vacuum; they are encountered alongside other environmental chemicals that may have overlapping or even opposing endocrine effects. This is where the gap between mechanistic plausibility and epidemiologic clarity becomes most visible. Animal studies can isolate a polymer, a dose, and a developmental window, but human cohorts must infer risk from biomarker snapshots that may poorly represent lifelong exposure. The result is a literature in which the same chemical family

can appear harmful in one cohort, null in another, and inconclusive when pooled into a broader review.

Discussions

Taken together, the evidence suggests a potentially meaningful association between plastic-related exposures to ASD-relevant neurodevelopmental outcomes, however most of the data has been produced in experimental models, with limited causal data in human populations. In the experimental literature, early-life exposure to microplastics and related plastic materials is repeatedly associated with social deficits, repetitive behaviors, neuroinflammation, oxidative stress, neurotransmitter disruption, gut-brain alterations, and vascular or barrier dysfunction. In human studies, the picture is more mixed: some reports of BPA and phthalate exposure suggest associations with autistic traits or related developmental outcomes, while others are null. The current evidence suggests that plastic-related exposures are biologically plausible contributors to neurodevelopmental outcomes, with effects that may vary by chemical type, exposure window, dose, and developmental susceptibility.

One explanation for divergent findings may be due to inconsistencies in exposure classification. Evidence directly examining microplastic particles remains much less developed than evidence on plastic-associated chemical additives such as BPA and phthalates, and those exposures should not be treated as interchangeable. Although they share a common origin, they may operate through different biological mechanisms: particles may contribute through tissue accumulation, barrier disruption, inflammation, or microbiome effects, whereas additives may act more clearly through endocrine signaling and developmental programming. This distinction matters because studies that group all “plastic exposures” together can blur mechanistic differences and make the human evidence harder to interpret. This raises an important classification issue: it remains unclear whether the literature is capturing a single exposure category or several partially overlapping exposure types.

The divergence between animal and human findings is also expected from a methodological perspective. Animal studies can more easily control timing, dose, route, and chemical composition, which makes them well suited for identifying pathways during sensitive developmental periods. Human exposure is far less orderly. Pregnant people and children are exposed to mixtures rather than single compounds, and exposure is usually chronic, low-dose, and difficult to reconstruct accurately from a single biomarker measurement. Diagnostic variability creates another challenge, because studies may measure formal ASD diagnosis, autistic traits, social responsiveness, or related neurodevelopmental outcomes that are not equivalent. These differences can produce apparent disagreement even when the underlying biology is not contradictory. Human null findings therefore do not necessarily contradict the animal literature; they may instead reflect measurement noise, insufficient exposure resolution, or failure to capture the most relevant developmental window.

A major strength of the animal literature is the consistency of its proposed mechanisms. Across different models and exposure paradigms, several pathways recur: neuroinflammation,

oxidative stress, altered neurotransmitter signaling, microbiome disruption, and vascular or blood-brain-barrier injury. That convergence supports a plausible developmental narrative in which early exposure to microplastics can affect neural systems relevant to social behavior and repetitive phenotypes. At the same time, these models do not fully capture the clinical and biological heterogeneity of human ASD. Rodent behavior assays provide useful approximations, but they remain indirect measures of human neurodevelopmental outcomes. Accordingly, the animal literature should be interpreted as evidence supporting biological plausibility rather than as direct evidence of human causation.

Several limitations are worth mentioning. First, the number of human studies directly addressing microplastic particles remains small, especially compared with the broader literature on BPA and phthalates. Second, exposure assessment is inconsistent across studies, ranging from chemical metabolite biomarkers to indirect proxies and differing experimental doses. Third, outcome definitions for ASD vary substantially, which limits cross-study comparability. Fourth, the literature remains heavily weighted toward animal models, and that imbalance makes translation to human risk uncertain. Publication bias and selective reporting cannot be excluded, particularly in a field where positive mechanistic findings may be more likely to appear in the published record than null results. Finally, residual confounding from socioeconomic factors, diet, maternal health, and co-exposures may complicate interpretation of observational associations. These limitations mean that the current literature is informative but not yet definitive.

Future research should address these gaps more directly. Human studies would benefit from repeated exposure measurements across pregnancy, infancy, and early childhood rather than isolated snapshots. Mixture-based models are essential, because real-world exposures to microplastics, BPA, phthalates, and other plastic-derived chemicals occur together. Longitudinal birth cohorts with standardized developmental and diagnostic outcomes would help clarify whether observed associations persist over time or are limited to narrow developmental windows. Sex-specific analyses should also become routine, since several studies suggest that vulnerability may differ by sex. Finally, integrating epidemiology with mechanistic biomarkers such as inflammatory markers, endocrine endpoints, microbiome measures, and barrier function would help connect observed associations to biologically meaningful pathways.

From a public health perspective, the findings justify caution without overstating causality. Public health strategies may reasonably prioritize minimizing unnecessary plastic exposures during pregnancy and infancy as a precautionary approach, especially because these are periods of rapid neurodevelopment and heightened susceptibility. That framing is more defensible than a direct policy claim of harm, because the evidence does not yet establish that plastic exposures cause ASD. Still, the combination of developmental vulnerability and repeated mechanistic signals makes the topic important for prevention-oriented research and risk reduction.

Conclusion

In conclusion, the current literature supports a cautious but serious interpretation. Evidence directly linking microplastic particles to ASD-relevant outcomes remains limited, while evidence on plastic-associated chemical additives such as BPA and phthalates is more developed but still mixed. Animal studies provide a coherent mechanistic foundation, whereas human studies remain constrained by exposure measurement, outcome heterogeneity, and causal uncertainty. The most balanced conclusion is that plastic-related exposures are biologically plausible contributors to neurodevelopmental risk, but the field still needs stronger human evidence before any causal claim can be made with confidence.

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Evaluating Music-Based Interventions as a Public Health Approach to Depression
By He Liu

Introduction

Depression is one of the leading causes of health burden worldwide, with an estimated 332 million people suffering from this disorder and placing a significant strain on society and the economy (WHO). Despite the fact that there are established clinical treatments for depression, each of which has proven effective, the social causes of the disease cannot be ignored. This is why music therapy can be considered an innovative method of health promotion that addresses the issue from a different angle and helps decrease levels of depression within a community by increasing social engagement and involvement.

Examining Depression through a Public Mental Health Lens

Depression as a Population Health Issue

Depression is a public health problem since it affects the population at large, not just individuals. The clinical medicine perspective focuses on understanding how to diagnose and treat the condition. However, public health encompasses a broader perspective that includes studying the occurrence of the illness and its impact on society. The World Health Organization (WHO) reports that it is among the leading causes of disability globally and has a significant health burden (Moussavi et al.). Additionally, it brings indirect costs, including lowered educational and work performance, increased use of healthcare resources, and overload of the social services system.

Social Determinants of Depression

There is a close link between mental health problems, including depression, and social determinants of health (SDOH). Individuals who experience social isolation, loneliness, unemployment, poverty, and discrimination face higher risks of developing the illness (Kirkbride et al.). At the same time, strong social connections and an active social life protect against it. This evidence demonstrates that public health addresses mental health issues from a different angle, focusing on ensuring people lead healthy lives rather than managing the consequences of poor mental health.

The Value of Health Promotion in Addressing Depression

Clinical treatment of mental illnesses plays a crucial role in health care. It is mainly aimed at managing the symptoms of the disease (Funk et al.). However, it is only one part of the comprehensive approach that public health takes to address the problem. In contrast, health promotion activities enable people to increase control over and improve their lives and increase their capacity to take care of their health ("Ottawa Charter for Health Promotion"). Thus, they address the root causes of health issues, including mental ones. For depression, this means

creating conditions that prevent it from occurring rather than focusing solely on those that help a person with the illness return to normal life. It involves various interventions at different levels, including school, workplace, and community. These interventions promote social inclusion and engagement, which are critical areas in preventing mental illnesses.

Music in Public Mental Health Promotion

When it comes to considering music as an instrument for promoting health, it is essential to keep in mind that it mostly occurs in populations. That is why it fits perfectly into the public health perspective (Sheppard and Broughton). Instruments for music play or singing are generally available in communities since this art is often promoted in schools and other institutions. It is easy to organize community events, engage people in collective actions, and ensure broad participation. Such activities create social connections, which are beneficial for mental health and help prevent illnesses like depression. It is not about treating those who suffer from the condition but promoting better mental health in general. Therefore, music is an excellent tool for public health to ensure social well-being and prevent individual health problems, such as depression, by engaging people in collective activities. It is a valuable addition to conventional clinical treatment, especially since the latter cannot address society-wide health and social concerns, including discrimination, social isolation, and poverty.

Music therapy has been used in hospitals, rehabilitation centers, schools, residential care facilities, and other settings for people of all ages. It is used as an effective method for promoting well-being, preventing mental and social illnesses, and improving psychological adaptation (Feng and Wang). Community-based music interventions are also an emerging field that seems to have a significant impact on the population's mental health. In addition, they are often low-cost and can be implemented in diverse cultural contexts.

The rising prevalence of depression worldwide has prompted the need to find and study new ways of improving mental health. However, the availability of mental health services remains inadequate, and the stigma around mental illness is still severe, which means that many people cannot get the treatment they need. Moreover, there is an insufficient number of health professionals, long waiting lists for accessing services, and social inequity (Baeten et al.). Thus, it is evident that the health issue cannot be addressed successfully on a global scale through traditional treatment methods alone.

Various clinical studies have demonstrated that music interventions could help individuals with depression and other mental health issues. However, there is limited and inconsistent evidence on how music interventions could be used to improve mental health in a community-based setting. Therefore, music interventions should be evaluated from a public health perspective to demonstrate their ability to promote mental well-being and reduce the burden of depression.

The Value of Music in Mental Health Promotion and Prevention

One of the frameworks that can be used in understanding the role and value of music as a

health promotion tool is the Ottawa Charter for Health Promotion (1986). It defines health promotion as a process that enables people to take control of their lives and improve their health, which goes beyond the biomedical view of health. This is an innovative approach because it recognizes the importance of building individual, communal, and social resources for achieving better health outcomes.

From the perspective of the Ottawa Charter, music is not primarily a health intervention for treating depression. Instead, it is a health promotion activity that can be used to create favorable conditions for achieving mental well-being for everyone. Specifically, music can help create opportunities for socialization and interaction, increase the level of personal development through self-expression and self-disclosure, and build social networks and supportive relationships. All three of these areas can be used to facilitate good mental health. In other words, music contributes to mental health promotion by helping individuals cope with mild or moderate levels of stress, anxiety, depression, and other psychological issues.

The Ottawa Charter also states that there are five priority areas for health promotion. Music interventions can be used in all of them. For instance, music builds social support networks in communities by creating opportunities for socialization, interaction, and bonding. It also facilitates the development of personal skills, such as self-esteem, self-confidence, and assertiveness. In addition, by helping people engage in recreational and enjoyable activities, music promotes a culture of health. Thus, it is evident that music plays an important role in mental health promotion. It should be considered a vital aspect of individual health, as well as a component of a wider strategy for reducing the impact of mental illness.

In relation to the topic of mental health promotion, music can be categorized as a primary prevention measure. It targets the risk factors that could contribute to the development of mental illness and aims at reducing their impact (Gold et al.). Given the rising levels of depression around the world and the significant individual and societal burden caused by this disorder, there is an immediate need for effective preventative measures.

Theoretical Frameworks for Music and Community Mental Health

The Biopsychosocial Model: Music as an Alternative Pathway to Mental Well-being

The Biopsychosocial Model (BPS) by George Engel (Lugg) can be applied to understand how music can influence a person's mental state from several angles. It is an alternative to the biomedical model, which explains health issues and illnesses in terms of bodily functions and defects only. Engel's model claims that a person's health status is determined by biological, psychological, and social factors.

First of all, music has a positive impact on a person's biological functions and thus improves well-being. The calming effect of music on heart activity, as well as its ability to lower stress hormones, has been proven scientifically. Therefore, music can be conducive to a person's physical condition, which consequently improves their mental state.

However, the psychological benefits of music have a much broader impact on a person's

health status. Music gives a person an opportunity to express their emotions and helps them cope with stress and negative feelings. Furthermore, engaging in a musical activity is beneficial for developing effective adaptive behavior, which results in an individual's overall positive psychological mood (Croom). The public health aspect of music is connected to the psychological benefits, although it may have an even more substantial influence on an individual's health status. People who participate in musical events, such as choir singing or other performing arts, have stronger social connections and better adjustment, which leads to improved well-being.

The social aspect of music engagement plays a prominent role in the context of public health. Depression is commonly related to isolation from society, which leads to poorer health outcomes. Moreover, social support is crucial for individuals with depression to recover successfully. Thus, music gives people a chance to build social connections and engage in activities that positively affect their mood. In terms of the BPS model, music influences people from all three closely related aspects. An individual's adaptation to society is essential for better health status from a public health perspective.

Therefore, it is evident that music can have a profound and positive effect on individuals' health status. The BPS model highlights the importance of social connections and public health aspects of music engagement, which may be critical for developing effective interventions aimed at improving well-being.

Social Capital Theory: Music as a Means for Strengthening Communities

While the biopsychosocial model helps understand the various ways music affects health, Social Capital Theory helps explain how music builds social capital in society. As the sum of relationships and networks that allow individuals to collaborate for mutual benefit, social capital has been positively correlated with good mental well-being and better mental health outcomes for the community (Bhandari and Yasunobu). Moreover, areas with higher social capital have displayed increased abilities to cope with challenges, including outbreaks of mental health problems.

Music is an especially effective tool to generate social capital because it is inherently interactive and collaborative. Many musical activities are aimed at building connections between people through music. Moreover, musical activity often presupposes collaboration and joint effort, thereby enhancing trust, friendship networks, and social cohesion.

There are three types of social capital that music can help create within a community. First, bonding social capital is created when people engage in music with their existing social networks, for example, singing in the local choir. Bonding social capital strengthens the community network, enabling it to provide greater social and emotional support to its members (Aldrich and Meyer). Second, music helps create bridging social capital by bringing together people who may not have interacted due to different ages, cultures, or socioeconomic status. Third, when community music groups begin cooperating with local businesses and organizations, they create linking social capital that helps the community access much-needed

resources and services.

The creation of social capital through music is essential to this study, as it highlights the various ways music can enhance mental well-being. This is because social capital often displays protective factors for developing depression, including higher social support, social engagement, and social cohesion. By focusing on building social capital, music programs do not just address the individual with mental health problems. Instead, they attempt to affect the larger community and alter social structures to make society more mentally healthy and resilient.

Music Programs as Community-Based Public Health Interventions

Drawing together the principles of health promotion, the biopsychosocial model, and social capital theory allows for considering music programs as valuable community-based public health interventions. They are beneficial because they can address a wide range of mental health problems and risk factors, being easily accessible, available in multiple settings, and relatively inexpensive.

The key advantage of music programs as public health interventions is that they can be integrated into the daily life of a community (Hallam et al.). The programs can be organized in various settings, including schools, places of employment, healthcare institutions, senior centers, and other organizations and venues. Moreover, they can be developed for different populations and can be led by professional specialists or volunteers. In this way, music programs can be made accessible to the population groups that do not want to address their mental health issues in a medical setting because of associated stigma, lack of mental health providers in the community, or financial constraints. Music programs can also facilitate engagement with mental health issues in a general setting, thereby reducing the treatment gap and providing access to care.

Being community-based and directed at enhancing social capital, music programs correspond to the principles of community engagement and community empowerment. They can help providers meet the real needs of the population and allow participants to contribute to the development of the intervention actively. In addition, the empowerment approach used in music programs can improve social cohesion and help community members achieve collective benefits.

As a result, when considering music programs as community-based mental health interventions, it is essential to focus not only on the reduction of depressive symptoms. The primary outcomes of such programs should be collective empowerment and social capital, which can manifest as increased social participation, resilience, and social cohesion, reduced loneliness, and improved quality of life. These factors are of particular significance for public health since they reflect the concept of healthy societies.

The theoretical basis described in the previous section provides a ground for arguing that music can be a community-building tool that promotes health (Vaillancourt, 2012). In addition to theoretical considerations, public health interventions also require practical evaluations. Unlike the clinical field, which is primarily concerned with demonstrating efficacy in terms of reduced symptoms in a controlled environment, the public health domain encompasses a wider range of issues related to accessibility, outreach, social inclusion, sustainability, and overall well-being.

Evaluating Community-Based Music Interventions: Evidence from Existing Public Health Promotion Practices

Case Study: The Sing Your Heart Out Project

"The Sing Your Heart Out" (SYHO) is a singing initiative developed in the United Kingdom that focuses on enhancing the well-being of individuals with mental health problems (Sing Your Heart Out). Contrary to standard music therapy, which is usually provided in clinical settings by professional healthcare providers, SYHO is an open community choir. All individuals who are interested in joining can participate regardless of their musical skills, and their involvement is voluntary (Shakespeare and Whieldon).

This approach to tackling mental health issues is different from the mainstream in several ways. First of all, people who join SYHO do not view themselves as patients but rather as members of a community who enjoy singing in a choir. The objectives fit into the Ottawa Charter's framework because they are about creating a supportive environment and advocating for community action. This is an important difference because it reduces the stigma around mental health problems and increases the likelihood that individuals will seek help.

1.Public Health Objectives of SYHO

While enhancing individuals' well-being is a critical goal, the program has additional objectives of public health significance. They include:

1) Reducing social isolation by providing frequent opportunities for interaction;
2) Raising awareness of belonging to a particular group;
3) Increasing social inclusion by facilitating access to cultural events;
4) Building self-confidence and self-esteem;
5) Engaging the community.

2. Intervention Mechanisms of SYHO

SYHO does not seek to improve mental health through medical intervention but by targeting a range of social determinants. First, weekly rehearsals provide interaction opportunities, which reduce social isolation and loneliness. Attending choir practices regularly helps the participants build social networks and obtain emotional support, which is essential for mental well-being. Second, the intervention fosters a feeling of belonging to a group. Unlike traditional individual therapies, singing in a choir is a collective activity that promotes harmony and cooperation. Such an approach helps prevent depression and builds social capital. The social capital is evident in how the choir members support each other, both emotionally and practically, and how they engage with the wider community. Finally, the intervention promotes active aging by encouraging the choir members to participate in the rehearsals and performances. It empowers the community by enabling it to take ownership of its well-being. In other words, the participants are not recipients of services but contributors to the development and implementation of the intervention.

3.Public Health Outcomes and Impact of SYHO

Several public health outcomes can be associated with the SYHO program. The most apparent ones are enhanced well-being, confidence, and socialization among the choir members. While the intervention's impact on reducing depressive symptoms is notable, its capacity to build individual-level resilience is more significant to public health. Essential outcomes that reflect the intervention's potential to improve mental health at a population level include increased social participation, strengthened peer support, reduced loneliness, and enhanced community cohesion.

SYHO is an innovative intervention that leverages the power of music to enhance mental health. It is a prime example of how an upstream approach can be used to address a public health issue. By targeting social isolation and other social determinants, the program prevents mental health problems from arising in the first place. Additionally, the intervention is scalable because it does not require substantial resources. Thus, it has the potential to become a critical asset in mental health strategy at a local and national level.

Case Study: Community Music Therapy

Community Music Therapy (CoMT) is the further development of the role of music in medicine (Aigen). Music therapy practice is usually carried out in hospitals and other treatment settings, while Community Music Therapy (CoMT) is about bringing the benefits of music into the community. It highlights the importance of music in people's everyday lives and the contribution of social, cultural, and environmental factors to health.

From a public health perspective, music therapy is about moving from an individual to a population health approach. This implies that the responsibility for health is not only on the healthcare provider but also on community members and various organizations.

1.Public Health Objectives of CoMT:

The objectives of Community Music Therapy align with public health goals of participation, equity, and empowerment. It aims to:

1) raise community engagement;
2) increase social inclusion;
3) reduce barriers to participation;
4) achieve community empowerment;
5) promote healthy communities.

2. Intervention Design of CoMT:

Community Music Therapy interventions are highly flexible and can be tailored to the specific community and setting. They can be implemented in a variety of places, such as schools, day centers, nursing homes, residential homes, or refugee organizations. Community members can collaborate with music therapists to co-produce music-making, singing, performances, and other interventions. CoMT interventions are designed to be participatory and engaging, which is essential in promoting public health.

3. CoMT's Mechanisms of Influence:

Community Music Therapy can promote mental health in several ways:

First, it can increase community participation and engagement. Encouraging people to become involved in a shared activity such as singing or playing an instrument can enhance social connections and reduce feelings of isolation.

Second, music can bring people together regardless of their social, cultural, or economic background. This can promote social cohesion by creating common goals and increasing social capital.

Third, community singing and performances can help to raise the profile of mental health issues in the community. Music-making can provide a platform to share experiences and highlight the strengths of people with mental health problems, challenging stigma and promoting social inclusion.

4. CoMT's Public Health Significance:

Community Music Therapy shows how mental health issues can be addressed outside the traditional health and social care sectors (Stige et al.). CoMT interventions have the potential to improve population health and well-being by increasing social cohesion, participation, and empowerment. This approach fits into a public health perspective by targeting community capacity, social cohesion, and collective well-being.

Evaluation

In general, the case studies show that music-based programs can offer powerful tools for enhancing social cohesion, involvement, and mental health. However, when selecting health promotion and disease prevention strategies, it is essential to consider their appropriateness, effectiveness, accessibility, equity, and sustainability.

1. Appropriateness and Population Coverage

The most significant advantage of music intervention is its broad applicability. Indeed, such programs can be easily integrated into the existing community structures, including schools, organizations, and places of employment. Moreover, compared to health-focused activities, music-based initiatives tend to be available to more people because they can be embedded in regular social events. Another benefit is that music intervention often takes place outside the formal mental health care system, thus being more approachable and less stigmatizing.

2. Health Equity and Community Involvement

Health equity and community participation are essential components of public health, and music intervention has the potential to offer substantial benefits in both domains. On the one hand, such programs can be introduced in diverse community settings and address the needs of different population groups. On the other hand, music-based activities inherently involve social bonding, making these programs beneficial for a wide range of social and cultural contexts. By

fostering well-being through social connection, music interventions respond to many mental health problems, most of which are linked to social isolation and disengagement.

3. Sustainability and Cost-Effectiveness

Compared to other mental health interventions, music programs have the potential to offer substantial cost-offsetting benefits due to their extensive coverage. They can use existing community infrastructure and be implemented in collaboration with local authorities and cultural institutions. Indeed, such programs appear to be a good value for public health **budgets as they operate outside the formal health service system.**

4. Limitations and Disadvantages

Despite these advantages, several limitations must be acknowledged. Music programs should not be considered a replacement for traditional mental health services. Most music interventions are designed to complement the existing services, addressing the social rather than medical aspects of mental health problems. There is a risk that vulnerable groups would use music programs as the sole treatment for severe mental illnesses, which should be avoided because comprehensive medical care is critical for their management.

In addition, certain community groups may have limited access to music-based programs. Since such initiatives are usually community-driven, they may be unavailable in areas with weak social cohesion. An inadequate policy framework may also limit the ability to deliver music programs to vulnerable populations, thus creating inequities in health outcomes.

Finally, there is a need for more studies evaluating the long-term impact of music intervention, including its cost-effectiveness. Without consistent evidence, it is challenging to estimate their impact on a national level. Most of the reviewed studies used small-scale samples and were conducted in high-income countries, limiting the generalizability of their findings.

Conclusion

Depression is not merely a clinical emergency but also a social and economic one. Given the rising prevalence of poor mental health, it is not enough to rely only on clinical treatment. The public health approach to managing mental illness emphasizes health promotion, prevention, and intervention, seeking to enhance protective factors and promote general well-being.

The present essay argues that music can be used as a community-based alternative to complement the existing evidence-based clinical approaches to managing depression. Drawing on the Biopsychosocial Model and Social Capital Theory, this essay explains how music can help improve mental well-being. Music facilitates connections between individuals while encouraging social support and engagement, thus promoting mental well-being. The analysis of two case studies—The Sing Your Heart Out Project and Community Music Therapy—highlights music's ability to enable an inclusive, engaging environment that reduces isolation and enhances social capital.

It is important to recognize that music is not a substitute for evidence-based clinical

treatments but can help augment them. For music to realize its potential as a public health measure, it requires adequate funding and multisectoral support. Additionally, future research endeavors should seek to establish the efficacy and sustainability of music interventions. This essay concludes by arguing that addressing a social issue such as depression requires a public health approach that goes beyond the healthcare sector.

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Biological Pathways Linking Preeclampsia and Gestational Diabetes to Autism Spectrum Disorder Risk By Lexie Zhao

Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental condition influenced by both genetic and environmental factors. Preeclampsia and gestational diabetes mellitus (GDM) are pregnancy complications that can disrupt fetal neurodevelopment through inflammatory and vascular changes. As the prevalence of ASD has increased, the search for prenatal risk factors has become increasingly important. This review examines the biological mechanisms that may explain the association between preeclampsia, GDM, and ASD risk. Existing literature was reviewed to identify shared and condition specific pathways for preeclampsia and GDM. One shared pathway is maternal immune activation (MIA), in which increased pro-inflammatory cytokine exposure can alter gene expression in neurodevelopmental pathways, NF-κB and ERK, potentially leading to regulatory issues in ASD related gene expression. Another shared pathway is oxidative stress, where excessive reactive oxygen species (ROS) may damage antioxidant defenses through the suppression of SOD2, a neutralizing enzyme of superoxides. In preeclampsia, disruption of angiogenic signaling through vascular endothelial growth factor (VEGF) may additionally affect cerebral blood flow and function. In GDM, disruption of the gamma-aminobutyric acid (GABA/GABAergic) system may contribute to an imbalance between excitatory and inhibitory signaling and alter activity within the prefrontal cortex. These findings suggest that maternal pregnancy conditions may influence the developing fetal brain through multiple biological pathways. Although ASD has no single underlying cause, these mechanisms represent potential biological pathways through which maternal conditions may influence ASD risk.

Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition that affects social communication and interaction while also involving restricted or repetitive behaviors and interests (American Psychiatric Association). The presentation and severity of these traits often vary from person to person, explaining why ASD is recognized as a spectrum. While ASDs exact origins are unknown, it is believed that multiple factors in early fetal stages can contribute to the onset of ASD. Current evidence suggests that ASD develops through a complex interaction between genetics and environmental aspects, but because there is no single known cause, identifying prenatal risk factors has become an important focus of research. In the United States, approximately 3.2% of children are diagnosed with ASD by age 8, and males are 3.4 times more likely to be diagnosed than females. ASD diagnoses have increased by roughly 300% over the past 20 years (Shaw). Therefore, understanding the risk factors and mechanisms that may contribute to ASD development has become increasingly vital.

The search for prenatal risk factors has progressively focused on the period of fetal development, since many biological processes involved in brain formation occur throughout

gestation. ASD is associated with altered brain development, and pregnancy is a critical period where the intrauterine environment can have lasting effects on fetal growth (Botelho et al.). During gestation, the fetal brain undergoes rapid neuronal migration, neural differentiation, and other developmental processes that establish neural networks (Ackerman). Because these processes happen in a tightly regulated environment, disturbances such as maternal stress, inflammation, poor nutrition, and high blood pressure have the potential to produce long term consequences. Therefore, maternal health conditions and pregnancy complications may play a significant role in shaping neurological effects and influencing future outcomes in offspring (Fitzgerald et al.). Consequently, maternal health has emerged as an important area of research for understanding neurodevelopment. It is thus crucial to investigate potential prenatal factors that may lead to disruptions in neural systems, which has been observed in individuals diagnosed with ASD (Rubenstein and Merzenich).

ASD risk has been associated with several prenatal conditions, including preeclampsia, which affects nearly 3% to 8% of pregnancies worldwide. The prevalence of preeclampsia is steadily rising, with rates nearly doubling over the past decade (Abalos et al.). Preeclampsia is a serious inflammatory pregnancy condition defined by high blood pressure and signs of damage to major organs, particularly the liver and kidneys. This condition originates from abnormal placental development, resulting in reduced blood flow between the mother and fetus (Staff). Since the placenta regulates essential aspects of fetal development, including oxygen and nutrient transport, placental dysfunction has the potential to have a significant impact on the developing fetus. Due to reduced placental blood flow, preeclampsia has various effects, including maternal inflammation, that directly influence fetal growth. Stress responses and cytokine expression increase with preeclampsia duration, resulting in prolonged fetal exposure to stressors. This extended exposure can increase risk for long term adverse effects (Koulouraki et al.). Because of preeclampsia's substantial effects on the intrauterine environment and fetal brain development, preeclampsia is considered a significant risk factor for ASD.

Gestational diabetes mellitus (GDM) is another common pregnancy complication, affecting 6% to 10% of all pregnancies in the United States. Rates of GDM have steadily risen among pregnant women (DeSisto et al.). GDM is a form of diabetes that occurs only during pregnancy, as hormones produced by the placenta cause insulin resistance (Catalano et al.). As maternal blood glucose levels rise, the fetal environment is altered, increasing exposure to inflammatory processes that may interfere with development (Xuan et al.). If left unmanaged, high blood sugar may cross the placenta and lead to oxidative stress in the fetus which is associated with numerous health conditions including, ASD, obesity, and a diabetes risk (Szmuilowicz et al.). Given the potential for unmanaged GDM to harm a developing fetus, GDM is also considered a risk factor for ASD.

Although preeclampsia and GDM are two distinct conditions, both pregnancy complications involve abnormalities in placental function, and can cause inflammation and oxidative stress in the mother and fetus (Aouache et al.). These effects suggest that preeclampsia and GDM may share similar biological pathways capable of increasing the risk of developing

ASD in affected offspring. While numerous studies have identified an association between preeclampsia, gestational diabetes, and an increased risk of ASD, the biological mechanisms connecting these conditions remain largely unknown. A better understanding of the underlying mechanisms may improve risk identification, inform future prevention strategies, and provide greater insight into the origins of ASD. As such, the purpose of this review is to synthesize existing evidence regarding the biology of both conditions and their relation to ASD, identify gaps in current literature, and guide future research by answering the question: what biological factors may explain how preeclampsia and gestational diabetes are associated with ASD risk in offspring?

To develop the narrative review, a literature search was conducted to identify peer reviewed studies examining the relationship between preeclampsia, gestational diabetes, and ASD risk in offspring. Searches were performed using PubMed and Google Scholar and search terms "preeclampsia biology autism risk," "gestational diabetes oxidative stress," and "maternal inflammation neurodevelopment." Inclusion criteria included a focus on the biological mechanisms of preeclampsia or gestational diabetes and an examination of ASD risk, fetal neurodevelopment, or related pathways. Exclusion criteria included studies written in languages other than English, lack of discussion of biological mechanisms of preeclampsia or gestational diabetes, and no ASD risk assessment. Included studies consist of cross-sectional and longitudinal original research, systematic reviews, and meta analyses.

Discussion

Immune Responses in Preeclampsia and Gestational Diabetes

During pregnancy, inflammatory processes are normal and serve different roles at different stages, including facilitating implantation and labor. However, there is an important distinction between normal controlled, and abnormal systemic inflammation, as an abnormal inflammatory state can affect the maternal fetal environment adversely (Mor et al.). Normal inflammation involves the body's natural release of cytokines and other signaling molecules, which respond to stress or injury. When inflammatory signaling becomes excessive, increased cytokine activity can disrupt normal cellular function and the homeostasis of the placenta. Both preeclampsia and GDM are associated with increased inflammatory activity, and this response can be initiated through different biological processes (Garcia-Morales et al.).

Healthy fetal development requires proper placental formation early in pregnancy. In normal placental development, cells providing nutrients to the embryo that eventually become a part of the placenta called fetal trophoblast cells invade the maternal uterus. When these cells remodel the uterine spiral arteries, which are small blood vessels supplying blood to the endometrium, into large and low resistance blood vessels, this allows the placenta to receive an adequate amount of oxygen and nutrients. In preeclampsia, abnormal placental development occurs in which the remodeling of the uterine spiral arteries is incomplete, resulting in rigid and narrow blood vessels. This in turn leads to placental ischemia, a decrease in blood flow and

oxygen to the placenta, places tissues under stress, and contributes to endothelial dysfunction (Gathiram and Moodley). When the cells lining the blood vessels no longer function properly, a variety of events capable of damaging the placenta can occur, such as a decrease in nitric oxide, a gas molecule that widens blood vessels, which leads to an increase in blood pressure (Possomato-Vieira and Khalil). This can contribute to a cascade of placental abnormalities, such as increased release of pro-inflammatory cytokines and extracellular vesicles into the maternal circulation (Tucker et al.). Extracellular vesicles are small, membrane bound particles that carry proteins, fats, RNA, DNA, and inflammatory signals which can cross the placenta and reach the fetus. As these inflammatory molecules continue to accumulate, they activate the maternal immune system and create a persistent inflammatory intrauterine, which can disrupt the tightly regulated conditions required for fetal development (Morelli and Yoel Sadovsky).

While preeclampsia is characterized primarily by abnormalities in placental vascular development, GDM involves metabolic disruption that can similarly alter the intrauterine environment. During pregnancy, the placenta produces hormones like human placental lactogen (hPL) and estrogen to support fetal growth. These hormones decrease maternal sensitivity to insulin, creating an insulin resistance that allows more glucose to remain in the blood stream. In a typical pregnancy, the mother's body compensates for this by producing more insulin, but in GDM, the insulin resistance becomes too excessive, causing chronic maternal hyperglycemia (Rassie et al.). This persistent high blood sugar is particularly significant as glucose can cross the blood placenta barrier, whereas maternal insulin cannot. As a result, the fetus is exposed to increased glucose amounts and must produce its own insulin to regulate blood sugar, increasing the stress within the intrauterine environment (Carpita et al.). Elevated glucose levels also stimulate the placenta to release pro-inflammatory cytokines, contributing to chronic inflammation. These inflammatory cytokines interfere with insulin signaling, worsening insulin resistance and creating a cycle where hyperglycemia and inflammation continue to reinforce each other (Rains and Jain). Although GDM begins as a metabolic disorder, it is triggered by placental factors that continuously create an inflammatory environment similar to placental changes observed in preeclampsia.

Although preeclampsia and GDM originate from different biological abnormalities, both conditions ultimately create similar inflammatory conditions during pregnancy. In preeclampsia, impaired placental development and reduced blood flow contribute to placental stress and the release of inflammatory markers, while GDM produces inflammation through metabolic dysfunction and chronic hyperglycemia (Botelho et al.). Despite these different origins, both conditions involve persistent activation of the maternal immune system and elevated levels of cytokines, otherwise known as maternal immune activation (MIA) (Mei et al.). One important pathway in MIA is the increased production of interleukin-6 (IL-6), a pro-inflammatory protein that acts as a chemical messenger between immune cells (Smith et al.). IL-6 promotes the formation of T helper 17 (Th17) cells, which are immune cells defending against infections and producing interleukin-17A (IL-17A). IL-17A is another cytokine capable of contributing to the development of chronic inflammation and fetal immune exposure. When inflammatory signaling

is elevated throughout pregnancy, cytokines such as IL-6 and IL-17 can disrupt the normal intrauterine balance and promote MIA (Kimura and Kishimoto). In addition to circulating cytokines, the placenta may contribute to fetal exposure through the release of extracellular vesicles (EVs). These small membrane bound particles can transport proteins, RNA, DNA, and other harmful molecules across the placenta, allowing placental inflammatory signals to directly influence fetal development (Fazeli and Godakumara). Together, MIA and EVs create an altered fetal environment that may interfere with normal neurodevelopmental processes. This shared inflammatory pathway provides a biological connection between preeclampsia, GDM, and their association with increased ASD risk in offspring.

In utero, the fetal blood brain barrier is developing, making the fetal brain susceptible to maternal inflammatory signals (Erlebacher). As MIA elevates inflammatory cytokines, these immune signals can alter the fetal environment and influence brain development (Rudolph et al.). Maternal IL-17A may reach developing brain tissue before the blood-brain barrier is fully formed, and activate signaling pathways NF-κB and ERK, both of which are involved in the expression of genes in neurodevelopment, such as the synaptic gene Grin2a. A mouse model showed that chronic IL-17A exposure alters gene expression in the fetal forebrain, with ASD related genes becoming overrepresented and synapse related genes unregulated. These changes were accompanied with reduced cortical development and altered social behavior, reinforcing the possibility of maternal inflammatory signaling producing neurodevelopmental changes relevant to ASD (Gumusoglu et al.). Therefore, MIA as produced in preeclampsia and GDM may influence ASD risk through cytokine signaling that alters developmental processes in the fetal brain.

Oxidative Stress in Preeclampsia and Gestational Diabetes

Oxidative stress is a condition in which the production of reactive oxygen species (ROS) exceeds the body's antioxidant defenses, preventing free radicals from being neutralized effectively. Although small amounts of ROS are normally produced during cellular metabolism and play an important role in cell signaling, excessive ROS become harmful (Pizzino et al.). One of the primary targets of oxidative stress is DNA, which is responsible for storing genetic information and regulating normal cellular function. ROS can modify DNA bases and break bonds within the backbone, which reduces the cell's ability to repair and maintain normal gene expression (Gonzalez-Hunt et al.). In addition, DNA damage from oxidative stress can impair mitochondrial function, which results in the production of more ROS and maintains the preexisting state of oxidative stress (Guo et al.). As this damage accumulates, placental cells become less efficient at carrying out important functions such as energy production, cell signaling, and overall creating a healthy environment for fetal growth (Voros et al.). This cycle of cellular damage contributes to placental dysfunction and establishes oxidative stress as a shared biological mechanism in both preeclampsia and GDM.

As observed in preeclampsia, abnormal remodeling of the uterine spiral arteries in preeclampsia reduces placental blood flow and creates a hypoxic environment in the placenta.

Because placental cells rely on a constant supply of oxygen to carry out cellular respiration, oxygen availability is a crucial factor in adenosine triphosphate (ATP) production, the main source of energy for cellular use. During hypoxia, oxygen is limited and unable to serve as the final electron acceptor in the electron transport chain. As a result, ATP production becomes less efficient and electrons begin to accumulate. Instead of following through with oxidative phosphorylation, some excess electrons prematurely react with oxygen molecules to form ROS (Aouache et al.). One example of an ROS is superoxide, which is produced when oxygen gains an extra electron (Ray et al.). Under normal conditions, enzymes break down these superoxides and maintain cellular homeostasis. However, an overabundance of ROS in preeclampsia overwhelms these defenses and results in oxidative stress (Chiarello et al.). Persistent oxidative stress damages lipids, proteins, and both nuclear and mitochondrial DNA, further impairing placental cell function (Jauniaux et al.). Damage to mitochondrial DNA can also worsen mitochondrial dysfunction, creating a cycle where abnormalities in energy production and ROS generation continue to strengthen the effects of one another (Hollensworth et al.).

The increased intracellular glucose seen in GDM places a greater metabolic demand on placental cells, forcing the mitochondria to work harder in order to produce ATP. As mitochondrial activity increases, electrons are more likely to leak from the electron transport chain and react with oxygen, creating excessive ROS, such as superoxide radicals (Murphy). Under normal conditions, antioxidant systems neutralize these free radicals and prevent significant cellular damage. However, chronic hyperglycemia results in ROS production that exceeds the placenta's capabilities to neutralize, resulting in a state of oxidative stress (Aye et al.). In addition, oxidative stress can interfere with insulin signaling pathways, further contributing to insulin resistance and elevated glucose levels (Rains and Jain). This iteration allows hyperglycemia and oxidative stress to continually reinforce each other and contribute to an increasingly impaired intrauterine environment (Gonzalez et al.).

Given that normal fetal development depends on a healthy placenta and tightly regulated cellular processes, prolonged oxidative stress can create conditions that may interfere with fetal neurodevelopment (Park et al.). Excessive ROS can overwhelm antioxidant defenses and suppress enzymes such as superoxide dismutase 2 (SOD2). SOD2 is involved in neutralizing superoxide radicals. In a prenatal mouse model, increased oxidative stress and reduced SOD2 expression produced ASD-related behavioral abnormalities in offspring. Increasing SOD2 expression subsequently reduced oxidative stress and unregulated SOD2 gene expression in the amygdala and hypothalamus, which partially reduced ASD behavior abnormalities. This suggests that oxidative stress may influence ASD related behavior through disruption of antioxidant regulation within the brain (Zhang et al.). These findings provide a potential mechanism through which oxidative stress associated with pregnancy complications such as preeclampsia and GDM may influence neurodevelopment.

Other Mechanisms for Autism Spectrum Disorder Risk in Preeclampsia and Gestational Diabetes

Beyond these shared inflammatory and oxidative stress pathways, preeclampsia may influence fetal neurodevelopment through disruptions specific to its pathology. In a healthy pregnancy, vascular endothelial growth factor (VEGF) is a signaling protein that promotes angiogenesis, the growth of new blood vessels, and supports the survival of endothelial cells. In preeclampsia, however, the placenta releases excess amounts of soluble fms-like tyrosine kinase-1 (sFlt-1), an antiangiogenic protein that binds to VEGF and placental growth factor (PIGF) which inhibits these growth factors from reaching cell receptors. By preventing the growth factors from binding to receptors on endothelial cells, sFlt-1 reduces angiogenic signaling, promoting decreased placental perfusion and hypertension (Jena et al.). In addition to placental function effects, VEGF is also important in fetal brain development. In the developing fetus, VEGF promotes endothelial cell migration and proliferation, supporting the formation of the brain's vascular network. When VEGF signaling is disrupted, cerebral angiogenesis may become impaired, potentially affecting the development and function of the blood brain barrier and other components of the vascular network (Halim et al.). Because the neurovascular unit helps coordinate communication between neural activity and cerebral blood flow, disruption of this system may affect normal regulation of the developing brain. Abnormalities including altered blood brain barrier function, angiogenesis, cerebral blood flow, and neurovascular uncoupling, have also been reported in individuals with ASD, suggesting a potential pathway connecting impaired angiogenesis with ASD related neurodevelopmental outcomes (Ahirwar et al.).

Whereas preeclampsia may influence fetal neurodevelopment through disrupted angiogenic signaling, GDM has additional effects that may play a role in the disruption of the fetal gamma-aminobutyric acid (GABA/GABAergic) system, the brain's main inhibitory neurotransmitter. During normal fetal brain development, GABA acts as an excitatory chemical messenger and plays a role in regulating neural cell proliferation, synapse formation, and establishing neural circuits (Allen et al.). However, chronic maternal hyperglycemia may affect gene expression through epigenetic modifications, such as hypermethylation of the glutamate decarboxylase 2 (GAD2) promoter. This gene produces an enzyme that synthesizes GABA, so reduced expression slows the development of GABAergic neurons and reduces overall GABA levels. Studies have shown that these changes are evident in the prefrontal cortex (PFC), which is involved in decision making, planning, self-control, and emotional regulation (Liu et al.). Reduced GABA signaling disrupts the excitatory/inhibitory balance that consistently presents in ASD (Madia et al.). This altered neural activity may impair communication between brain regions and contribute to the behavioral and cognitive aspects associated with ASD, suggesting that disruptions to the GABAergic system may be another pathway that links GDM to influencing fetal neurodevelopment.

Limitations

This review has several limitations that should be considered. First, many of the proposed biological mechanisms identified possible explanations for the association between preeclampsia

or GDM, and ASD. However, the relative contribution of each mechanism is unknown. This is because multiple pathways likely interact throughout fetal development making it challenging to disentangle a singular ASD risk factor. A second limitation is that much of the current evidence is the result of retrospective observational studies and animal models. Because it is not ethical to induce pregnancy complications in humans, studies cannot fully capture the extent of how these mechanisms contribute in pregnancy and neurodevelopment. Finally, ASD is a disorder influenced by a complex interaction between genetic and environmental factors, therefore it is likely that pregnancy complications such as preeclampsia and GDM represent a small component of a much larger biological process.

Future Directions

Future research should focus on longitudinal human studies that follow pregnancies and offspring from the prenatal period through early childhood to monitor long term neurodevelopmental outcomes in offspring. Unlike retrospective studies, longitudinal designs would allow biological changes to be observed from their early emergence through development. This long term observation may also help identify critical windows during fetal brain development in which pregnancy complications have the greatest influence on ASD risk. Furthermore, tracking biomarkers associated with the biological mechanisms identified here, such as inflammatory cytokines, oxidative stress markers, and angiogenic factors, could provide a better understanding of how these pathways change and interact throughout pregnancy. Measuring these biomarkers over time may help determine which mechanisms are most strongly associated with altered fetal neurodevelopment and whether certain biological changes occur consistently before an ASD diagnosis.

Conclusion

In conclusion, this review explored the biological mechanisms that may explain how preeclampsia and GDM are associated with increased ASD risk in offspring. Current evidence suggests that preeclampsia and GDM may influence fetal neurodevelopment through multiple connected pathways. Maternal inflammation may contribute to MIA, resulting in cytokine exposure that can alter signaling pathways that control genes related to ASD. Oxidative stress may have an effect by disrupting antioxidant regulation through reduced SOD2 expression. Additional pathways, including angiogenic imbalance in preeclampsia and GABAergic dysfunction in GDM, provide further insight into how abnormal prenatal environments associated with these conditions may influence ASD risk. However, these mechanisms represent only potential contributors to the development of ASD, and continued research is needed to clarify how these pathways interact and influence differences in fetal neurodevelopment.

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Investigating Whether Thymol Pre-Exposure Improves Regeneration Capacity in *Hydra vulgaris* Following Hydrogen Peroxide (H_2O_2) Induced Oxidative Stress

By Arya Mamidyala and Rachel Doraiswamy

Abstract

Regeneration in Hydra vulgaris is driven by the proliferation and differentiation of interstitial stem cells. When exposed to 1 mM hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) that induces oxidative stress, Hydra experience DNA damage. This study investigated whether pretreatment with thymol, an antioxidant, is able to preserve the regenerative capacity of Hydra vulgaris under oxidative stress (Tsarkova, Elena, et al, 2023). It was hypothesized that thymol would mitigate oxidative damage and improve regeneration in the presence of H_2O_2. Hydra were starved for 7 days before experimentation. Five Hydra per trial were trisected around the mid-gastric region to induce regeneration. A toxicity test identified that 10μM thymol was the ideal non-toxic working concentration (Mann-Whitney U Test, $p < 0.01$, compared with 20μM). Four experimental groups were evaluated: a positive control (thymol), a negative control (H_2O_2), a control (Hydra Medium), and the treatment (H_2O_2+ thymol). Regeneration was assessed at 24, 48, and 72 hours using a standardized 0-5 regeneration scale. There was a statistically significant difference in mean regeneration scores between Hydra exposed with H_2O_2 and Hydra pretreated with H_2O_2 + thymol (Mann-Whitney U Test, $p < 0.01$). Hydra pretreated with thymol under oxidative stress conditions demonstrated improved regeneration compared to the 1mM H_2O_2 only group. These findings suggest that thymol protects regeneration by reducing ROS damage and preserving stem cell-mediated regeneration. Hydra vulgaris provides a cost-effective screening model for evaluating compounds that protect regenerative capacity under oxidative stress and supports further investigation of thymol and related antioxidants in regenerative biology.

Introduction

Tissue regeneration is a critical biological process found in most organisms. While humans have limited regeneration abilities, *Hydra vulgaris*, a freshwater cnidarian, possesses extraordinary regenerative capabilities(Lovas, J. R., & Yuste, R., 2022; Vogg, M. C., Galliot, B., & Tsiairis, C. D., 2019). Hydra are capable of regenerating full bodies from just a small fragment of tissue due to the huge concentration of interstitial stem cells (Reddy et al., 2019). Highly conserved molecular signaling pathways, such as the Wnt/β-catenin signaling pathway, are the primary inducers for regeneration. Furthermore, the evolutionarily conserved trait of this signaling pathway allows it to play the same role in human wound healing and repair (Haval et al., 2020). Oxidative stress plays a key role in tissue and cellular regeneration. Oxidative stress occurs in the presence of excess reactive oxygen species (ROS). ROS are byproducts of cellular metabolism; they play important roles in cell signaling, immune responses, and wound healing (Kozlov et al., 2024). In particular, ROS act as signaling molecules to initiate the regenerative process, or more specifically, activate the Wnt/β-catenin signaling pathway (Kozlov et al., 2024).

However, ROS can become harmful in large concentrations. When ROS levels become excessive, they can lead to cellular damage and inhibit regenerative processes altogether by impairing stem cell functions (Haval et al., 2020). This damage occurs because ROS are highly reactive molecules and often oxidize other cellular components to stabilize themselves, leading to molecular damage. To counteract this imbalance and restore cellular homeostasis, biological systems rely on antioxidant mechanisms to donate an electron to the unstable ROS molecule (Muscolo et al., 2024). However, as humans age, antioxidant systems degrade and ROS can lead to neurodegenerative diseases (Manoharan et al., 2016). For example, in Alzheimer's disease, excessive ROS production contributes to neuronal damage, protein misfolding, inability to regenerate neuronal cells, and progressive loss of cellular function (Manoharan et al., 2016). One potential approach involves utilizing antioxidant compounds to effectively restore redox balance and reduce excess ROS concentrations. Thymol, a naturally occurring phytochemical, has been shown to have strong antioxidant and free-radical-scavenging properties in previous studies (Muscolo et al., 2024). Thymol was heavily explored and its biochemical properties as an antioxidant have been shown(Chroho, M., Rouphael, Y., Petropoulos, S. A., & Bouissane, L., 2024). However, its impact on tissue regeneration under oxidative stress has not been fully studied in regenerative organisms and on the conserved cell signaling pathways. This study will investigate whether thymol pretreatment will improve the regenerative capacity of *Hydra vulgaris* under oxidative stress induced by hydrogen peroxide. The null hypothesis states that there will be no significant difference between the mean regeneration scores of Hydra exposed to 1 mM of H_2O_2 with thymol pretreatment and Hydra exposed to 1 mM of H_2O_2 alone. The alternate hypothesis is that Hydra pretreated with 10µM thymol and then exposed to 1 mM of H_2O_2 will exhibit significantly higher mean regeneration scores following H_2O_2 exposure due to thymol's antioxidant properties that aid in reducing oxidative damage. To test this hypothesis, three experimental phases were conducted: a thymol concentration/toxicity test to determine a safe optimal concentration, an H_2O_2 validity test to confirm oxidative stress-induced regenerative impairment, and the primary treatment, which compared H_2O_2, Thymol, H_2O_2 +Thymol, and a control. Controlled variables across all groups included a 7-day starvation period to standardize metabolic state, trisection of the mid gastric region, equal group sizes of 5 Hydra per dish, a consistent rinsing mechanism between treatments, and regeneration was assessed at identical timepoints using the same 0-5 scoring rubric.

Methods and Materials

Creation of Solutions

To create the Thymol 50 µM stock solution, 7.51mg of thymol was dissolved in 0.1mL of 95% ethanol in a beaker. Then Deer Park natural Spring Water was mixed in and added to bring the total volume of the stock solution to 1L. Working solutions of 10µM, 20µM, 30µM, and 40µM were created by using 20mL, 40mL, 60mL, and 80 mL, respectively, and adding spring water to make the total volume 100mL. Each solution was stored in an airtight container and

remade on a weekly basis. A 1mM H_2O_2 solution was prepared by diluting 4.5 mL of 3% hydrogen peroxide into 1 L of Deer Park natural Spring Water in a beaker. This solution was freshly prepared as needed due to its instability and short shelf life.

Thymol Toxicity/Concentration test

All Hydra were sourced from Carolina Biological Supplies. Sixty *Hydra vulgaris* were randomly selected and starved for 7 days. They were then divided into six groups (n = 10 per group) and exposed to thymol concentrations ranging from 0–50µM (increments of 10) for 24 hours. Between all treatments, the Hydra were rinsed by placing them in a cell strainer and gently pouring water over them. All Hydra were trisected to isolate the mid gastric region(Figure 2), and redistributed into groups of 5 per dish with 2 initial trials at each concentration. After 24, 48, and 72 hours, regeneration was assessed based on Figure 1 and recorded. Due to toxicity and Hydra death at 40µM and 50µM, the subsequent trials(n=6) were conducted using concentrations from 0-30µM(increments of 10) using 40 Hydra with 5 per concentration.

Hydrogen Peroxide Validity Test

Sixty *Hydra vulgaris* were randomly selected after 7 days of starvation, with 30 pretreated in hydrogen peroxide for 4 hours, and 30 serving as controls. Between all treatments, the Hydra were rinsed by placing them in a cell strainer and gently pouring water over them. All were then trisected to isolate the mid gastric region (Figure 2) , distributed in groups of 5 into 6 petri dishes per group (H_2O_2 and Control), and assessed for regeneration at 24, 48, and 72 hours based on the scoring rubric in Figure 1.

Effect of Thymol on Hydra Regeneration Under Oxidative Stress

200 *Hydra Vulgaris* were randomly selected, with 100 pretreated in 10µM thymol for 24 hours, after which subsets were either immediately trisected following pretreatment or subsequently exposed to hydrogen peroxide for 4 hours before trisection or exposed to hydrogen peroxide for 4 hours alongside a non-thymol pretreated group; between all treatments, the Hydra were rinsed by placing them in a cell strainer and gently pouring water over them. All samples were trisected to isolate the mid-gastric region (Figure 2), distributed in groups of 5 across 10 petri dishes per condition (control, H_2O_2 only, thymol only, H_2O_2 + thymol). All were evaluated for regeneration at 24, 48 and 72 hours using the standardized scoring rubric (Figure 1). Experimental conditions were all constant during this time in a dark cabinet at room temperature, 22°C.

Score:	0	1	2	3	4	5
Relation to regeneration	No regeneration	Initial wound closure: Cut edges are beginning to close	Early Bud formation: A small mound appears at the location of the wound	Partial regeneration : The body column reforms	Advanced regeneration : Tentacles are mostly formed	Complete regeneration : Fully regenerated hydra

Figure 1. Regeneration Scoring Criteria for *Hydra Vulgaris*
Hydra regeneration was assesong using a 0-5 scaled based on the observable recovery following amputation. Scores were assigned per Hydra and averaged per petri dish
(created by researcher)

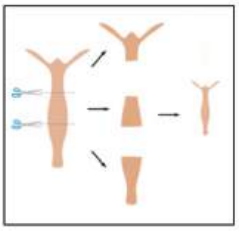

Figure 2. Model for Trisection Hydra to measure regeneration. Image adapted from Reddy et al. (2019)

Results

Thymol Concentration Test Data

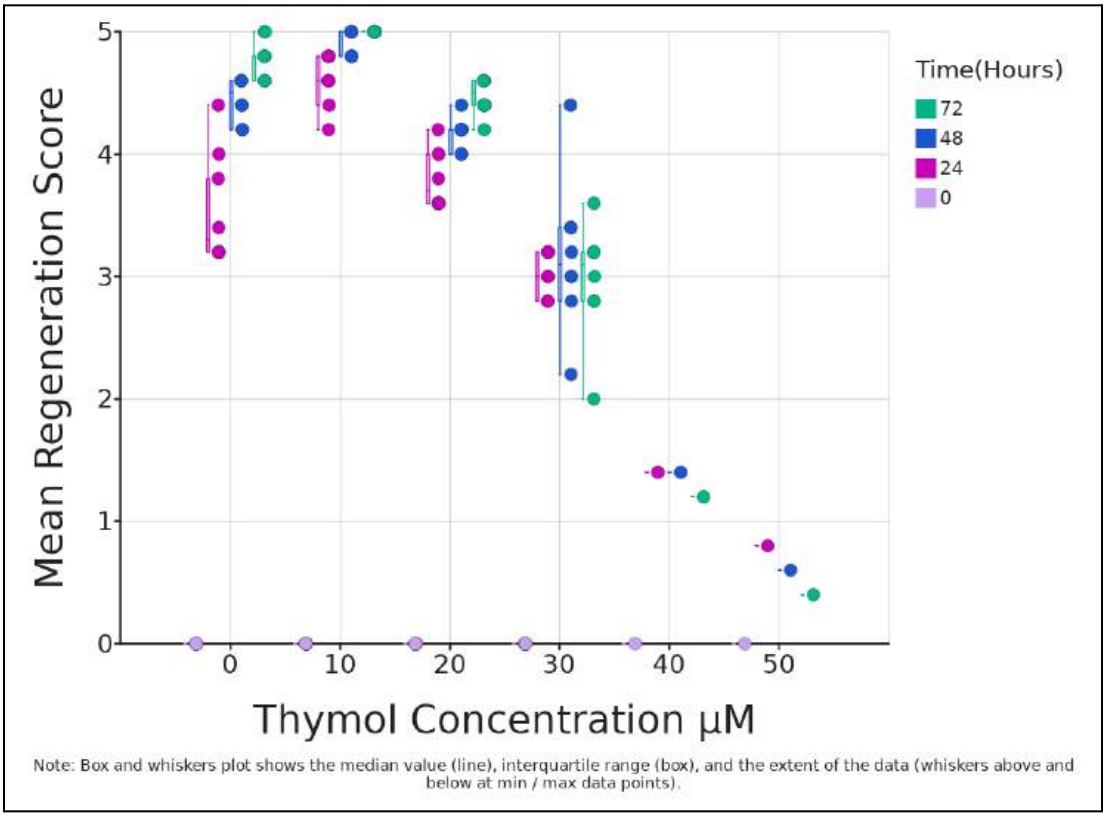

Figure 3. This shows the concentration-dependent effect of thymol on Hydra regeneration under oxidative stress. Regeneration scores increased over time across all groups. The 10µM demonstrated the most rapid and complete regeneration by 72 hours while 20µM-30µM showed partial recovery with 20µM being more efficient. Higher concentrations 40-50µM, ended up being toxic to the Hydra. 40µM and 50µM killed the Hydra
(created by researcher in DataClassroom)

Effect of Thymol on Hydra Regeneration Under Oxidative Stress

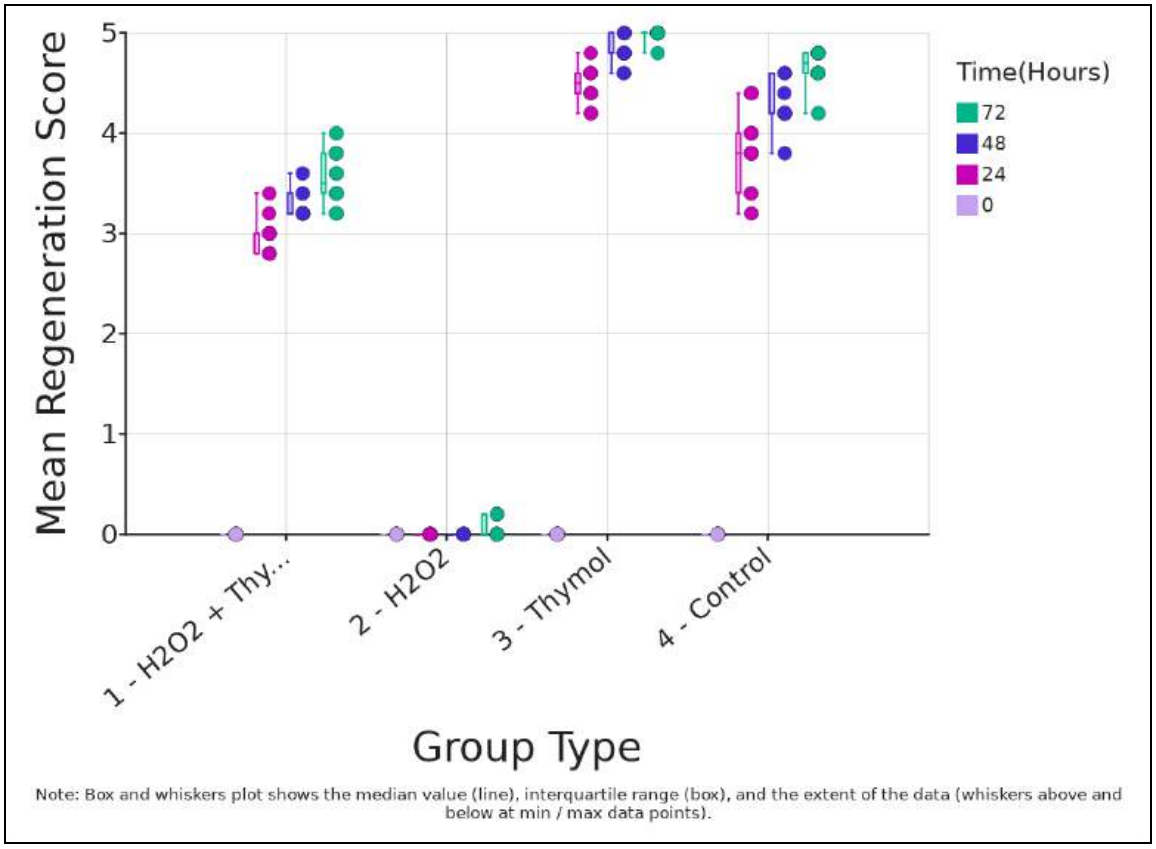

Figure 4. Analysis of regeneration in *Hydra vulgaris* across 4 treatment groups: 4 - control (no H_2O_2 no thymol), 3 - thymol only, 2 - H_2O_2 only, and 1 - H_2O_2 & thymol. Mean regeneration scores (0-5) were calculated solely from original cut fragments. H_2O_2 exposure suppressed regeneration when also pretreated with thymol the regenerative capacity was increased over the course of 72 hours.
(created by researcher in DataClassroom)

H_2O_2 Validity Test Data

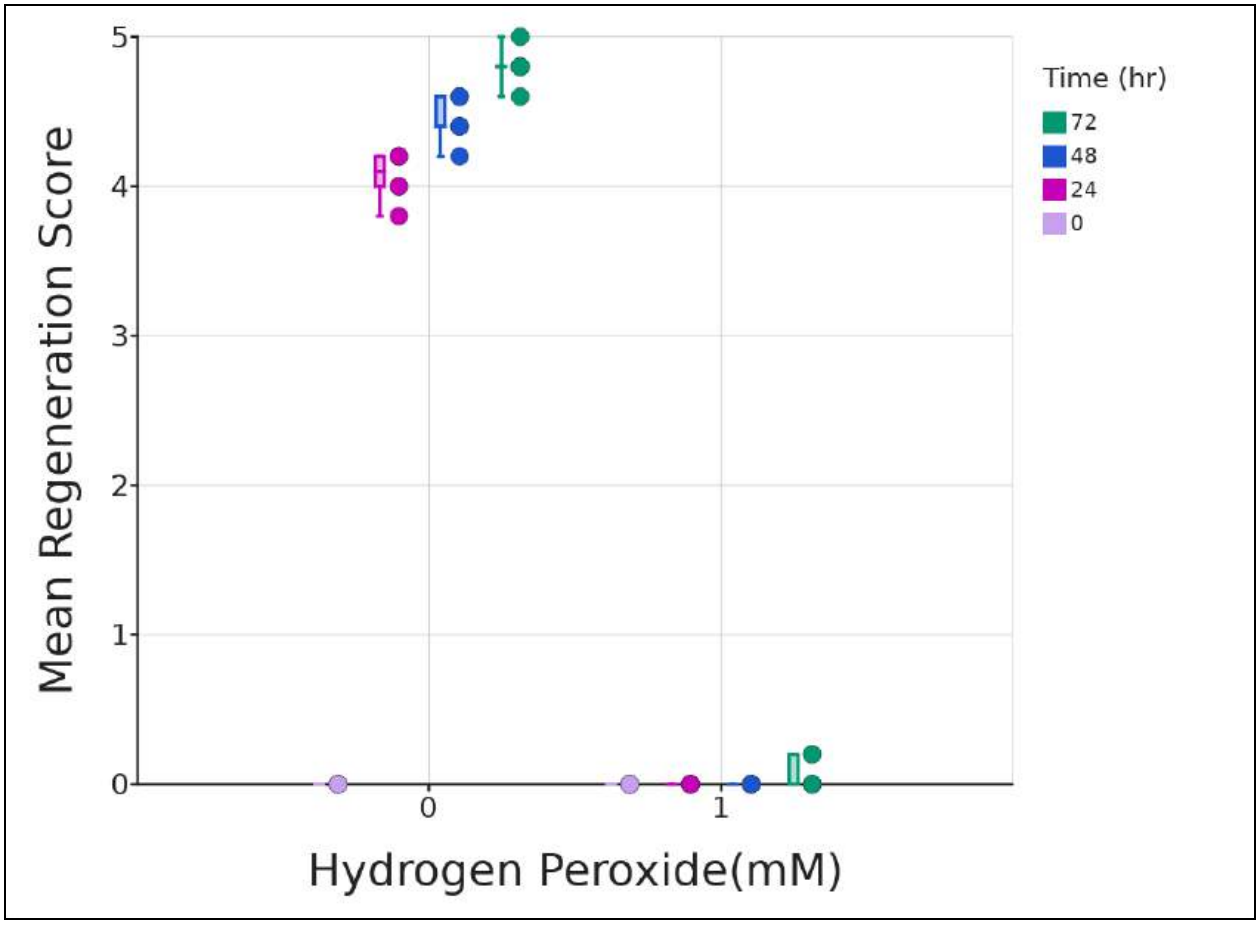

Figure 5. Validation of oxidative stress model in *Hydra vulgaris* using 1mM of H_2O_2. Hydra exposed to H_2O_2 showed suppression of regeneration across all timepoints (0-72 hr) while the untreated controls showed continued regeneration and achieved near complete regeneration by 72 hours. (created by researcher in DataClassroom)

Mann-Whitney U Test - Concentration Test

Time = 24 Hours	10µM	20µM	30µM
0µM	P < 0.01	P =0.16	P =0.01
10µM		P < 0.01	P < 0.01
20µM			P < 0.01
30µM			

Time = 48 Hours	10µM	20µM	30µM
0µM	P < 0.01	P = 0.01	P <0.01
10µM		P < 0.01	P < 0.01
20µM			P = 0.01
30µM			

Time = 72 Hours	10µM	20µM	30µM
0µM	P < 0.01	P < 0.01	P <0.01
10µM		P < 0.01	P < 0.01
20µM			P < 0.01
30µM			

Figure 6. This shows the results of the Mann- Whitney U Test comparing each concentration with each other. 40µM and 50µM were dropped because they were killing the Hydra

(created by researcher in DataClassroom)

Mann-Whitney U Test - Treatment Test

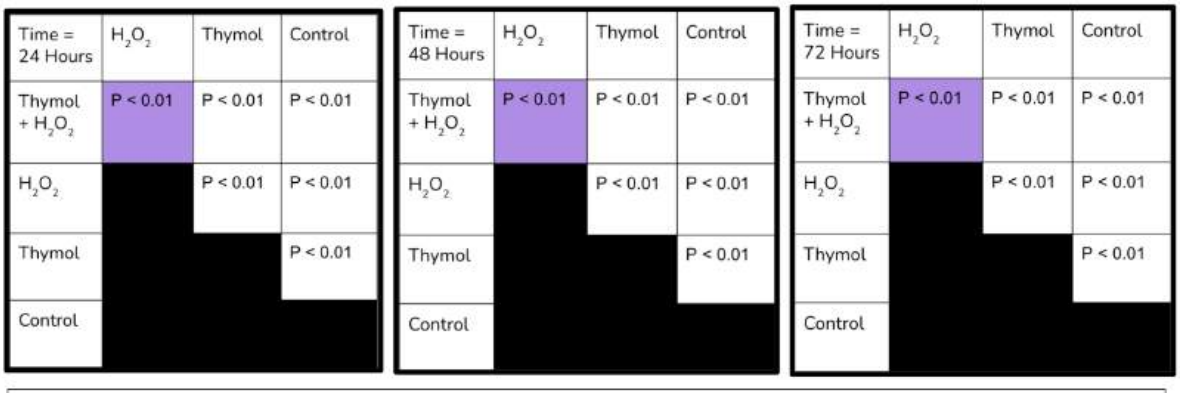

Figure 7. This shows the results of the Mann- Whitney U Test comparing the mean regeneration score of each group. The purple region indicates there was a significant difference between the H_2O_2-only group and the H_2O_2 + Thymol groups
(created by researcher in DataClassroom)

Discussion

Our results indicate that exposure to 1 mM hydrogen peroxide significantly impairs head and foot regeneration in *Hydra vulgaris*(Figure 5). Hydra exposed to solely hydrogen peroxide consistently exhibited lowered regeneration scores in comparison to untreated Hydra with no pretreatment (Figure 5), which suggests oxidative stress disrupted stem cell function and tissue regeneration. In contrast, Hydra pretreated with 10μM of thymol prior to 1 mM hydrogen peroxide exposure demonstrated increased and improved regeneration under oxidative stress(Figure 4). Although these Hydra were still exposed to the same concentration of hydrogen peroxide, their regeneration scores were significantly higher. This suggests that thymol effectively preserved the regenerative capacity of Hydra even under excessive ROS concentrations. A Mann-Whitney U test comparing regeneration scores between the thymol + H_2O_2 group and the H_2O_2-only group revealed statistically significant differences at 24, 48, and 72 hours ($p < 0.01$) (Figures 4 and 7). Furthermore, the usage of a 10μM concentration of Thymol was found to be the best choice to promote regeneration without inducing toxicity. The concentration test results showed that higher thymol concentrations were associated with reduced regeneration and increased mortality, whereas 10 μM thymol maintained significantly improved regeneration outcomes compared to both higher concentrations and controls ($p < 0.01$) (Figures 3 and 6). Thymol is suggested to have reduced the oxidative damage during hydrogen peroxide exposure. The excessive accumulation of ROS can damage critical cell components such as DNA, proteins, membranes, etc. These are essential for cell division and differentiation to occur in the wound. Thymol scavenges ROS and limits oxidative damage; through its antioxidant properties, it may have helped to preserve stem cell viability and efficiency in Hydra.

Regeneration in *Hydra vulgaris*, as previously mentioned, is heavily dependent on specific signaling pathways such as the Wnt/β-catenin pathway (Chatterjee S & Sil PC, 2022). Oxidative stress may interfere with these pathways and impair proper wound regeneration. The addition of a thymol pretreatment, through its antioxidant properties, may have helped stabilize intracellular redox conditions and maintain the integrity of these signaling mechanisms. The increase in regeneration scores when exposed to Thymol suggests that it can play a vital role in restoring redox balance and in cellular regeneration as a whole. Overall, the data rejects the null hypothesis and supports the alternative hypothesis that thymol pretreatment at 10 μM significantly improves regeneration in *Hydra vulgaris* under oxidative stress conditions. These results are consistent with Haval et al. (2020), who demonstrated that 1mM H_2O_2 inhibited head and foot regeneration in Hydra by impairing the DNA repair mechanisms, and with Muscolo et al. (2024), who identified thymol's free-radical scavenging properties as a key mechanism for reducing oxidative cellular damage.

Evaluation

Experimental tests demonstrated that thymol improves regeneration under oxidative stress; several limitations and potential sources of error may have influenced the results. One of the biggest possible limitations was the qualitative nature of the study. It introduces subjectivity and bias without a clear set of guidelines to score on. Differences in interpretation can be problematic. Furthermore, despite attempts to standardize metabolic rates through a 7-day starvation period, Hydra regeneration could have been affected by size differences or the physiological state of individual Hydra. This added variability could have affected the mean regeneration scores. Additionally, thymol was dissolved in 0.1mL of 95% ethanol before dilution into 1L of stock solutions. While this concentration is minimal, the absence of an ethanol-only control means its potential effect on regeneration cannot be entirely ruled out. Furthermore, regeneration scoring was not conducted blind to group assignment, meaning the observer knew which treatment each Hydra received during scoring. This introduces the possibility of observer bias influencing the qualitative regeneration scores. The sample size of 5 Hydra per dish also limits statistical power and may reduce the ability to detect small differences between each group.

Conclusion

The results shown by the experiment further strengthen the concept that Regeneration requires a tightly modulated balance in ROS. ROS bursts are crucial for regeneration, but also harmful at excessive amounts. Thymol is able to stabilize the unstable ROS and preserve homeostasis in the intracellular environment. It acts as a modulator by keeping ROS levels in check without eliminating the necessary ROS. The usage of hydra provides insight into the role oxidative stress plays in regeneration, tissue repair, and stem cells in more complex organisms due to the signaling pathways present being evolutionarily conserved. It helps contribute to a

broader understanding of how redox reactions affect regeneration. Also shows how antioxidant based therapies can be developed to be applied and used in more complex organisms.

Future Work

Rather than using Regeneration as a measure of ROS levels, fluorescent oxidative stress markers could be utilized to directly quantify ROS levels. PCR(polymerase chain reaction) could also be used to quantify the expression of key signaling pathway genes, namely Wnt/β-catenin, which would directly show the relation between ROS levels and antioxidant activity in the Hydra. This would further show and prove the protective effect of Thymol and how antioxidants play a role in preserving regeneration. Furthermore, it would be a quantitative measurement rather than qualitative, which would further support the results due to the lack of data collector bias or any other confounding factors during data collection. In addition to the fact that the Wnt/β-catenin pathway is found in humans, Hydra could be used as a pre-screening model for possible regenerative treatments due to the vast similarity between humans and Hydra regenerative processes.

These findings have broader implications for regenerative medicine as a whole. Thymol and other antioxidants have the potential to serve as therapeutic candidates for preserving cellular regeneration under stress conditions. Additionally, the use of *Hydra vulgaris* enables early-stage testing in an organism that shares conserved regenerative processes with humans, paving the way for future treatments.

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Growth of Neuroinflammation Research in Autism Spectrum Toward a Mechanistic and Interdisciplinary Model: A Bibliometric Analysis By Alicia Sapon Say[1], [2]Hansika Gokavarapu, [3]Aryan Gupta, [4]Dhyey Patel, Angeline [5]Nguyen, Rajata [6]Palicherla, [7]Ameera Nehal, [8]Katherine Puac, [9]Aditi Banerjee, [10]Gokul R. Jala

Abstract

Objectives: Analyze how research on neuroinflammation in ASD has developed over time by mapping publication trends and emerging topics.

Background: Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition that has become a major focus of scientific research due to its increasing prevalence and significant impact on communication and social behavior. Neuroinflammatory processes may contribute to ASD development and underlying mechanisms; As global ASD research expands, identifying influential authors and emerging directions on neuroinflammation in ASD is essential.

Methods: A bibliometric analysis was performed with the n=100 most-cited publications (n= initial records) in the Web of Science Core Collection using the query: analyzed using the R package *bibliometrix* and Biblioshiny for publication trends, citation patterns, influential authors, institutions, prevalent keywords, and collaboration networks.

Results: A noticeable upswing in publications after 2018, with a stronger increase during 2021-2023. It can be deduced that along the way noticeable emerging themes came up, like gut microbiota immune signaling, genetic plus epigenetic regulation of glial activation (for example CHD8), and anti-inflammatory treatment pathways. The literature base is moving forward. mechanistic, but also translational, ASD research.

Conclusion: Research findings on neuroinflammation in ASD greatly increased since 2018, peaking in 2021–2023. This upward trend reflects the emerging causal themes of gut microbiota immune signaling, genetic plus epigenetic regulation of glial activation (for example, CHD8), and anti-inflammatory treatment pathways. This evolution reveals the field is moving away from descriptive work towards mechanistic and multi-system framework, integrating neural, immune, and metabolic processes. By mapping this progression, the analysis demonstrates a cross-disciplinary study of biological systems guides to precise approaches to ASD intervention. The field is increasingly oriented toward biomarker development and targeted therapies, though collaboration remains fragmented and greater interdisciplinary integration is needed to advance clinically meaningful progress.

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1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by irregularities in social behaviors, and an individual's ability to register sensory input. Research links ASD to neuroinflammation and immune dysregulation, specifically glial signaling, epigenetic control, and gut-brain-immune crosstalk (Hughes et al., 2023). However, there is no clear analysis showing how these studies have evolved. This bibliometric analysis fills this gap by quantifying the publication growth, and emerging themes.

2. Methods

A literature search was conducted in Web of Science Core Collection using three keywords to obtain 199 records: "autism spectrum disorder (ASD)", "neuroinflammation" and "neurodevelopment/brain-development". The top 100 most-cited works were selected for bibliometric analysis. Each team member summarized 3-5 publications to identify major findings, recurring neuroinflammation-ASD themes. The final data pool was processed using Microsoft Excel, RStudio, and Bibliometrix/Biblioshiny to generate visualizations that highlighting major research trends, influential research and emerging research themes.

3. Results

Figure 1 displays the most productive authors among the top 100 cited papers on neuroinflammation in ASD. Li Y. published the most papers (9 publications), followed by Wang H. who published 8. Both Bjorklund G. and Sadek B. each contributed 6 publications, while several other authors published 5 papers. Figure 2 shows the institutions with the most top-100 cited publications. Central South University contributed the most with 4 publications, while several other institutions published 2 papers each. Figure 3 shows the top-7 journals that had multiple top-100 publications. *The International Journal of Molecular Sciences* had the most papers with 10, followed by *Brain, Behavior, and Immunity* with 9. Figure 4 visualizes the process of how papers were selected for analysis. The Web of Science search found 199 papers, and the top-100 cited papers were chosen. Out of the 100, 52 were articles and 48 were reviews. Figure 5 displays a map of the main keywords that were used together throughout the top 100 papers. Some highly interconnected keywords included microglial activation, inflammatory cytokines, oxidative stress, mitochondrial dysfunction, gut microbiota, and the gut-brain axis. Figure 6 shows the countries with the most citations. The United States had the most citations, followed by other highly-cited nations such as Italy and China. Figure 7 shows how publication counts changed over time. The amount of papers published in a year increased from 1 in 2013 to a peak of 14 in 2021. Publication counts were highest from 2018-2023, followed by 8 papers in 2024 and 4 in 2025.

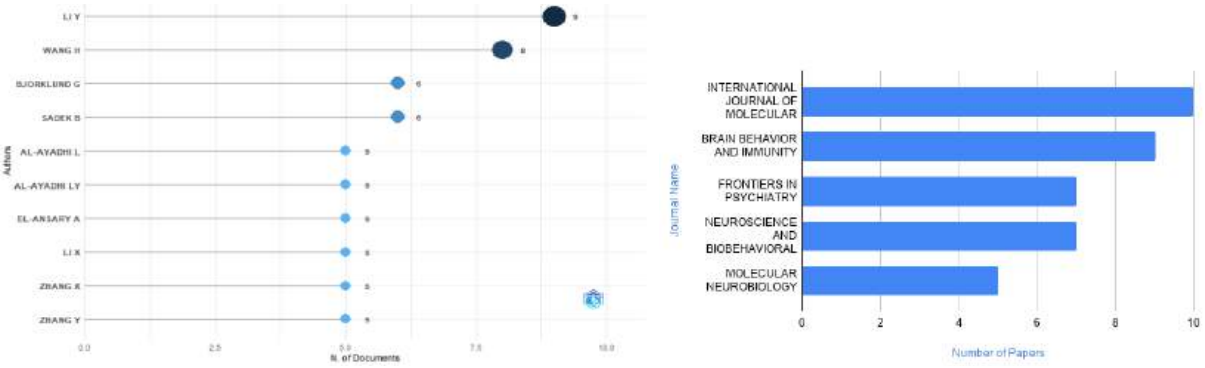

Figure 1. Senior Authors with Multiple Top 100 cited papers

Figure 2. Senior Author Institution with Multiple Papers of Top-100 Most Cited Papers

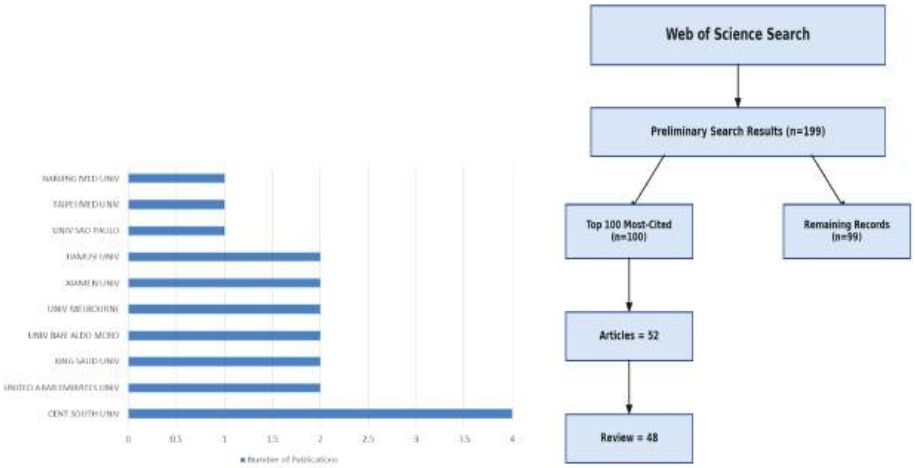

Figure 3. Journals with Multiple Top-100 cited Articles

Figure 4. WOS publication extraction showing eligible and excluded record

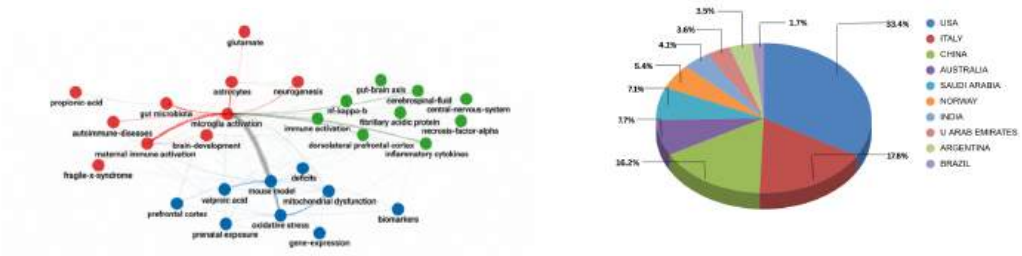

Figure 5. Top-30 Keyword Co-Occurrence Network

Figure 6. Countries with Most Top-100 Citations

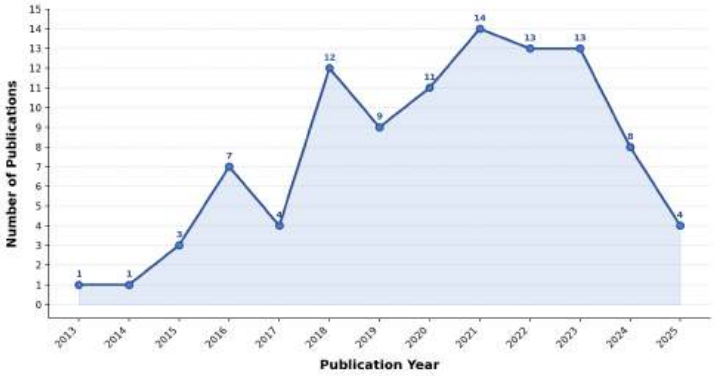

Figure 7. Topic Evolution Top-100 Articles

4. Discussion

Neuroinflammation research in ASD is shifting from broad correlations toward specific mechanistic pathways. Rising publication activity reflects growing interest and improvements in neuroimmunology and genetics (Figure 7). Keyword patterns show consistent causal themes: microglial activation, oxidative stress, mitochondrial dysfunction, inflammatory cytokines, and the gut-brain signalling (Figure 5). Studies examined microglia-astrocyte interactions and intercellular communication, ASD-related gene CHD8 linkage to astrocyte inflammation, and gut-brain axis, showing research expanding beyond brain-only inflammation (Liao et al, 2020). However, citation patterns show uneven global participation, led primarily by the United States, Italy, and China (Figure 6). The field is focusing on concrete mechanistic routes and is moving into a more translational, treatment-oriented direction with implications for better biomarkers and treatments. However, collaboration is concentrated to few institutions, and with little current translational progress for therapies.

5. Conclusion

Neuroinflammation research in ASD has expanded steadily, with top-cited studies converging on key mechanistic themes such as microglial activation, oxidative stress, mitochondrial dysfunction, and gut-brain signaling. The field is increasingly oriented toward biomarker development and targeted therapies, though collaboration remains fragmented and greater interdisciplinary integration is needed to advance clinically meaningful progress. Overall, these findings suggest that neuroinflammation in ASD is a growing body of international research. Future research should continue to investigate biological mechanisms, potential biomarkers, and the pathogenesis to deepen the understanding of ASD and develop more effective treatments.

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Applications of CRISPR Gene Editing in Regenerative Medicine and Space Biology

By Brian Lam

Abstract

In the 21st century, CRISPR-Cas9 has transformed regenerative medicine by allowing precise genetic modifications in stem cells, with applications including anti-aging, tissue repair, and gene therapy (Zhang et al., 2023; Lei and Wang, 2026). However, these applications are typically studied alone, and no existing review has explored how CRISPR-mediated rejuvenation, tissue repair, and space-based biomanufacturing can be integrated to support both Earth medicine and deep space exploration. This review synthesizes three interconnected themes to highlight how gene editing can advance regenerative medicine and sustain astronaut health during deep space missions. This review follows the PRISMA framework, and a systematic literature search was conducted across PubMed, Science, Nature, and Springer Nature (2014-2026) using Boolean keywords linked to each theme. After title and abstract screening and full-text review, 23 studies were selected from an initial pool of 412 papers. CRISPR activation screening has identified SOX5 as a factor that restores regenerative capabilities in aged mesenchymal stem cells MSCs without changing cell identity (Jing et al., 2023), while partial reprogramming extended lifespan in aged mice (Cano Macip et al., 2024). In tissue repair, CRISPR-based treatments have achieved clinical success in sickle cell disease (Frangoul et al., 2021) and preclinical efficacy in Duchenne muscular dystrophy (Nelson et al., 2016). And in space, microgravity allowed 3D tissue formation without scaffolds on the ISS, with the Redwire facility successfully bioprinting functional constructs, including a human knee meniscus (Klarmann et al., 2024; Redwire, 2024). These findings suggest that integrating gene editing with space-based biomanufacturing could produce enhanced tissue for both terrestrial and extraterrestrial use (Kim et al., 2024). Ultimately, integrating CRISPR-mediated rejuvenation, repair, and space-based biomanufacturing will be essential for advancing regenerative medicine and sustaining human life beyond the likes of Earth.

Keywords CRISPR-Cas9; gene editing; stem cell rejuvenation; tissue regeneration; tissue repair; cellular senescence; regenerative medicine; microgravity; 3D bioprinting; organoids; space-based biomanufacturing.

1. Introduction

In the 21st century, medicine is shifting from symptomatic treatment towards the restoration of damaged tissues and organs. At the center of this transformation is the use of stem cells and gene-editing technologies. Stem cells act as the body's raw materials, as they are able to grow into many different cell types, making them essential for tissue repair and regeneration. However, in order to improve their therapeutic potential, scientists need to be able to edit their genetic information. Gene-editing tools such as CRISPR have been one of the most transformative biotechnology advances in the 21st century. In essence, CRISPR-Cas9 uses a

small RNA molecule to find a specific sequence of DNA and make precise genetic modifications, allowing scientists to correct disease-causing mutations and modulate cellular functions at their source (Zhang et al., 2023). While CRISPR was initially revered for its gene editing capabilities, the application of CRISPR has expanded massively beyond simple knockouts to include base editing, epigenetic regulation, and transcriptional modulation (Xie et al., 2019). This versatility makes it an indispensable asset in regenerative medicine, offering scientists the ability to engineer stem cells that are both functional and resistant to diseases (Lei and Wang, 2026).

Despite these advances, several biological barriers still limit the clinical translation of CRISPR-based technologies. Stem cells, the basis of tissue repair, are also susceptible to intrinsic aging and extrinsic damage. As organisms age, their stem cell functions decline due to telomere attrition, genomic instability, and epigenetic drift, leading to a reduced capacity for tissue homeostasis, also known as stem cell exhaustion (Li et al., 2020). Furthermore, these acquired genetic defects can make cells dysfunctional, leading to degenerative conditions (Li et al., 2020). Similar effects can also occur during long-duration space missions, where microgravity and cosmic radiation accelerate cellular damage and mimic many features of natural aging on Earth (Conti et al., 2025). This poses a risk to astronauts, as stem cells are essential for maintaining tissue integrity during long space missions. However, the microgravity environment of space also offers a unique environment for tissue engineering because cells can grow into complex three-dimensional structures with less dependence on artificial scaffolds (Aldrich et al., 2021).

Recent literature has demonstrated a growing potential of CRISPR in stem-cell rejuvenation and tissue engineering and space biology. However, these applications are often studied separately, and few reviews examine how they can work together. This review aims to explore three interconnected themes: the use of CRISPR to rejuvenate aged stem cells, repair damaged tissues, and support the biomanufacturing of tissues and organoids in space. By bringing these areas together, this review highlights the potential of gene editing to advance regenerative medicine and support future long-duration space missions.

2. Methodology

This review follows the PRISMA framework. A comprehensive literature search was conducted across major scientific databases, including PubMed, Science, Nature, and Springer Nature. The search was focused on articles published between 2014 and 2026 to document the rapid evolution of CRISPR technology and the recent advancements in space biology.

The search strategy used targeted Boolean keywords linked to the three core themes of this review, respectively:

1. Theme 1 (Rejuvenation): CRISPR, gene editing, base editing, epigenetic editing, stem cells, ageing, cellular senescence, epigenetic regulation, Yamanaka factors
2. Theme 2 (Repair): CRISPR, Cas9, gene therapy, tissue repair, cellular damage, fibrosis, mitochondrial repair, gene correction

3. Theme 3 (Space Biology): microgravity, spaceflight, low Earth orbit LEO, biomanufacturing, 3D bioprinting, organoids, tissue engineering, stem cells, space biology

The initial screening process was done in 2 stages to ensure the relevance of the selected articles.

1. **Title and abstract screening:** All identified papers were initially screened based on their titles and abstracts. Studies were chosen if they were directly related to the investigation of the application of gene editing in the context of the 3 core themes.
   Exclusion criteria: studies not involving gene editing, papers focusing solely on predictions without experimental validation, articles published in languages other than in English, articles that fell outside the scope of the review
2. Full text assessment: The full texts of the selected articles were assessed against the inclusion criteria. During this phase, studies were further evaluated for their relevancy or rigor, and the clarity of their findings. Review articles were included to provide broader context, while original research articles were prioritized for functional insight.

The final selection consisted of studies that provided significant evidence or reviews regarding the utilization of gene editing; selected papers were then categorized into the 3 core themes to structure the review.

2.1 PRISMA flowchart

a. Identification: The initial database search yielded a total of n=412 records.

b. Screening: After removing duplicates, n=364 unique records were left. These records underwent title and abstract screening, of which n=305 records were excluded because they were irrelevant. This left n=59 records for full-text assessment.

c. Eligibility: Of the n=59 articles, n=34 articles were excluded because of lack of focus on the core themes or being purely theoretical without validation.
Selected: n=22 were selected for inclusion, forming the core of the review.

3. Discussion

3.1 CRISPR-Mediated Rejuvenation of Aged Stem Cells

The overlap of CRISPR technology and stem cell biology has created promising opportunities for combating aging. A core theme emerging from recent literature is the use of gene editing to restore the regenerative abilities of aged stem cells, effectively reversing the process of stem cell exhaustion. One of the most significant advances in this field comes from the work of Jing et al. (2023), who used a genome-wide CRISPR activation screen to identify the transcription factor SOX5 as a crucial factor of cellular rejuvenation. Unlike global cellular reprogramming, which can change cell identity, the targeted activation of SOX5 was found to be able to restore youthful characteristics to aged cells like MSCs, while preserving the cells'

original lineage (Jing et al., 2023). This is crucial for therapeutic applications. In a compelling in vivo demonstration, researchers delivered SOX56 via gene therapy to aged mice suffering from osteoarthritis. The treatment resulted in them having delayed joint aging, reduced inflammation, and improved cartilage regeneration (Jing et al., 2023). This study highlights the potential of CRISPR activation not just to silence harmful genes, but to also trigger factors that can repair and rejuvenate damaged tissues.

Often complementing these targeted approaches is the strategy of partial reprogramming, which involves the Yamanaka factors OCT4, SOX2, and KLF4 to reset the cell's biological clock without turning it all the way back into an embryo-like state. Cano Macip et al. (2024)showed that periodic induction of these factors in aged mice could extend their lifespan and reverse age-related changes across multiple organ systems. The treated mice showed improved metabolic function, reduced inflammation, and extended median lifespan, proving that the aging process is alterable and can be driven backwards (Cano Macip et al., 2024).

The efficacy of these treatments works through several connected mechanisms. A primary driver is the reversal of epigenetic drift, which is essentially the accumulation of epigenetic errors that occur with age and disrupt gene expression patterns (Li et al.,2020; Zhang et al., 2023). Through resetting these markers, gene editing can restore a more youthful pattern. Additionally, researchers are also focusing on how senescence in MSCs is often driven by mitochondrial dysfunction. Wan et al. (2020) explain that aging stem cells often have worn-out mitochondria, leading to low energy output. Through interventions like genetic editing, cellular bioenergetics can be improved and stem cell potency restored.

Another crucial mechanism is the prevention of cellular senescence. As stem cells age, they sometimes stop dividing and secrete inflammatory factors that damage surrounding tissue. To combat this, Cai et al. (2024) suggest a dual approach: by combining stem cell rejuvenation via reprogramming or gene therapy with therapies that target senescent cells. This approach addresses both the loss of functional stem cells and the accumulation of senescent cells, potentially extending healthy lifespan more effectively than either strategy alone.

Despite the promises of these new technologies, significant risks remain, particularly regarding the safety of gene editing in stem cells. Conti et al. (2025) highlight that the process of crispr-cas9/AAV6 mediated gene editing in HSCs can accidentally cause inflammation and senescence, potentially counteracting the benefits of rejuvenation. Their research suggests that transient p53 inhibition or blocking inflammatory pathways can mitigate these adverse effects, emphasizing the importance of optimized delivery methods and careful control of the cellular DNA damage response (Conti et al., 2025). Furthermore, Lei and Wang (2026) emphasize the need for rigorous safety checks when engineering stem cells to ensure that the editing doesn't accidentally cause tumours or other unintended problems.

Looking forward, the most viable path to clinical efficacy potentially lies in multi-omic approaches. Combining stem cell rejuvenation via targeted gene therapy with senolytic treatments to destroy senescent cells could resolve both stem cell exhaustion and the toxic senescence-associated secretory phenotype SASP (Cai et al., 2022; Ji et al., 2023). Ultimately,

refining these gene editing tools to safely fix the root causes of aging will be vital. Mastering these techniques on Earth will not only revolutionize how we treat age-related diseases, but it will also lay the groundwork for sustaining astronaut health during long-duration space exploration missions.

3.2 CRISPR-Based Gene Editing for Tissue Repair and Regeneration

While cellular rejuvenation focuses on reversing aging, gene editing also offers capabilities for repairing cells that have suffered genetic, structural, or metabolic damage. Unlike traditional treatments, which only manage symptoms, CRISPR-based treatments can address the root molecular causes of cellular dysfunction, effectively restoring the normal state. This approach has shown groundbreaking success in a variety of disease models, including blood disorders to muscular dystrophies and neurodegenerative conditions.

One of the most celebrated successes in this field is the treatment of Sickle Cell Disease (SCD), a condition caused by a mutation that makes red blood cells rigid and sickle-shaped, leading to severe pain and damage. Rather than directly correcting the mutation, Frangoul et al. used CRISPR-Cas9 to disrupt a gene called BCL11A — a suppressor that normally turns off fetal hemoglobin production after birth. They essentially tricked the patients' edited stem cells into producing fetal hemoglobin (Frangoul et al., 2021). This healthy protein compensated for the defective adult hemoglobin, effectively curing the cellular damage and eliminating disease symptoms in patients, highlighting how gene editing can be used to activate alternate pathways to repair broken genes.

Similarly, Nelson et al. (2016) used CRISPR to treat Duchenne Muscular Dystrophy (DMD), a condition where muscles lacked a crucial shock absorbing protein called dystrophin due to a missing piece of DNA. In a landmark study, researchers used viral vectors to deliver CRISPR directly into the muscles of living mice (Nelson et al., 2016; Tabedbordbar et al., 2016). By making precise cuts to remove the mutated section of the gene, the muscle cells stitched the remaining pieces back together. While this resulted in a shorter-than-normal protein, it was still a highly functional version of the dystrophin protein, successfully repairing the cells' structural integrity and restoring muscle function.

CRISPR is also showing promise in other difficult-to-treat areas, for example, neurodegenerative diseases like Huntington's disease. This is where mutant proteins accumulate and poison brain cells. Researchers have demonstrated that CRISPR can act like a "sniper", specifically targeting and disrupting mutant gene cells while preserving the healthy ones (Monteys et al., 2017). This precision is vital in the brain, as we want to protect as many healthy neurons as possible.

Furthermore, gene editing is also changing the way we think about scarring. When tissues are damaged, they often form scars tissues, which blocks healing. A study by Liu et al. (2019) used CRISPR not to cut DNA but to change the volume on a specific gene — CEBPA — in scar forming cells. By turning down this gene, the researchers blocked the cells from forming scar tissue and shifted the body's response towards true regenerative repair.

Despite these achievements, repairing damaged cells in vivo remains fraught with challenges. Delivering CRISPR to organs like the brain or heart without triggering immune responses remains a major hurdle. There is also risk of "off-target effects" where the editor cuts in the wrong place, potentially causing new problems. However, as technology advances, these risks are decreasing. Ultimately, CRISPR is able to repair more and more damaged cells at the genetic level. By addressing the fundamental causes of genetic diseases, CRISPR offers the potential for permanent cures rather than just temporary symptom management.

3.3 CRISPR and Space-Based Biomanufacturing of Tissues

While previous themes have focused on how CRISPR can rejuvenate and repair cells on Earth, the future of regenerative medicine lies beyond it. The microgravity environment of LEO allows cells to self-assemble into complex 3D structures without the need for scaffolds. On Earth, researchers must rely on these scaffolds to prevent the cellular layers from collapsing under their own weight, but in microgravity, tissues can form shapes that more closely resemble their real counterparts in the human body (Sharma et al., 2021). This fundamentally changes what kinds of tissues can be engineered. As humanity prepares for longer space missions, the ability to manufacture functional tissues becomes more vital than ever.

The ISS has become an unlikely but integral part of stem cell biology. Recent investigations on the ISS have demonstrated that stem-derived organoids behave differently in microgravity compared to on Earth. Similarly, Marotta et al. (2024) reported that human-induced pluripotent stem cell (iPSC)-derived neural organoids cultured on the ISS began showing maturing signs faster than those on Earth, suggesting that the absence of gravity may accelerate certain aspects of tissue growth.

The broader implications of these findings go beyond simple tissue growth. Mozneb et al. (2025) reviewed how spaceflight influences stem cell biology and organoid formation, noting that microgravity affects how cells grow, differentiate, and signal in ways that can be used for biomanufacturing. For instance, MSCs grown in microgravity maintain their multipotency over long periods of time while forming larger and more resilient 3D groups (Kim et al., 2025). These cells also show increased production of anti-inflammatory and pro-regenerative factors, potentially enhancing their therapeutic value upon return to Earth (Kim et al., 2025).

The Redwire Corporation's 3D BioFabrication Facility BFF, installed on the ISS in November 2022, has pioneered the ability to print living cellular structures in microgravity (Redwire, 2024). They have successfully printed the first human knee meniscus on the ISS, demonstrating that functional tissue constructs could be fabricated in orbit and returned to Earth intact (Klarmann et al., 2024). Following this success, Redwire launched materials to bioprint cardiac tissue in late 2024, and more recently announced plans to test 3D bioprinted liver tissue in space (Redwire, 2024).

The advantages of bioprinting in microgravity are not trivial. On Earth, printing tissues that are complex and delicate — such as cardiac tissue — is extremely difficult because of

gravity (Sharma et al., 2021). In microgravity, bioprinting can be without scaffolding, and the printed constructs can be grown in specialized bioreactors for weeks to months until they develop into functional tissue (Kim et al., 2024). The ESA is also developing its own bioprinting capabilities for the ISS, with researchers at TU Dresden pioneering bioinks derived from human blood plasma and plant cellulose — which could be sourced locally during long space missions (van Ombergen et al., 2023). This represents a critical step for deep space exploration, where resupply from Earth is unlikely.

Currently, tissues manufactured in space are derived from unmodified stem cells, meaning they carry whatever defects the donor cell possesses. However, as Redwire (2024) has noted, the integration of gene editing to enhance bioprinted cells remains a critical next step. Imagine a future where an astronaut's own cells are first rejuvenated using CRISPR-mediated activation of factors such as SOX5 (Jing et al., 2023), then corrected for any mutations using base editing, and finally used as bioink to print replacement tissue in microgravity environments in spacecraft. This would effectively close the loop between the three themes.

Marotta et al. (2025) emphasize that the future of LEO biomanufacturing will depend on the integration of rapidly advancing technologies like AI, machine learning, and advanced automation with stem cell therapies. Kim et al. (2024) project that space-based biomanufacturing will evolve from small-scale research demonstrations to commercially viable production platforms, potentially supplying patients on Earth with superior tissue grown in orbit. The ISS National Laboratory has already identified this, with strategic partnerships between government agencies and private companies accelerating the integration of space research into clinical applications (Marotta et al., 2025)

Despite the remarkable progress, many hurdles remain before space-based biomanufacturing can achieve its full potential. The most pressing challenge would be the effect of space radiation. The ISS experiences radiation levels ~100 times higher than Earth's background, and beyond the magnetosphere, cells would be exposed to galactic cosmic rays and solar particle events that cause clustered DNA damage and genomic instability (Kim et al., 2025; Conti et al., 2025). CRISPR-based DNA repair treatments could theoretically mitigate this damage, but delivering gene editing tools reliably in spacecraft environments, where resources are limited and there is no margin for error, remains an engineering challenge.

Regulatory and ethical frameworks also lag behind the technology. As Mozneb et al. (2025) note, the governance of space-based biomedical research raises questions about IP, patient safety, and the ethical use of gene-edited tissues manufactured beyond national jurisdictions. The current regulatory landscape will likely require international cooperation and new policy frameworks. Additionally, the high cost of access to space remains a barrier to scaling these technologies.

Ultimately, the biomanufacturing of tissues and organoids in space represents the convergence point of all three themes explored. Mastering the integration of all three will not only revolutionize regenerative medicine for patients on Earth but will also be indispensable for

sustaining human health during deep space missions that will define humanity's future beyond the stars.

4. Conclusion

This review explored CRISPR-based gene editing in stem cell biology across three interconnected themes: rejuvenation, repair, and space-based biomanufacturing. CRISPR activation screening has identified factors like SOX5 that restore regenerative capacity without changing cell identity (Jing et al., 2023), while partial reprogramming has extended lifespan in animals (Cano Macip et al., 2024). For tissue repair, CRISPR treatments have demonstrated clinical success in blood disorders (Frangoul et al., 2021) and preclinical efficacy in correcting root causes of blood disorders (Frangoul et al., 2021; Nelson et al., 2017). In space, microgravity has enabled scaffold-free 3D tissue formation on the ISS (Marotta et al., 2024; Klarmann et al., 2024), with facilities such as the Redwire BioFabrication Facility already printing functional constructs (redwire, 2024). This suggests that integrating gene editing with space-based tissue engineering could produce enhanced tissues for both Earth medicine and deep space missions (Kim et al., 2024). Altogether, these themes converge toward a vision of engineered, rejuvenated, and space-manufactured tissues.

Key challenges still remain before clinical integration. CRISPR induced senescence and off target effects require optimized delivery methods (Conti et al., 2025; Lei and Wang, 2025), and space radiation's random impact on gene edited tissues is poorly understood (Mozneb et al., 2025). Future research should prioritize multi omic approaches combining rejuvenation with senolytic treatments (Cai et al, 2022; Ji et al., 2023), scalable in-orbit biomanufacturing platforms (Marotta et al., 2025), and international regulatory frameworks for gene edited tissues manufactured in space (Mozneb et al., 2025). Ultimately, integrating all three themes together will be essential for sustaining human life beyond Earth.

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Investigating TNF and IL1B as Candidate Biomarkers for Vaping-induced Neuroinflammation By Dev Trivedi[1]

Abstract

Electronic cigarette usage has increased drastically over the past decade, raising concerns regarding its potential neurological consequences. Although both tumor necrosis factor (*TNF*) and interleukin-1 beta (*IL1B*) are recognised as inflammatory mediators during neuroinflammation, their suitability as biomarkers for vaping-induced neuroinflammatory responses remains unclear. This study aimed to evaluate TNF and IL1B as candidate biomarkers for vaping-associated neuroinflammation by using in silico tools and transcriptomic analysis. Through the transcriptomic analysis of publicly available GEO dataset GSE142400 containing the bulk RNA sequence of mouse brain microvessels following chronic exposure to nicotine-free electronic cigarette aerosols, differential gene expression analysis using DESeq2, and in silico protein properties related analyses through TMHMM, SignalP, Clustal Omega, STRING was done. The study also proposes a CRISPR/Cas9 gene editing strategy that can be applied to establish a framework to study the role of TNF. Both TNF and IL1B were not significantly differentially expressed according to the analysis in this paper. However, over 4,453 genes were predicted to be differentially expressed according to the DESeq2 analysis overall. The findings indicate strong biological relevance despite limited transcriptomic support within the analysed dataset. These findings highlight the significance of combining transcriptomic and computational approaches to improve biomarker discovery, particularly in complex pathological processes involving the activation of numerous pathways simultaneously.

Keywords vaping, e-cigarette, brain inflammation, cytokines, CRISPR/Cas9, TNF-α, IL-1β

1. Introduction

Over the years, vaping has become an increasingly prevalent aspect of adolescent's lives. Electronic cigarettes, commonly known as e-cigarettes or vapes, are battery-powered/electronic nicotine delivery systems **(ENDS)** designed to heat up a liquid solution, referred to as an e-liquid or "vape juice" and convert it into an inhalable aerosol. Unlike conventional cigarettes, vapes do not burn tobacco. Instead, they vaporize a liquid containing propylene glycol, vegetable glycerin, nicotine, and a wide variety of flavoring chemicals constituents. This study aims to identify the plausibility of both *TNF* and *IL1B* genes as potential candidate biomarkers for vaping induced neuroinflammation by investigating its response and differential expression following exposure to nicotine-free inhalable aerosols (Novaleski et al. 2023). Whenever microglia detect cellular stress signals, damage-associated molecular patterns (DAMPs), or inflammatory stimuli generated by toxic compounds, they become activated and release pro-inflammatory cytokines.

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These cytokines cause a low-grade swelling of the brain tissue altering neural pathways in teenagers. The nicotine present in vapes also causes the release of dopamine. The cytokine which will be studied in this research paper is TNF-α (Tumor necrosis factor alpha) and IL-1β encoded by genes TNF and IL-1B (Fock and Parnova 2023).

Although originally developed as an alternative to traditional cigarettes and other combustible tobacco products, e-cigarettes have converted into highly developed products capable of delivering high concentrations of nicotine alongside thousands of other flavoring compounds. Their sleek designs, adapting flavors, and ability to trigger the release of dopamine have made them popular primarily among adolescents and young adults (Hamann et al. 2023). While people are becoming more aware about the harms of conventional cigarettes, the concept of vaping being a more "healthy" alternative to combustible tobacco products has become increasingly more popular primarily among adolescents and teenagers. Compared to traditional cigarettes, vapes deliver nicotine, a highly addictive substance linked to long-term lung diseases. In 2019 alone, tobacco companies spent more than $8.2 billion alone, an attempt to target the rising popularity of e-cigarettes among young populations. Nearly 76% of high school students were exposed to tobacco advertisements (approximately 3 out of 4 students) in 2021 and about 74% of students had seen tobacco advertisements through social media posts and related content. Many young people said that they tried e-cigarettes due to their appealing flavors. Among the youth currently using vapes, approximately 9-10 are flavored ones (Sachdeva et al. 2023)

Regardless of the type, both cigarettes and vaping provide severe harms to consumers and their surroundings. From the presence of nicotine in their delivery systems to the countless flavoring agents responsible for long-term addiction and severe lung diseases, both conventional and e-cigarettes have played a pivotal role in taking the lives of millions all around the world (Sachdeva et al. 2023)**.** While recognized as safe for food colorings, flavoring agents like ethyl butyrate can pose severe harms in the form of inhalable aerosolized liquids, especially in the respiratory tract. Inhaling aerosolized vapors are known to irritate the eyes, mucous membranes, nose, and throat. The chemical constituents act as tussigenic agents and studies show that inhaling it at even small concentrations can induce a strong urge to cough among most users (CDC 2025)**.** While recognized as safe for ingestion by the FDA, possibilities of decomposition at highly abnormal temperatures in "dry" vaping conditions remain largely unstudied. Understanding of these risks remains highly extrapolated from standard chemical safety assessments rather than long-term vape epidimielogy (Traboulsi et al. 2020)**.**

Nicotine is a highly addictive substance responsible for the release of dopamine and other reward-related neurotransmitters, they differ substantially in the way they are delivered and the other chemicals that users inhale during the process. It is a naturally occurring chemical formed in the nightshade family of plants (*Nicotiana, Solanaceae)*. It triggers adrenaline release causing a spike in blood pressure, heart rate, vasodilation while stimulating the release of dopamine in the brain's reward centers. It is found in both e-cigarettes (vapes) and traditional cigarettes (Www.Heart.Org, 2026). Conventional cigarettes burn tobacco at a temperature exceeding 600°C, producing smoke that contains more than 7000 chemicals, out of which 70 are known

carcinogens such as tar, formaldehyde, carbon monoxide, and benzene. E-cigarettes contain largely understudied chemicals, particularly based on their impacts on the central nervous system. All these chemicals combine under high temperatures to create an inhalable aerosol rather than smoke, generally resulting in lower levels of combustion-related intoxicants. However, this does not mean that vaping is completely harmless, as the aerosol still contains multiple potentially harmful compounds such as ultrafine particles, volatile organic compounds, heavy metals, aldehydes, and flavoring chemicals that may induce oxidative stress and inflammation. Although vaping is generally considered less harmful compared to traditional combustible tobacco products, neither product is safe, and both present significant health risks through nicotine addiction and exposure to toxic substances **(**"Difference between Conventional Cigarettes and Vape - 2014).

Research suggests that long-term vaping can result in severe neuroinflammation, the brain's innate immune system that activates in response to injury, infection or toxins. Blood-brain barrier represents one component of neuroinflammation which also involves the activation of microglia and astrocytes, increased cytokine production, oxidative stress, and activation of peripheral immune cells. Unlike other forms of inflammation that cause visible swellings, neuroinflammation occurs at a molecular level. Vaping aerosols and toxic byproducts from the thermal degradation of flavoring agents degrade the "blood-brain barrier," a highly packed and selective network of cells (endothelial cells, astrocytes, pericytes and microvascular cells) and blood vessels that act as a protective layer between the bloodstream and the central nervous system (da Fonseca et al. 2014). These byproducts loosen the tight junctions of proteins and fibers (particularly claudin-5 and occludin) that hold the blood-brain barrier, increasing its permeability. When they are inhaled, they trigger brain endothelial cells to release MMP enzymes (MMP-2 AND MMP-9) that physically digest claudin and occludin proteins. They also cause oxidative stress that causes lipid peroxidation, which chemically alters the cell membrane and ZO-1 anchor proteins to detach from the cytoskeleton. Microglial cells detect cellular stress signals through damage-associated molecular patterns (DAMPs) and inflammatory stimuli generated by vaping-associated compounds triggering the transcription and subsequent translation of pro-inflammatory cytokines like TNF-α (tumor necrosis factor alpha) and IL-1β which signal the endothelial cells to reduce the expression of claudin-5 while also disrupting tight junction organization and increased endothelial permeability. Many studies have linked increased e-cigarette exposure to higher TNF-α and IL-1β expression in the cerebral cortex. This inflammatory response has resulted in severe memory, language, and comprehension impairments, particularly within adolescents (Kim and Lee 2024)**.**

Neuroinflammation, commonly referred to as brain inflammation is triggered by a complex neurological pathway as an excitatory response to foreign toxins and chemicals, starting from the respiratory tract. The thermal degradation of numerous flavoring agents into carbonyls like acetaldehyde, formaldehyde, and acrolein act as systemic irritants. This irritation activates pattern recognition receptors such as Toll-like receptor 4 (TLR4), stimulating the IKK complex to phosphorylate and degrade IkB. This releases NF-kB, allowing it to translocate into the

nucleus of microglial cells. This triggers transcription in the TNF and IL1B gene where RNA polymerase II synthesizes complementary mRNA from the DNA template. In the IL1B gene, this involves the formation of pro-IL1β (an inactive precursor of IL1β), the second mechanism involves the activation of the inflammasome NLRP3 (Eibl et al. 2012) that activates caspase-1 which cleaves pro-IL1β into its mature form (more heavily linked to systemic neuroinflammation). In the TNF gene, NF-κB stimulates the transcription of the TNF gene, producing membrane bound TNF-α. The extracellular domain is subsequently cleaved by ADAM17 (TACE) releasing soluble TNF-α into the extracellular environment, where it can bind to TNFR1 and TNFR2 expressed on brain microvascular endothelial cells (BMVC). TNF-α and IL-1β bind to specialized receptors present on brain endothelial cells (TNFR1 and IL-1R1) suppressing the production of Claudin-5, an essential protein that holds the brain blood barrier together. This occurs when foreign toxins like aldehydes trigger the release of MMP enzymes (MMP-2 and MMP-9) which physically digest claudin and occludin proteins. When claudin-5 production is suppressed, there is a systemic loosening of the cells holding the barrier together resulting in increased permeability and subsequent inflammation (Liu et al. 2024)

Both TNF (tumor necrosis factor) and IL1B (interleukin-1 beta) are genes that encode TNF-α and IL1β proteins respectively. These pro-inflammatory cytokines along with a few others (IL-6, IL-8, MCP-1, IFN-γ, TGF-β) are secreted in response to acute immune stress in the brain-blood barrier. TNF is primarily specialized for rapid, high volume transcription in the presence of foreign toxins. It spans a short region of approximately 2.76 kilobases in chromosome 6. In the brain, it is primarily transcribed by activated microglia and astrocytes (brain immune cells). When microglia detect damage-associated molecular patterns (DAMPs) or inflammatory signals generated by toxic compounds, the protein complex NF-κB binds to the regulatory DNA sequences within the promoter regions of the TNF and IL1B genes triggering rapid pro-inflammatory cytokine transcription ("TNF (Tumor Necrosis Factor (TNF Superfamily, Member 2))," 2024)**.** IL1B gene (interleukin-1 beta) encodes for IL1-β. It contains a strict two tier mechanism as described above to ensure that its activation does not cause physical tissue damage. The iL1B gene is first transcribed into mRNA, which is then translated into an inactive precursor protein known as pro-IL-Iβ. To activate a matured form of the precursor, the NLRP3 inflammasome activates caspase-1, which cleaves pro-IL-Iβ into mature IL-1β which then leaves the cell causing visible neuroinflammation ("IL1B Interleukin 1 Beta [Homo Sapiens (Human)] - Gene - NCBI," 2026).

The aim of the study is to investigate the potential biological relevance and plausibility of both *TNF* and *IL1B* in being candidate biomarkers for vaping-associated neuroinflammation through the analysis of transcriptomic datasets and other forms of in silico analyses through publicly available algorithms. While electronic cigarettes are considered to be comparatively more safe relative to traditional combustible tobacco products, it is largely understudied. Several flavoring agents are deemed safe by the FDA but do not have enough data to support the claim, therefore, this study will aim to give a comprehensive overview on complex neuroinflammatory pathways and will investigate the toxicity of aerosolized solvents in dry vaping conditions.

Despite wide-scale use, very little is known about its effects on the CNS and its potential to activate neuroimmune responses in microglial cells. Excess activation of microglial cells can lead to increased concentration of TNF-α cytokines (linked to oxidative stress, neuroinflammation and long-term neurological distress).

2. Materials and Methods

NCBI (National Center for Biotechnology Information): The National Center for Biotechnology Information (NCBI) is a publicly accessible bioinformatics resource developed by the U.S. National Library of Medicine. It provides a comprehensive collection of biological and biomedical databases. Rather than functioning as a single source of information, it relies on data produced by several other resources, including GenBank, Gene, Protein, PubMed, GEO (Gene Expression Omnibus), and dbSNP. These databases contain information of nucleotide sequences, protein sequences, and gene expression profiles. In this study, GEO (Gene Expression Omnibus) was used to analyse publicly available gene expression datasets to determine changes in TNF and IL1B expression following exposure to vaping-associated compounds.

Ensembl: Ensembl is a comprehensive genome database and browser that provides detailed annotations for genes across numerous species. It contains complete genome assemblies, chromosome maps, exon and intron sequences, transcription variants, regulatory elements, and comparative genomic information. Ensembl helps researchers understand our exact genomic organization and examine evolutionary relationships between different species. In this study, Ensembl was used to examine the genomic structure of the TNF gene, including its chromosomal location, exon-intron structure, regulatory elements, nucleotide sequences and how protein complex NF-kB activates it in microglial cells.

TMHMM: TransMembrane Hidden Markov Model (TMHMM) is a bioinformatics algorithm designed to predict the presence and location of transmembrane helices within proteins. The algorithm is based on the Hidden Markov Model (HMM), a statistical model that predicts the probability of each amino acid belonging to one of three states, inside the cell, embedded within the cell membrane (transmembrane helix), or outside the cell. By examining the physicochemical properties of amino acids and calculating the most probable arrangement of its regions between these three states, TMHMM predicts membrane topology. Following TMHMM analysis, SignalP was used to determine whether the protein also contains a classical N-terminal signal peptide. By doing so, TMHMM predicts the number and position of each membrane-spanning region within a protein.

Signal P. : SignalP is a bioinformatics algorithm used to predict the presence and location of signal peptides in newly synthesized proteins. Signal peptides are short amino acid sequences, typically 15-30 amino acids located at the amino terminus (N-terminus) of the protein. The algorithm analyzes the C-domain of the signal peptide to see if it contains small neutral amino acids (alanine, glycine, or serine) where signal peptidase can make its cut during protein maturation in the secretory pathway. Based on the information gathered during this analysis, the machine learning model comes to a conclusion on whether the protein contains a signal peptide.

This machine learning model was used on proteins in this study to analyze their transmembrane domains.

Clustal Omega: Clustal Omega is a multiple sequence alignment algorithm used to compare DNA, RNA, and protein sequences among different species and organisms. Unlike typical biological databases, Clustal omega analyzes data and sequences provided by the researcher and aligns them based on the sequence similarity using progressive alignment methods. The software first performs pairwise comparisons to calculate percentage similarities, constructs a guide tree representing evolutionary relationships, and then aligns all the sequences to produce a final multiple sequence alignment. This final product helps in identifying mutations, deletions, insertions, and evolutionary important amino acids responsible for pivotal functions inside cells and tissues. In this study, Clustal Omega can help in identifying similarities between gene sequences in humans and other laboratory animals, for understanding whether signalling pathways relating to neuroinflammation are common among animals carrying different genetic information. It also allows an assessment of the evolutionary conservation of inflammatory pathways among different organisms.

STRING Protein: The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database is an online resource that contains protein-protein interactions from experimental studies, computational predictions, co-expression analyses, curated biological pathways, and scientific literature. Rather than focusing on individual proteins, STRING helps one understand how different proteins and genes interact with one another to produce a particular response through a signalling pathway. In the process of neuroinflammation, it will help one understand what interactor proteins for TNF and IL1B are in a broader context.

UniProt: UniProt (Universal Protein Resource) is the world's largest curated protein database, providing information on protein structures, sequences, biological functions, molecular interactions, and disease associations. Each protein entry combined with experimentally validated findings provides a detailed overview of the entire protein biology. In this investigation, UniProt was used to obtain detailed information about the structure, function, and sequence of the TNF-α and IL-1β proteins. It helps in identifying a protein's amino acid sequence, biological function, receptor-binding properties, cleavage sites, and role in regulating inflammatory responses. These data contribute to an understanding of how increased TNF and IL1B transcription may promote microglial activation and neuroinflammation following exposure to inhalable aerosolized chemicals.

CRISPR Finder: CRISPR Finder is a bioinformatics tool designed to identify Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and associated sequence patterns within genomic data. The algorithm scans DNA sequences to detect characteristic repeat-spacer-repeat arrangements that form CRISPR arrays, enabling researchers to identify potential CRISPR-associated regions and the guide RNAs. Although CRISPR finder is primarily used in microbial genomics, it remains relevant in biomedical research since it supports the development of new CRISPR related gene modification strategies. In this investigation, CRISPR based approaches could be applied in future studies to suppress the TNF and IL1B gene in

microglial cells, allowing researchers to directly investigate their function and importance in vaping-induced neuroinflammation.

SnapGene: SnapGene is a molecular biology software package used to simulate DNA manipulation experiments. Unlike regular bioinformatics databases that store genomic information, SnapGene allows you to import DNA sequences from publicly available resources like NCBI and Ensembl and perform virtual molecular biology procedures on the DNA, including primer design, polymerase chain reaction (PCR) simulation, restriction enzyme analysis, plasmid construction, DNA cloning, and CRISPR guide RNA design. In this research, SnapGene will be used to visualize the DNA sequence of the TNF and IL1B gene, design PCR primers for analyzing gene expression in response to foreign toxins, and develop molecular constructs for future functional studies investigating how vaping-associated chemicals influence inflammatory gene regulation.

3. Results

3.1. Transmembrane domain analysis for TNF and IL1B gene

To investigate the structural properties of TNF-α protein, a transmembrane domain analysis was performed using the TMHMM (Transmembrane Hidden Markov Model) server. For this analysis, the amino acid sequence of the human (homo sapien) TNF-α protein (UniProt accession P01375) was entered into the TMHMM server. The server used machine learning and information from the Hidden Markov Model (HMM) to generate probability plots demonstrating the likelihood of each amino acid residue belonging to either a transmembrane region, the intracellular side, or the extracellular side of the membrane. The results shown in Figure 1 suggest that one highly probable transmembrane region is located between amino acid residues 30 and 55, as seen through a strong purple peak between the blue and yellow line as the probability peaks to 1.0. Amino acid residues before this peak are predicted to be on the intracellular side within the cell membrane as they exhibit hydrophilic properties (blue line), while the majority of the remaining protein (aa residues 55 to 233) is predicted to be located outside the cell membrane (orange line). This topology is consistent with the known physical structure of TNF-α since it is a type II transmembrane protein, in which a short N-terminal domain, also known as a transmembrane helix anchors the protein to the membrane while the larger C-terminal domain extends outside the cell. This analysis was included since the membrane spanning of TNF-α is systemically related to its biological function in neuroinflammation.

Unlike other transmembrane proteins, TNF-α does not contain a signal peptide (a sequence of 15-30 amino acids that direct the protein into the endoplasmic reticulum for processing and protein maturation in the secretory pathway), however, it uses its transmembrane domain as a signal anchor for the long extracellular C-terminal before it is cleaved off by ADAM17. Understanding how TNF-α is processed and subsequently released from the cell is essential in predicting its activation and function following exposure to any vaping-related

compounds. Since this research investigates whether exposure to the vaping-related flavoring agent ethyl butyrate may increase TNF gene expression and promote microglial activation, confirming the biological structure of TNF-α helps improve the interpretation of the inflammatory signalling pathway.

RecName: Full=Tumor necrosis factor; AltName: Full=Cachectin; AltName: Full=TNF-alpha; AltName: Full=Tumor necrosis factor ligand superfamily member 2; Short=TNF-a; Contains: RecName: Full=Tumor necrosis factor, membrane form; AltName: Full=N-terminal fragment...

UniProtKB/Swiss-Prot: P01375.1

GenPept Identical Proteins Graphics

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>sp|P01375.1|TNFA_HUMAN RecName: Full=Tumor necrosis factor; AltName:
Full=Cachectin; AltName: Full=TNF-alpha; AltName: Full=Tumor necrosis factor ligand
superfamily member 2; Short=TNF-a; Contains: RecName: Full=Tumor necrosis factor,
membrane form; AltName: Full=N-terminal fragment; Short=NTF; Contains: RecName:
Full=Intracellular domain 1; Short=ICD1; Contains: RecName: Full=Intracellular
domain 2; Short=ICD2; Contains: RecName: Full=C-domain 1; Contains: RecName:
Full=C-domain 2; Contains: RecName: Full=Tumor necrosis factor, soluble form;
Flags: Precursor
MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQREEFPRDLSLI
SPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLF
KGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSA
EINRPDYLDFAESGQVYFGIIAL
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# sp|P01375.1|TNFA_HUMAN Length: 233
# sp|P01375.1|TNFA_HUMAN Number of predicted TMHs:  1
# sp|P01375.1|TNFA_HUMAN Exp number of AAs in TMHs: 22.92024
# sp|P01375.1|TNFA_HUMAN Exp number, first 60 AAs:  22.90493
# sp|P01375.1|TNFA_HUMAN Total prob of N-in:        0.94794
# sp|P01375.1|TNFA_HUMAN POSSIBLE N-term signal sequence
sp|P01375.1|TNFA_HUMAN   TMHMM2.0        inside       1    34
sp|P01375.1|TNFA_HUMAN   TMHMM2.0        TMhelix     35    57
sp|P01375.1|TNFA_HUMAN   TMHMM2.0        outside     58   233
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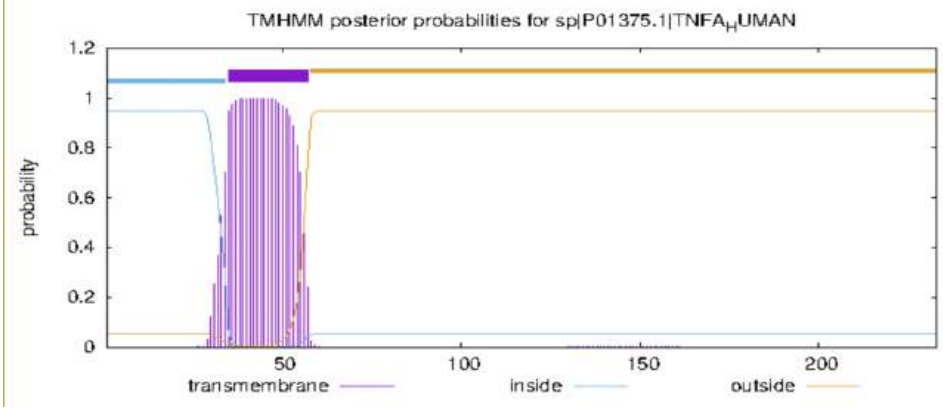

Figure 1. Transmembrane domain prediction for TNF-alpha shows one predicted transmembrane region. The graph shows the probability calculation for the prediction. X-axis: number of the amino acids that the query protein contains. Y-axis: probability calculation for the amino acids being predicted as inside a transmembrane, inside or outside domains of the protein. Purple Line: Shows that the amino acids in this part of the sequence are located within the membrane structure as they are hydrophobic. Blue line: Shows that the amino acids in this part of the sequence are located on the other side of the membrane. Yellow line: Shows that the amino acids in this part of the sequence are located outside of the membrane structure.

To further develop an understanding on the structural features of TNF-α protein, a signal peptide analysis was conducted using the SignalP 6.0 database. Signal peptides are short sequences of amino acids, typically 15-30 amino acid residues that direct newly synthesized proteins through the secretory pathway before being cleaved off by signal peptidase, allowing the protein to be transported outside the cell or incorporated within the cell membrane. For this analysis, the amino acid sequence of the human TNF-α protein was submitted to the SignalP

server. The algorithm analyzed each amino acid residue and calculated the likelihood of the aa residue being part of a classical Sec/SPI signal peptide across the N-terminal region.

The results are shown in Figure 2 classified TNF-α as “other” with a prediction likelihood of 1.0, while the probability of each amino acid being part of a signal peptide remained as 0.0. Throughout the amino acid sequence, the prediction remained consistently classified as “other,” indicating that TNF-α does not contain a classical N-terminal signal peptide. The result confirms the known biological characteristics of TNF-α as a typical type II transmembrane protein. Rather than being secreted directly through the conventional secretory pathway using an N-terminal signal peptide, TNF-α is anchored within the cell membrane by its transmembrane domain before being cleaved of by TACE/ADAM17 releasing the soluble, biologically active inflammatory cytokine into the extracellular region. This analysis was performed because the TMHMM analysis suggested that TNF-α contains a transmembrane region which is a characteristic exhibited by proteins containing a signal peptide. Since the SignalP analysis showed no probability for any classical n-terminal signal peptide, the protein can be successfully classified as a typical type II transmembrane protein. The absence of a classical signal peptide confirms that increased TNF gene expression does not immediately result in direct extracellular secretion. Instead, TNF-α must first be synthesized as a membrane-bound protein before undergoing enzymatic cleavage to produce its active soluble form, which then binds to TNF receptors (TNFR1 and TNFR2 on BMVE cells) and initiations inflammatory signalling.

The SignalP analysis (Figure 2) complements the TMHMM membrane topology analysis (Figure 1) by providing additional structural evidence to the unique processing and secretion pathway of TNF-α. While TMHMM suggested the presence of a transmembrane domain, SignalP confirmed that this membrane insertion was not mediated by a classical signal peptide. Together, these findings strengthen the structural idea of TNF-α and provide a clearer understanding of the mechanisms involved in its production, activation, and extracellular release.

Protein type	Other	Signal Peptide (Sec/SPI)
Likelihood	1	0

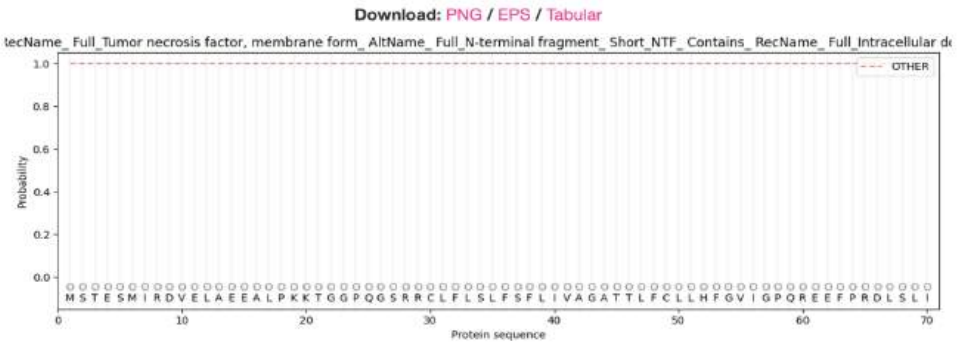

Figure 2. TNF-alpha does not contain a signal peptide. The graph shows the prediction of the potential signal peptide presence in the protein. X-axis: Shows the entire protein sequence of TNF-alpha. Y-axis: Shows the probability of the amino acids being part of a signal peptide chain. Red line: Shows that all the amino acids part of the protein sequence exhibit high probability of not being part of a signal peptide.

To further investigate the structural characteristics and properties of Interleukin-1 beta (IL-1β**)**, another transmembrane domain analysis was conducted (Figure 3) using the TMHMM server. For this analysis, the amino acid sequence of the human IL-1β protein (NCBI Protein accession P01584.2) was obtained from the National Center for Biotechnology Information (NCBI) protein database and was submitted to the TMHMM server. The program completed the analysis of 269 amino acid residues and generated predictions regarding the likelihood and presence of transmembrane helices in the overall membrane topology of the protein. The results shown in figure 3 predicted zero transmembrane helices (Predicted TMHs=0) within the IL1B protein. The expected number of transmembrane regions in this protein was extremely low (0.00017 amino acids), indicating that the protein does not contain any membrane-spanning regions. Regardless, the software predicted that the entire protein sequence (aa residues 1-269) is located outside the membrane, with no probability of intracellular membrane localization. The probability graph supports this finding, as the extracellular probability consistently remains as 1.0 throughout the sequence (yellow line).

This analysis was conducted to determine whether IL-1β functions as a membrane-bound protein or as a soluble cytokine. Unlike TNF-α, which is initially produced as a membrane-bound protein before being cleaved off by TACE to release its soluble, biologically active form into the extracellular region, IL-1β does not contain a transmembrane helix that anchors its C-terminal onto the plasma membrane. Instead, it is produced within the cytoplasm as an inactive precursor (pro-IL-Iβ) and is subsequently cleaved off by caspase-1 following the activation of NLRP3 inflammasome. This produces the biologically active, soluble cytokine which is released to pass on the inflammatory signal.

RecName: Full=Interleukin-1 beta; Short=IL-1 beta; AltName: Full=Catabolin; Flags: Precursor

UniProtKB/Swiss-Prot: P01584.2

GenPept Identical Proteins Graphics

>sp|P01584.2|IL1B_HUMAN RecName: Full=Interleukin-1 beta; Short=IL-1 beta; AltName: Full=Catabolin; Flags: Precursor
MAEVPELASEMMAYYSGNEDDLFFEADGPKQMKCSFQDLDLCPLDGGIQLRISDHHYSKGFRQAASVVVA
MDKLRKMLVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEAYVHDAPVRSLNCTLRDSQQKSLVMSGPY
ELKALHLQGQDMEQQVVFSMSFVQGEESNDKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDPKNYPKKK
MEKRFVFNKIEINNKLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFVSS

```
# sp|P01584.2|IL1B_HUMAN Length: 269
# sp|P01584.2|IL1B_HUMAN Number of predicted TMHs:  0
# sp|P01584.2|IL1B_HUMAN Exp number of AAs in TMHs: 0.00017
# sp|P01584.2|IL1B_HUMAN Exp number, first 60 AAs:  0
# sp|P01584.2|IL1B_HUMAN Total prob of N-in:        0.01019
sp|P01584.2|IL1B_HUMAN  TMHMM2.0       outside     1   269
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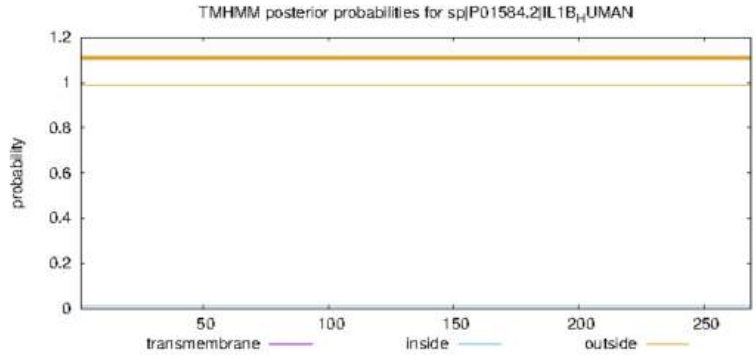

Figure 3. Transmembrane domain analysis of IL-1β protein in TMHMM. The graph shows the probability calculation for the prediction. X-axis: number of the amino acids that the query protein contains. Y-axis: probability calculation for the amino acids being predicted as inside a transmembrane, inside or outside domains of the protein. Purple Line: Shows that the amino acids in this part of the sequence are located within the membrane structure as they are hydrophobic. Blue line: Shows that the amino acids in this part of the sequence are located on the other side of the membrane. Yellow line: Shows that the amino acids in this part of the sequence are located outside of the membrane structure.

3.2. Clustal Omega Analysis of TNF AND IL1B gene

To investigate the evolutionary conservation of TNF-α, a multiple sequence alignment was conducted using Clustal Omega. For this analysis, the amino acid sequences of TNF-α from human (homo sapien), chimpanzee (Pan troglodytes), mouse (Mus musculus), and pig (Sus scrofa) were taken from the NCBI database and submitted onto the Clustal omega algorithm. The resulting product of the analysis compared each amino acid residue position across all four species. The alignment shown in figure 4 shows that TNF-α is highly conserved among these four animals. Several regions of this protein contain continuous stretches of asterisks (*) showing that all four amino acid residues at that particular position match each other. This is especially prevalent in the extracellular helix of the protein, suggesting that these residues are essential in maintaining structural integrity and continuing the biological activity of TNF-α. Since TNF-α functions in the inflammatory signalling pathway by binding to receptors (TNFR1 and TNFR2) on brain microvascular endothelial cells, conservation of these amino acids indicates that these receptor-binding regions and functional helices have remained largely unchanged across mammalian evolution. Additionally, it suggests that these amino acids are essential in the function of the protein even across different species.

Although the overall sequence similarity is high, several residue substitutions can also be seen throughout the protein sequence, particularly when the mouse (Mus musculus) and pig (Pan troglodytes) was compared with humans. Many of these changes are represented by colons (:) or periods (.), indicating that even though these amino acids differ, they possess similar biochemical and physical properties such as charge, polarity, or size. These conservative substitutions are unlikely to significantly affect the biochemical functionality of the protein since proteins often

tolerate chemically similar amino acids as they can undergo similar changes and perform similar functions. A small number of gaps (-) are also present in the multiple sequence alignment, representing insertion or deletion occurrences that have taken place during evolution.

The highest sequence similarity is seen between human and chimpanzee TNF-α, with very few amino acid differences. This highlights the close evolutionary relationship between these two species and suggests that the protein sequence has been conserved regardless of evolutionary changes. In contrast, the mouse sequence exhibits relatively greater variation, although the majority of important residues remain conserved, demonstrating that TNF-α has retained its essential inflammatory role among mammals. This analysis was conducted because the investigation aims to assess the role of TNF-α in vaping-induced neuroinflammation. Demonstrating that TNF-α is highly conserved among mammalian species strengthens the reliability of using evidence obtained from animal models to understand inflammatory mechanisms in humans.

Figure 4. Multiple protein alignment of TNF-Alpha. Multiple sequence alignment of TNF-α between human, mouse, pig, and chimpanzee generated through Clustal Omega. Each line represents a different amino acid sequence from a different mammalian species. The number on the side represents the position of the amino acid sequence within the protein. The colors signify amino acids with similar biochemical properties. Red: Hydrophobic amino acids, Blue: Positively charged amino acids, Purple: Negatively charged amino acids, Green: Polar amino acids. Asterisk (*): Every species has exactly the same amino acid in that position (Perfectly conserved) shows that the amino acid is functionally crucial to the protein. Colon (:): The amino acids are different but they behave similarly chemically (Strongly conserved substitution). Period (.): The amino acids are slightly similar (weak conservation) Dash (-): There is no amino acid in that position (insertions or deletions).

To gain a greater understanding on the evolutionary conservation of interleukin-1 beta (IL-1β), a multiple sequence alignment was performed using Clustal Omega (Figure 5). For this analysis, the amino acid sequence of IL-1β of human, chimpanzee, mouse and pig were taken from the NCBI protein database and submitted onto the Clustal Omega server. The generated

product showed the similarity in amino acid sequences residue by residue across all four species. The alignment shown in figure 5 shows that IL-1β is highly conserved across mammalian species, although slightly more variation is present in comparison to TNF-α. As shown in the figure, numerous amino acid positions are marked with asterisks (*) which indicates identical aa residues across all four species. There are several substitutions visible across all four species, indicated by the periods (.) and colons (:). This demonstrates that although the amino acid sequence differs, their biochemical properties remain similar as seen through the same colors across numerous residue positions. This suggests that the protein sequence can maintain structural and functional integrity regardless of minor changes in amino acid sequences. The human and chimpanzee proteins display the highest sequence similarity, while the pig and mouse protein sequence display the highest number of substitutions and gaps, reflecting increased evolutionary divergence.

This analysis was conducted because IL-1β is another crucial protein in inflammatory signalling and therefore, it plays a pivotal role in vaping-induced neuroinflammation. Determining whether the amino acid sequence and chemical properties of IL-1β are conserved across mammalian species helps confirm its role as a pro-inflammatory cytokine in neuroinflammation. Such conservation is particularly important as several investigations on neuroinflammation are performed using animal models before findings are translated to humans. The results suggest that IL-1β retains a highly conserved amino acid sequence across mammals, supporting the use of published animal studies while interpreting the inflammatory pathways investigated in this research. These findings support the biological relevance of IL-1β as a biomarker for neuroinflammation and strengthen its importance alongside TNF-α in assessing the neuroinflammatory effects of vaping flavored chemicals.

```
pig_sp|Q9N2I1.1|CASP1_PIG       MADKVLKEKRRLFVRSVAMGTINGLLDELLETRVLNQEEVEIVRGENATVMDKARALIDS  60
mouse_NP_032387.1               ------------------MATVPELNC----------EMPP-FDSDENDLF---------  22
human_sp|P01584.2|IL1B_HUMAN    ------------------MAEVPELAS----------EMMAYYSGNEDDLF---------  23
chimpanzee_AAN76443.1           ------------------------------------------------------------  0

pig_sp|Q9N2I1.1|CASP1_PIG       VIRKGPQACQICINHI--CGDDPHLAG-V--LELSTGSQSGKCLTVQESQAVVPPFPAPQ  115
mouse_NP_032387.1               FEVDGPQKMKGCFQTFDLGCPDESIQLQISQQHINKSFRQAVSLIVAVEKLWQLPVSFPW  82
human_sp|P01584.2|IL1B_HUMAN    FEADGPKQMKCSFQDLDLCPLDGGIQLRISDHHYSKGFRQAASVVVAMDKLRKMLVPCPQ  83
chimpanzee_AAN76443.1           ------------------------------------------------------MLVPCPQ  7
                                                                                        .  *

pig_sp|Q9N2I1.1|CASP1_PIG       TVQDNPVKPASSEPRGSLKLCPPDIAQRLWKEKSAEIYPIMGKSIRTRLALIICNTEFEN  175
mouse_NP_032387.1               TFQDEDMSTFFS-----FI--------------FEEEPILCDSWDDDDNLLVCDVPI--  120
human_sp|P01584.2|IL1B_HUMAN    TFQENDLSTFFP-----FI--------------FEEEPIFFDTWDNEA--YVHDAPV--  119
chimpanzee_AAN76443.1           TFQENDLSTFFP-----FI--------------FEEEPIFFDTWENEA--YVHDAPV--  43
                                *.*:: :.        :               *  **: .:       : :. .

pig_sp|Q9N2I1.1|CASP1_PIG       LPRRDGADVDIRDMKILLEDLGYSVDVRENLTASDMAIELKAFAARPEHKSSDSTFLVLM  235
mouse_NP_032387.1               -----------RQLHYRLRDEQQ-----KSLVL-SDPYELKALHLNGQNINQQVIFSMSF  163
human_sp|P01584.2|IL1B_HUMAN    -----------RSLNCTLRDSQQ-----KSLVM-SGPYELKALHLQGQDMEQQVVFSMSF  162
chimpanzee_AAN76443.1           -----------RSLNCTLRDSQQ-----KSLVM-SGPYELKALHLQGQDMEQQVVFSMSF  86
                                          *.::  *.*        :.*.  .   ****:  . :. ..:  * : :
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Figure 5. Multiple protein alignment of IL-1B. Multiple sequence alignment of IL-1β proteins from human, chimpanzee, mouse and pig generated using Clustal Omega.The number on the side represents the position of the amino acid sequence within the protein. The colors signify amino acids with similar biochemical properties. Red: Hydrophobic amino acids, Blue: Positively charged amino acids, Purple: Negatively charged amino acids,' Green: Polar amino acids. Asterisk (*): Every species has exactly the same amino acid in that position (Perfectly conserved) shows that the amino acid is functionally crucial to the protein. Colon (:): The amino acids are different but they behave similarly chemically (Strongly conserved substitution). Period (.): The amino acids are slightly similar (weak conservation) Dash (-): There is no amino acid in that position (insertions or deletions).

3.3. STRING Protein database analysis of TNF-alpha and IL-1beta

To investigate the biochemical interactions of TNF-α (TNF), a protein-protein interaction network was generated using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database. For this analysis, the human TNF gene was entered into the STRING database with the organism set to homo sapiens. STRING generated an interaction network showing which proteins have strong physical and functional associations with TNF-α based on previous studies and data regarding NF-kB signalling pathways (Figure 6). The figure gives insights that the role of TNF-α goes far beyond neuroinflammation, influencing systemic shifts in apoptosis, cell survival, angiogenesis, and metabolism. By interacting with receptors TNFR1 and TNFR2, it probably cascades NF-kB, MAPK, and caspase pathways. TNF-α predicted to be interacting with several proteins involved in inflammatory signalling, including NFKB1, CHUK, MAP3K7, TNFRSF1B, TNFRSF1A, TRADD, CASP8, RIPK1, TAB2, and TRAF2. Together, these proteins collectively maintain and regulate essential biological processes such as the activation of the NF-kB signalling pathway, inflammatory cytokine production, apoptosis, and immune responses. The dense connectivity between TNF-α and other regulatory proteins indicates that it functions as an important central regulatory molecule within numerous inflammatory pathways. This analysis was performed to identify the downstream molecular pathways that may be activated when TNF expression increases following exposure to vaping-related toxins. Rather than functioning independently, TNF-α is part of a complex

signalling network involving several different adaptor proteins, receptors, and transcription factors. Gaining a comprehensive understanding on how TNF-α functions in other inflammatory pathways allows an accurate interpretation of the biological consequences of increased TNF-α expression. The STRING analysis demonstrates that TNF-α plays a pivotal role in inflammatory signalling networks associated with neuroinflammation. This supports the hypothesis that alterations in TNF-α expression following exposure to foreign toxins may influence several other protein signalling networks and downstream pathways involved in microglial activation, cytokine production and neuronal damage, strengthening its selection as one of the primary biomarkers in the study.

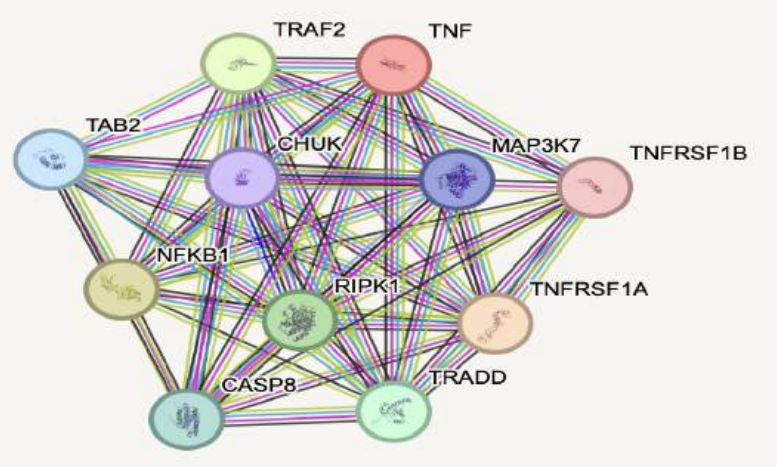

Figure 6. Protein-protein interaction map of TNFAlpha. STRING protein-protein interaction network of homo sapiens TNF-α. Predicted interactor proteins: NFKB1, CHUK, MAP3K7, TNFRSF1B, TNFRSF1A, TRADD, CASP8, RIPK1, TAB2, TRAF2, and TNF-α. Each colored node represents individual proteins, while connecting lines represent predicted or experimentally validated interactions between proteins. Different colored lines (edges) represent different sources of supporting evidence present within the STRING database. The network illustrates TNF-α as a central protein in several downstream signalling pathways, apoptosis, and NF-kB pathway activation.

To investigate the functional interaction network of Interleukin-1 beta (IL-1β), a protein-protein interaction analysis was performed (Figure 7) using the STRING database. For this analysis, the human (homo sapien) IL1B gene was entered into the STRING database with homo sapiens selected as the reference organism. STRING then generated a protein interaction network illustrating inflammatory pathways and signalling networks that IL-1B interacts in. The interaction network shown in figure 7 demonstrates that IL-1β interacts with several different inflammatory and adaptor proteins, including L4, IL1RAP, IL1R1, IFNG, IL5, IL1A, IL2, IL1R2, CASP1 and MYD88. These proteins regulate a variety of different functions responsible for immune signalling, inflammasome activation, cytokine production, and adaptive immune responses. In particular, the biological interaction between IL-1β is biologically significant since caspase-1 cleaves inactive pro-IL-1β into its mature, biologically active form in response to NLRP3 activation. The biochemical interactions with IL1RAP, IL1R1, and MYD88 play a key role in the activation of the downstream inflammatory signalling pathway leading to NF-kB activation when its inhibitory backup, the IkB protein, is photophosphorylated, and degraded by

the proteasome. This stimulates further cytokine production in the inflammatory pathway. This analysis was performed to understand the wider molecular pathways through which IL-1β contributes to neuroinflammation. Since this study investigates the potential relationship between ethyl butyrate exposure and inflammatory cytokine expression, identifying the other proteins that interact with IL-1β provides strong insight into how elevated IL1B expression may elevate inflammatory responses within activated microglial cells.

The string analysis demonstrated that IL-1β functions as a pivotal signalling molecule within the inflammatory network as seen through the dense, interconnected illustration. Its interactions with caspase-1 (conversion of pro-IL-1β into biologically active IL-1β) and other proteins (IL1RAP, IL1R1, and MYD88) suggests its role in regulating several downstream inflammatory pathways. These findings reinforce the selection of IL-1β as one of the good candidates to be examined in this study and provide additional mechanistic evidence linking cytokine expression with microglial activation and neuroinflammation.

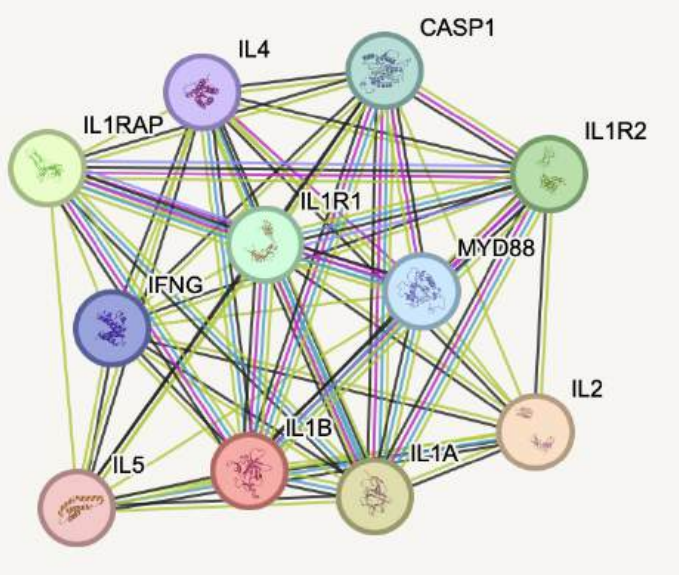

Figure 7. Protein-protein interaction map of IL-1B. STRING protein-protein interaction network of homo sapiens IL-1β. Predicted interactor proteins: IL4, IL1RAP, IL1R1, IFNG, IL5, IL1B, IL1A, IL2, IL1R2, CASP1 and MYD88. Each colored node represents individual proteins, while connecting lines represent predicted or experimentally validated interactions between proteins. Different colored lines (edges) represent different sources of supporting evidence present within the STRING database.

3.4. Generating CRISPR/Cas9 gene editing design for TNF-Alpha

To generate a gene editing strategy based on CRISPR/Cas9 technology the chromosomal location of TNF-alpha gene was checked (Figure 8) and the Snapgene gene editing view has been generated (Figure 9). TNF is positioned alongside numerous functionally related genes, including LTA (Lymphotoxin Alpha), LTB (Lymphotoxin Beta), NFKBIL1 (NF-kB Inhibitor Like 1), LST1 (Leukocyte Specific Transcript 1), and NCR3 (Natural Cytotoxicity Triggering

Receptor 3). However there are no clashing sequences between genes in the close proximity therefore a gene editing strategy was considered as safe in this position.

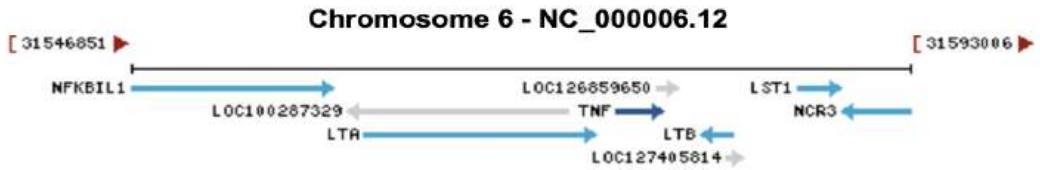

Figure 8. Chromosomal location of TNF gene. Chromosomal location of the human TNF gene on chromosome 6 (NC_000006.12). There are no clashing sequences with the TNF gene. The arrow indicates the orientation of gene transcription and the closely packed region suggested a highly structured and coordinated relationship between the genes which supports the role of TNF as a central mediator of immune responses relevant to neuroinflammation.

To further investigate the potential for targeted manipulation of the TNF gene, CRISPR/Cas9 guide RNA (gRNA) target sites were detected via CRISPR Finder (Welcome Sanger Institute) and visualized using SnapGene (Figure 9). Using CRISPR Finder Search to identify the best possible guide RNA sequence targeting the TNF genomic region by focusing on the off target scores (0=1, 1=0, 2=0 3 and 4 as low as possible). The sequences are displayed across two lines due to the formatting of the algorithm. The gRNA is highlighted in red and is flanked by the Protospacer Adjacent Motif (PAM) sequences (NGG), highlighted in cyan, which are required for the recognition and cleavage by the Streptococcus pyogenes Cas9 (SpCas9) nuclease. The presence of the correctly positioned PAM sequences confirms that the selected region satisfies the requirements for Cas9-mediated DNA cleavage. The guide RNA is responsible for directing Cas9 onto a specific location within the TNF gene, enabling precise introduction of double-stranded DNA breaks for potential gene disruption or modification. Although there was no experimental genome editing in this investigation, the successful identification of a suitable CRISPR target site proves that the TNF gene is susceptible towards targeted genetic manipulation. This provides fundamental information for future investigations studying how altered TNF expression influences the production of pro-inflammatory cytokines, as well as the downstream inflammatory pathways associated with vaping-induced neuroinflammation.

The direct identification of a CRISPR/Cas9 target site within the TNF gene indicates that it can be modified and selectively edited in future studies. By enabling targeted disruption or modification to the gene, future experimental studies can focus on how gene expressions are physically disrupted following exposure to toxic compounds like inhalable aerosols. Consequently, this analysis complements the sequence conservation, protein localization, and interaction analyses by demonstrating the amenability of the gene to mutation.

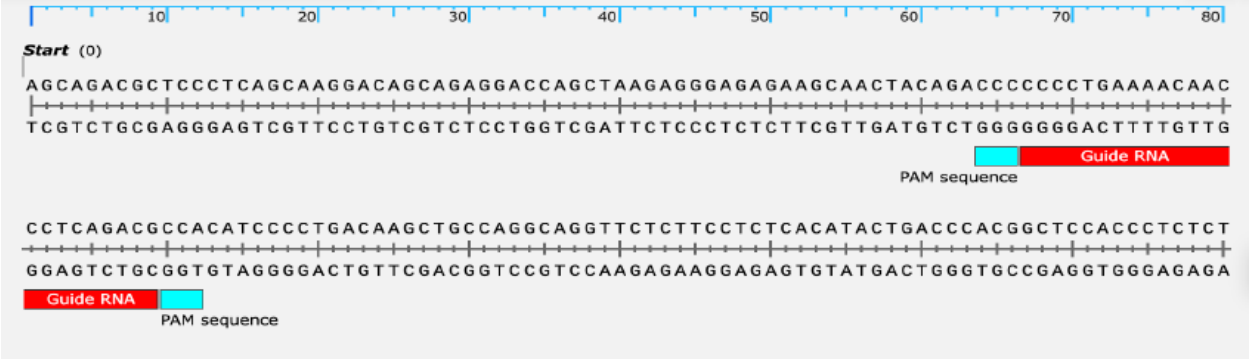

Figure 9. Snapgene view of CRISPR/Cas9 gene editing for TNF gene. CRISPR/Cas9 guide RNA design for the human TNF gene obtained using SnapGene. The target region for the guide RNA is highlighted in red, spanning a wrapped genomic sequence displayed across two lines for formatting purposes. The Protospacer Adjacent Motif (PAM) sequences required for recognition of the SpCas9 nuclease are highlighted in cyan on either side of the guide RNA display.

3.5. Differential gene expression analysis done by using publicly available dataset

The normalized RNA-sequence expression levels of TNF and IL1B in mouse brain microvessels following chronic exposure to nicotine-free electronic cigarette aerosol compared with room-air controls were tested by using a publicly available Gene Expression Omnibus dataset GSE142400 investigating the effect of nicotine-free e-cigarette aerosols on the gene counts in mouse (mus musculus) brain microvessels. It contained bulk RNA sequencing data.

For TNF, samples exposed to nicotine-free electronic cigarette aerosols exhibited a generally higher median expression level in comparison to the samples kept in room-air controls (Figure 10). However, considerable biological variability was observed between replicates preventing the difference from reaching statistical significance in terms of change in expression. Differential expression analysis results demonstrated that this apparent difference was not statistically significant (p=0.093). Although TNF expression demonstrated an upward trend in several examples, the variability shown proved too much limiting the trend from reaching statistical significance.

Similarly, IL1B expression showed overlapping distributions between the two experimental groups (Figure 10). While one room-air sample displayed relatively high expression levels, remaining samples exhibited comparable expression levels across both conditions. The differential expression analysis performed using DESq2 package in R confirmed that IL1B was not significantly differentially expressed following exposure to nicotine-free e-cigarette aerosols (adjusted p=0.614), indicating that the observed variation is similar to normal biological variability rather than the impact of an external treatment effect. Despite the lack of statistically significant transcriptional changes in these two genes, the overall RNA sequence analysis identified 4453 significantly differentially expressed genes, indicating that exposure to electronic cigarette aerosols has the ability to induce widespread transcriptomic alterations within mouse brain microvessels.

Therefore, the lack of expression changes in TNF and IL1B should not be interpreted as evidence proving that pro-inflammatory cytokines are uninvolved in neuroinflammatory processes. Instead, these results suggest that transcriptional regulation of these canonical inflammatory genes was not a dominant feature of the response made by the organism under the specific environmental conditions examined. Their established biological significance made in figures 1-9 collectively demonstrate the established biological importance of TNF and IL1B in neuroinflammatory signalling, microglial activation and cytokine-mediated immune responses. This supports their biological relevance as candidate neuroinflammatory biomarkers for future investigations assessing protein-protein interactions and gene expression changes in neuroinflammatory pathways.

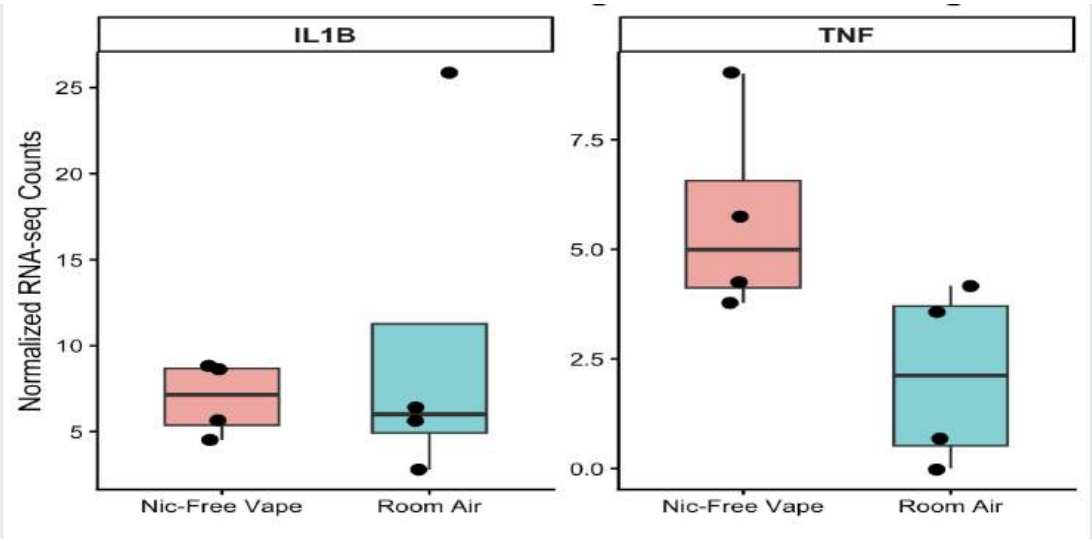

Figure 10. Differential gene expression of TNF and IL1B following chronic nicotine-free electronic cigarette aerosol exposure. Normalized RNA sequencing expression levels of TNF and IL1B were obtained from a publicly available GEO dataset GSE142400, comprising of bulk RNA-seq data from mouse brain microvessels following chronic exposure to nicotine-free electronic cigarette aerosols and room air controls. Boxplots represent the median, interquartile range and overall distribution of normalized gene expression. Individual data points represent the biological replicates (n=4 per group).TNF: $\log_2$FC=-1.454, p = 0.093, *IL1B*: $\log_2$FC = 0.55, adjusted p = 0.614.

4. Discussion

This study aimed to investigate whether current bioinformatic and transcriptomic evidence supports TNF and IL1B as candidate biomarkers for vaping-induced neuroinflammation. To do so, the investigation implemented numerous in silico and informatic analyses to evaluate the biological significance of these essential inflammatory mediators.

Structural analysis from TMHMM and SignalP suggested that TNF-α exhibited structural properties similar to a type II transmembrane protein and the signalP analysis confirmed no presence of a signal peptide. For IL-1β, a transmembrane domain analysis was conducted using TMHMM confirming that it did not contain any transmembrane helices or membrane-spanning regions. To gain a deeper understanding on the evolutionary conservation of both TNF-α and

IL-1β, a multiple sequencing alignment analysis was performed on both genes using Clustal Omega with amino acid sequences obtained from the NCBI database. The sequences were taken from four different organisms that exhibit similar evolutionary characteristics (human, chimpanzee, mouse, and pig). The analysis for TNF-α demonstrated that it was highly conserved across mammalian species through the numerous (*) located at multiple amino acid residue positions. On the other hand, the sequence for IL-1β demonstrated that the sequence was not heavily conserved across all four mammalian species with strong variation being exhibited by the amino acid sequences of the pig and mouse. The protein-protein interaction analysis generated by STRING showed that both pro-inflammatory cytokines occupy central positions within inflammatory signalling pathways highlighting their pivotal roles in cytokine regulation, neuroimmune communication, NF-kB mediated pathways, and autoimmune responses within the central nervous system. The transcriptomic analysis generated from the bulk RNA sequencing of TNF and IL1B gene counts in mouse brain microvessels following chronic exposure to nicotine-free electronic cigarette aerosols found that both genes were not significantly differentially expressed in the selected bulk RNA-sequencing dataset despite widespread transcriptomic changes occurring elsewhere in the genome.

The results demonstrate that biological relevance is not necessarily synonymous with statistical significance especially in the scenario of complex pathological processes involving multiple signalling pathways activated simultaneously. TNF and IL1B remain biologically plausible candidate biomarkers for early diagnosis of externally stimulated neuroinflammation even when one dataset fails to show significant transcriptional changes. Candidate biomarkers should therefore be evaluated using multiple complementary sources of evidence like heavily focused molecular approaches (single-cell RNA sequencing) rather than transcriptomic data alone. These findings alone provide the basis for examining the biological importance of both genes within the broader context of vaping-induced neuroinflammation and highlight several important considerations/limitations regarding early biomarker identification using in silico approaches like bulk RNA sequencing.

While the bulk RNA sequencing analysis represented both genes as not significantly differentially expressed, that does not necessarily imply that both genes do not play essential roles in inflammatory-mediated pathways. The transcriptomic evidence collected before the differential expression analysis all suggested that both proteins occupy central positions in several signalling pathways including cytokine production, microglial activation, and neuroimmune responses. As mentioned before, the transmembrane domain analysis suggested that TNF-α is a type II transmembrane protein while IL-1β lacks membrane-spanning regions. This is biologically relevant to their roles as signalling proteins since it dictates whether the cytokine acts through localized cell-to-cell contact or through freely diffusing emergency signals. Rather than containing a signal peptide necessary to direct the protein into the secretory pathway for extracellular use, TNF-α uses its transmembrane domain to anchor itself onto the plasma membrane where it is cleaved off by TACE/ADAM17 for further cell-to-cell contact to spread the inflammatory signal. On the other hand, IL-1β is originally synthesized as an inactive

precursor lacking a signal peptide or a transmembrane helix. It is then cleaved off by caspase-1 within the inflammasome complex through the activation of the NLRP3 inflammasome releasing the soluble, biologically active form of IL-1β. Their unique activation mechanisms are central to their incredibly potent, destructive nature. By synthesizing TNF-α directly onto the membrane, the body ensures that it acts primarily on a cellular level. IL-1β activation requires a two-step verification process involving caspase-1 and an inflammasome complex. This is because active, free floating IL-1β triggers profound, body wide inflammatory responses through high fever and tissue destruction. A single-step trigger may cause septic shock in response to acute environment exposure which would dilute its role as a pro-inflammatory cytokine.

As shown in the differential gene expression analysis of the bulk RNA sequencing dataset in figure 10, the transcriptomic analysis did not identify statistically significant changes in TNF or IL1B expression. However, this does not necessarily imply that these cytokines are biologically irrelevant and should not be interpreted as evidence that these cytokines are uninvolved in neuroinflammation. Neuroinflammation is a complex biological process involving the activation of several pathways, genes and proteins. Under specific environmental conditions and evolutionary characteristics, the process does not necessarily require all proteins to be activated simultaneously. The biological complexity of the process requires only certain molecular alterations and interactions to take place depending on the biological context, tissue type, exposure conditions, and the timing of sample conditions. The findings from the DESeq2 analysis observed that 4,453 genes were significantly differentially expressed in mouse brain microvessels following chronic exposure to nicotine-free electronic cigarette aerosols and neither TNF nor IL1B were shown in that list.

The biological relevance of both genes introduces a paradox in biomedical research. The specific experiment, looking at mouse brain microvessels (pericytes, basement membrane, and endothelial cells) following chronic exposure to nicotine-free electronic cigarette aerosols, highlights several technical and biological issues to why these two master cytokines failed to pass the DESeq2 significance threshold. Since the endothelial tissue is rich with pericytes and other endothelial cells, it has a low concentration of microglial cells who are primarily responsible for the secretion of these proteins. Therefore, DESeq2 may lack the computational sensitivity to analyze statistically insignificant gene expressions. On the other hand, bulk RNA sequencing is a technique that provides an absolute average transcript abundance within a pool of cells. After chronic, prolonged exposure, the microvascular cells have likely shifted into a state of chronic adaptation, remodeling, or localized endothelial dysfunction. The initial TNF and IL1B transcriptional spikes would have subsided due to this shift in state, leaving downstream genes permanently altered instead. Secondly, the experiment primarily utilizes nicotine-free aerosols. Nicotine is a highly potent neuroactive compound that actively stimulates nicotinic acetylcholine receptors (nAChRs) on neurovascular or immune cells. Master cytokines like TNF and IL1B require strong stimulation to be highly transcribed or differentially expressed and the absence of nicotine would have been a key reason for its low response in the experiment. The non-nicotinic components of e-cigarette aerosols cause severe structural and oxidative stress to

the BBB, explaining the 4,453 differentially expressed genes. However, they lack the biochemical trigger required to activate such potent, destructive master cytokines. Lastly, gene expression changes in microvascular tissues are dynamic so TNF expression may increase early after exposure to activate the NF-kB signalling pathway and return to its baseline value later. Interpretation in the DESeq2 differential gene expression analysis would not have captured minute expressional changes at the beginning of the bulk RNA sequence.

Previous studies have reported increased TNF and IL1B expression following exposure to cigarette smoke and nicotine-containing e-cigarette aerosols, supporting their role in neuroinflammation as central mediators of neuroinflammatory pathways. However, the absence of significant differential expression in the present dataset within mouse brain microvessels suggests that inflammatory responses may vary depending on external environmental conditions, aerosol composition, and tissue type. As mentioned in the transcriptomic analysis of the DESeq2 differential expression, the limitations studied highlight the context-dependent nature of inflammatory responses, particularly when stimulated by foreign toxins like the chemical constituents within inhalable aerosols. These findings align with previous literature recognizing TNF and IL1B as key inflammatory regulators while highlighting systemic limitations in studying complex pathological processes involving numerous mediatory pathways. According to a famous comparative study produced in 2024, bulk RNA sequencing was demonstrated as ineffective in comparison to precision molecular approaches like single-cell RNA sequencing. He recognized that each glioblastoma contained cells expressing different transcriptional programs related to proliferation, hypoxia, immune response, and oncogenic signaling (Patel et al. 2014). It showed that bulk RNA sequencing masks transcriptional heterogeneity by averaging gene expression across all cells within the tissue, whereas single cell RNA sequencing targets rare cell subpopulations and individual molecular interactions within a smaller pool of cells. A similar limitation may explain the present findings, as TNF and IL1B expression from the activated microglia could have been diluted by the stronger signals provided by the remaining endothelial cells, pericytes, and other microvascular cells within the sequencing dataset, limiting their biological relevance from reaching statistical significance.

Alongside this review, the study by Heldt et al. in 2020 demonstrated that chronic exposure to electronic cigarette aerosols disrupted blood-brain barrier integrity and produced widespread transcriptomic alterations within cerebral microvessels (Heldt et al. 2020). It identified 4,453 differentially expressed genes following chronic exposure but did not report both TNF and IL1B as significant DEGs, despite evidence of neurovascular dysfunction and transcriptomic data supporting their pivotal role in neuroinflammatory responses. This analysis provided the fundamental framework for this study that evaluated the biological relevance of both genes from multiple independent perspectives converting a negative result into a biologically meaningful discussion. The study proved that lack of statistical significance in one transcriptomic dataset does not necessarily indicate biological irrelevance. Instead, early biomarker discovery and evaluation requires a combination of our understanding of molecular structures, evolutionary conservation, interaction networks, and overall biological function.

Muthumalage et al., 2022 investigated the effects of chronic JUUL e-cigarette exposure on the mouse brain and found increased expression of TNF and IL1B along with the activation of several other inflammatory mediators such as HMGB1 and RAGE (Muthumalage et al. 2019). Further investigation offered evidence for high microglial activation, neuroinflammation in several cerebral regions, and strong support that chronic vaping can activate inflammatory pathways in the central nervous system. The findings made in Muthumalage et al. and the present study may look contradictory but it proposes a significant biological discussion establishing several considerations in early biomarker evaluation particularly in complex pathological processes. Rather than representing conflicting findings, these differences are likely due to the varying nature of both experiments, including the use of nicotine containing aerosols by Muthumalage et al. compared with nicotine-free aerosols analysed in the present study, as well as significant differences in tissue type and analytical methodology. While the previous study demonstrated cytokine production under the conditions of inflammatory stimulation, the present study extends these valuable findings by demonstrating that TNF and IL1B retain biological relevance as candidate biomarkers even in the absence of statistically significant differential expression.

Despite this extension of current biological knowledge, this study contains several limitations that need to be considered. The most significant limitation of this research is its reliance on a single, publicly available bulk RNA sequencing dataset. Although it was chosen with consideration based on its relevance to the theme of the research paper, it contained significant variation due to the method and environmental conditions in which the research was performed. As mentioned before, the chosen data depicted the effect of chronic nicotine-free electronic cigarette exposure on mouse brain microvessels and contains several systemic limitations stemming from the nature of the experiment. As established before, bulk RNA sequencing provides an absolute average diluting potential signals provided by smaller, more insignificant molecular alterations. Considering the opinions of previous studies, a signal provided by 1% of cells may represent differential expression but could be excluded from the average due to the signal provided by the 99% of remaining cells. Additionally, cerebral microvessels are rich in endothelial cells, and pericytes rather than microglial cells who are responsible for *TNF* and *IL1B* transcription. Additionally, everything performed in this study is purely computational, therefore the conclusions made are highly predictive rather than experimentally confirmed. Moreover, neuroinflammation is an incredibly complex pathological process containing several essential proteins (IL6, MCP-1, HMGB1, and TLR4), however, the study only examined *TNF* and *IL1B*. Additionally, although both genes are highly conserved proteins, particularly between humans and mice, mouse biology is not identical to human biology. Since species differences exist, translation to human biomedical intervention requires experimental validation and caution.

Building upon the limitations identified in the present study, future research should focus on experimentally validating these computational findings and implementing stronger, higher

resolution molecular approaches to further evaluate the biological suitability of both genes as biomarkers for vaping-associated neuroinflammation. Alongside this broad future direction, early biomarker discovery is highly essential, particularly for future biomedical intervention in translational medicine and other upcoming fields so they require a combination of both computational approaches and the interpretation of existing biological data. Since the study is entirely computational, molecular and experimental approaches are crucial to validate these findings. Quantitative PCR (qPCR) is necessary to validate differential gene expression. ELISA or Western blotting is required to measure TNF-α and IL-1β protein levels on a cellular level and other molecular approaches like immunohistochemistry or immunofluorescence are both required to determine where these cytokines are expressed within the central nervous system.

5. Conclusion

This study shows a strong biological support for transcriptomic analysis that both *TNF* and *IL1B* remain biologically plausible biomarkers for vaping-induced neuroinflammation. Improving biomarker discovery is essential to develop future therapeutic effects in medical interventions, and in understanding vaping-associated neuroinflammation as a complex neurological process that deeply varies depending on external and environmental conditions.

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The Relationship Between the Brain Development and the Heart Function in the Context of PHOX2B Mediated Signalling By Erin Chen[1]

Abstract

The human body has a very close connection between our brain and heart, which is called the brain-heart axis. If the brain cannot develop properly, the nerve signals sent to the heart will be disordered. This paper carries out comprehensive analysis on human PHOX2B homeobox protein, including transmembrane structure prediction, cross-species sequence alignment and protein-protein interaction network construction followed by a gene editing strategy. The PHOX2B acts as an intracellular transcription factor and according to the results of this study it is highly conserved among species, and it interacts closely with RET, GDNF, and ASCL1 to regulate autonomic neuron development within the brain-heart axis. CRISPR technology can be used to generate various mutations on the gene to establish a model that can be used to study the role of genes in this question in future research. However, this study only uses online database prediction and calculation, which create certain limitations for the reliability of all the conclusions. Overall, this paper provides theoretical evidence with PHOX2B structure to brain-heart balance, and gives future experimental verification in clear directions.

Keywords brain, heart, CRISPR/Cas9, PHOX2B

1. Introduction

The human body has a very close connection between our brain and heart, which is called the brain-heart axis (Figure 1) (Valenza et al. 2025a). The development of the brain builds up the autonomic nervous system. This system is basically responsible for controlling heart rate and heartbeat rhythm, and temperature regulation (Jr et al. 2023). If the brain cannot develop properly, the nerve signals sent to the heart will be disordered (Weese-Mayer et al. 2003). Therefore, the heart cannot work steadily. Most of us think heart related problems are only caused by heart damage. On the contrary, the irregular heart rhythm is actually caused by abnormal development of brain nerves that regulate cardiac activity (Pereira et al. 2013). This paper will focus on how PHOX2B gene expression influences heart function during development of the brain (Jinek et al. 2012), fully describe relevant diseases which are caused by gene mutation, and investigate a modern gene editing tool called CRISPR to establish a framework for future research

The brain is the main control organ in the central nervous system. It can be divided into four main parts, including cerebrum, cerebellum, hypothalamus and brainstem. The cerebrum is the largest part in the brain. It mainly controls how people feel, think, memory, and emotions. When people feel stressed and nervous, it can also cause changes in heart rate (Rhoton 2007). The cerebellum coordinates body movements and keeps body balance (Bradley et al. 1991). The hypothalamus manages the internal balance of the whole body, controls hormone release and

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helps people to adjust to different environments (Brooks 1988). The brainstem is located at the bottom of the brain. It includes midbrain, pons and medulla
oblongata. The medulla oblongata is the most important region that controls heart rate and blood pressure (Byczynski and Vanneste 2026).

There are 2 main types of cells inside the brain, neurons and glial cells. Neurons are nerve cells that can produce and pass electrical signals. Autonomic motor neurons carry commands from the brain all the way to the heart to change heartbeat speed (Shields 1993). Glial cells support neurons, including the providing of nutrients, wrapping nerve fibres for insulation (Kristjan R Jessen 2004)

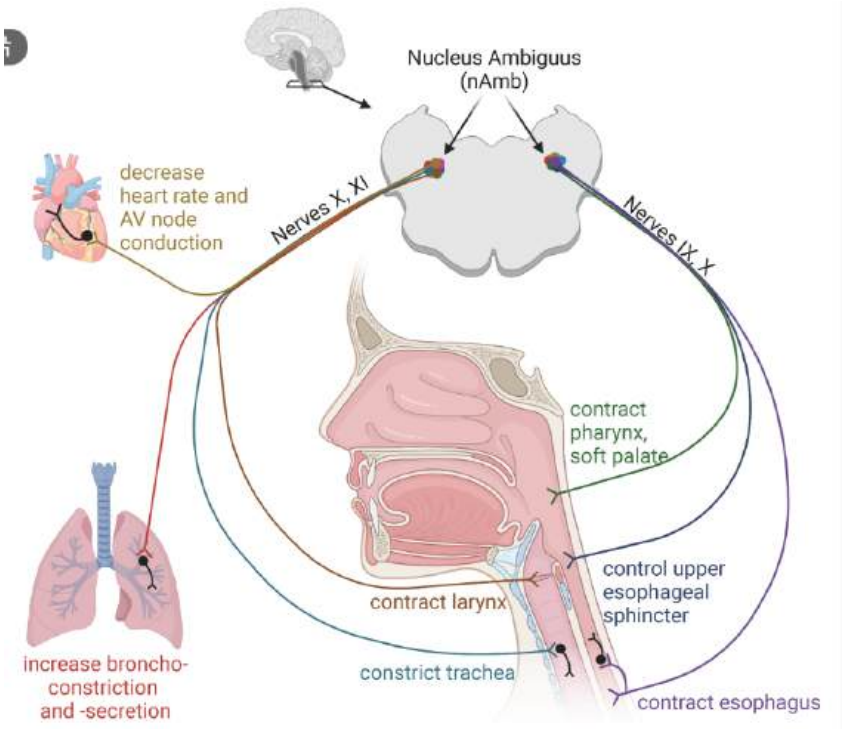

Figure 1. The brain-heart axis. diagram of the bidirectional brain-heart axis neural pathway regulated by the medulla oblongata. This anatomical drawing shows different parasympathetic signals generated from the nucleus ambiguous inside the medulla oblongata, that can be delivered to organs such as heart, lungs, pharynx, trachea, esophagus through cranial nerves IX and X. The brown lines on the left of the drawing are cranial nerves IX and X. It transmits parasympathetic signals that decrease heart rate and slow atrioventricular conduction. Other branches extend to the lungs to bronchoconstriction and secretion, as well as larynx, trachea and esophagus to regulate smooth muscle contraction. On the right, three sets of the colored lines transmit direct electrical nerve signals,hormonal pathway lines carry chemical messengers through blood circulation, and biochemical pathway lines deliver small molecular substances.

All the lines build a neurocardiac regulatory network, and mutations in the PHPX2B gene damage nerve growth and lead to heart disorders.

Medulla oblongata controls heart function. The brain controls the heart through the autonomic nervous system, which has two branches: the sympathetic nervous system and the parasympathetic nervous system (Gibbons 2019).The sympathetic nervous system becomes active during danger, stress or exercise. It releases a chemical called noradrenaline to speed up the heart rate and make the heart contract more strongly (Erik R. Swenson 2020). The parasympathetic nervous system is also known as the vagus nerve. It works during rest. It releases acetylcholine to slow down the heart rate and save energy for the body. The medulla keeps balancing these two systems to maintain a stable heartbeat (Martin 1977).

During early embryonic development, special cells called neural crest cells grow into autonomic neurons. This whole process is controlled by homeobox genes. These genes produce transcription factor proteins. The transcription factors act like switches to turn on or off the development of nerve cells. The key gene in this pathway is PHOX2B. It produces proteins that guide neurons to grow from the brainstem towards the heart and form complete nerve connections. Many ion channel proteins and neurotransmitter transport proteins are also needed to help electrical signals travel smoothly along nerves (Windels et al. 2024).

If the brain-heart control system fails because of gene mutation, people may develop Congenital Central Hypoventilation Syndrome (CCHS) (Ajay S Kasi 2024). Common symptoms include unstable heart rate, slow heartbeat during sleep, poor breathing and cardiac arrhythmia. Current treatments include regular heart monitoring and medicine to stabilise heartbeat. In the future, CRISPR/Cas9 gene editing technology may be used to repair the mutated gene (Ma et al. 2014).

PHOX2B is a homeobox gene located on human chromosome 4. It is known as the master regulator for the development of the whole autonomic nervous system.This gene is highly active during the embryonic stage. It controls the differentiation and growth of neurons that connect the brain to the heart. After birth, PHOX2B keeps a low level of expression to maintain the balance between sympathetic and parasympathetic nerves. If PHOX2B has a mutation, autonomic neurons cannot mature normally. The brain cannot send correct signals to regulate the heart, leading to irregular cardiac function (Windels et al. 2024).

This study aims to understand the structure of the brain and explain how different brain regions control heart activity, describe the two types of brain cells and their roles in signal transmission, clarify the working mechanism of the autonomic nervous system connecting the brain and the heart, introduce the molecular pathway together with the key regulatory gene PHOX2B, explore symptoms, diagnosis and treatment of diseases triggered by abnormal gene expression, and discuss the application of CRISPR/Cas9 gene editing technology in studying neuro-cardiac developmental disorders. Specifically the study focuses on PHOX2B and investigates the protein features and design a gene editing to establish a framework for future studies.

2. Materials and methods

The NCBI database is a free public bioinformatic resource storing verified standard gene and protein sequences across species. The amino acid sequence of human PHOX2B protein (accession number NP_003915.2, 314 amino acids) was retrieved in FASTA format from the NCBI Gene database. This protein sequence served as the fundamental raw data for all subsequent bioinformatic analyses, including transmembrane domain prediction and protein-protein interaction network construction.

Ensembl is an open-access vertebrate genomic repository that archives manually curated gene annotations, chromosomal coordinates, transcript variants and cross-species comparative genomics datasets based on the human reference assembly GRCh38.p14 shown in this study. The built-in database search browser was queried using the gene symbol PHOX2B, retrieving its stable gene ID (ENSG00000109132), precise chromosomal location on chromosome 4 reverse strand, official gene synonyms, full transcript records, orthologous and paralogous gene data across vertebrate species, and associated phenotype annotations. All extracted genomic metadata was retained to use in the gene editing strategy.

TMHMM is a web-based bioinformatic tool specialized in predicting transmembrane helical structures within full-length amino acid sequences by calculating residue hydrophobicity profiles. The human PHOX2B FASTA protein sequence (NP_003915.2, 314 amino acids) downloaded from NCBI>Protein>FASTA section was uploaded as the input dataset to the TMHMM server. This prediction workflow was carried out to screen potential membrane-spanning regions and infer the subcellular localization of the target protein, generating raw probability output graphs for subsequent result interpretation.

STRING is an open public bioinformatics database that integrates experimental, genetic and text-mining evidence to predict and visualize genome-wide protein-protein interaction networks. The human PHOX2B was selected in the database as the central protein and the database was asked to generate an interaction map based on built in prediction methods. . This analytical workflow was performed to identify all high-confidence interacting protein partners of PHOX2B, as well as the associated developmental signaling pathways, exporting the complete interactive network diagram for subsequent result analysis.

Clustal Omega is an automated multiple sequence alignment tool designed to evaluate evolutionary conservation across orthologous protein sequences from diverse vertebrate species. Human PHOX2B protein sequence and vertebrate orthologous sequences extracted from NCBI were imported into the Clustal Omega web server for multi-sequence comparison. The alignment operation aimed to detect conserved amino acid motifs, especially the functional homeobox domain, generating aligned sequence files and visual conservation plots for downstream interpretation.

CRISPR Finder is an online computational platform for screening valid single-guide RNA (sgRNA) target sites within gene coding sequences for CRISPR/Cas9 gene-editing design. The Human was chosen as a species and PHOX2B was selected as a gene of choice. Then the exon was defined and the algorithm showed the predicted guide RNAs with their coordinates and

off-target scores. Off-target scores were used to determine the best predicted guide RNAs possible. The selected guide RNA fits the criteria 0=1, 1=0, 2=0, and for 3 and 4 as low as possible. This screening workflow was carried out to locate specific editable target loci for generating PHOX2B mutations, collecting candidate sgRNA sequences to establish preliminary gene-editing frameworks for future studies

Snapgene is a desktop molecular biology software for sequence annotation, plasmid construction and gene map visualization. All PHOX2B genomic, transcript and protein sequences retrieved from NCBI and Ensembl were imported into Snapgene for unified sorting and manual annotation of functional domains such as the homeobox region. This software was also used to draft the schematic strategy of CRISPR/Cas9 based on sgRNA sequences screened by CRISPR Finder, organizing all sequence data for integrated bioinformatic analysis.

3. Results

3.1. PHOX2B does not contain a transmembrane domain

The TMHMM website judges proteins by checking each amino acid's hydrophobicity properties. Amino acids with strong hydrophobicity will insert into cell membranes. And the tool is to calculate the possibility score for each of the amino acids and generates a line graph. The complete amino acid sequence of human PHOX2B protein was downloaded from the NCBI>Protein>FASTA, and copied the whole sequence and pasted it into the TMHMM webpage to run the prediction test. The analysis generated a graph that shows there is no transmembrane domain in PHOX2B protein (Figure 2). According to this outcome it has been understood that PHOX2B can not anchor to the plasma membrane or any other membrane structure within the cell. Only a very small region around residue 260 can present a slightly elevated probability of being hydrophobic This finding can verify that PHOX2B belongs to intracellular proteins, and supports the conclusion that PHOX2B can regulate autonomic neuron development as a nuclear transcription factor.

RecName: Full=Paired mesoderm homeobox protein 2B; AltName: Full=Neuroblastoma Phox; Short=NBPhox; AltName: Full=PHOX2B homeodomain protein; AltName: Full=Paired-like homeobox 2B

UniProtKB/Swiss-Prot: Q99453.2

GenPept Identical Proteins Graphics

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>sp|Q99453.2|PHX2B_HUMAN RecName: Full=Paired mesoderm homeobox protein 2B;
AltName: Full=Neuroblastoma Phox; Short=NBPhox; AltName: Full=PHOX2B homeodomain
protein; AltName: Full=Paired-like homeobox 2B
MYKMEYSYLNSSAYESCMAGMDTSSLASAYADFSSCSQASGFQYNPIRTTFGATSGCPSLTPGSCSLGTL
RDHQSSPYAAVPYKLFTDHGGLNEKRKQRRIRTTFTSAQLKELERVFAETHYPDIYTREELALKIDLTEA
RVQVWFQNRRAKFRKQERAAAAAAAAAKNGSSGKKSDSSRDDESKEAKSTDPDSTGGPGPNPNPTPSCGA
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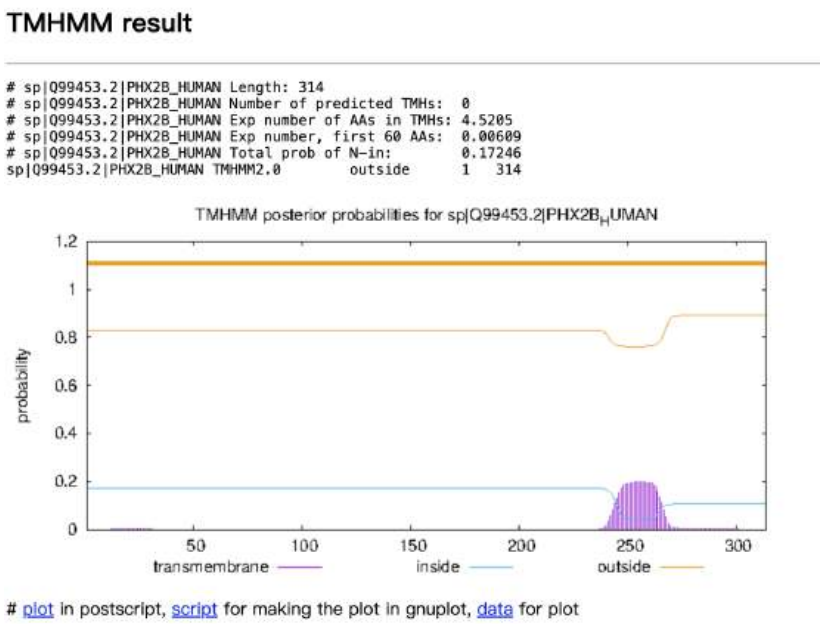

Figure 2. Transmembrane domain prediction result for PHOX2B protein. The horizontal x-axis displays amino acid positions 1 to 314 of the full-length PHOX2B polypeptide chain. The y-axis shows probability values ranging from 0 to 1.2, representing the likelihood of each amino acid segment belonging to three states, inside, outside, or crosses the cell membrane. Three colored lines are on the graph. The yellow line represents the protein parts outside of a membrane structure, , blue line is protein parts that cross to the other side of the membrane layer if the transmembrane region exists The purple line corresponds to the protein region that forms the transmembrane domain and hydrophobic amino acids. The purple shaded block near position 260 refers to a slight local increase in the probability of being hydrophobic.

3.2. PHOX2B is a highly conserved protein

Clustal Omega is an online tool for comparing different protein sequences. It first matches similar short amino acid segments from different species. Then it arranges all sequences in parallel lines to line up matching parts. The tool uses special marks and different colours to show how similar each amino acid position is across all sequences. The three complete amino acid sequences of PHOX2B protein from three different species such as human, mouse, zebrafish and chicken were aligned (Figure 3). Many symbols (*) appear continuously across the three lines in Figure 3 indicating that this protein is highly conserved. These marks mean most amino acid positions are identical in all four species. Only a small number of colon (:) and dot (.) symbols show slight differences at few positions. These segments that unchanged are key functional areas. If mutations happen in these conserved parts, they are very likely to break normal nerve and heart development, and finally cause neurocardiac diseases related to PHOX2B.

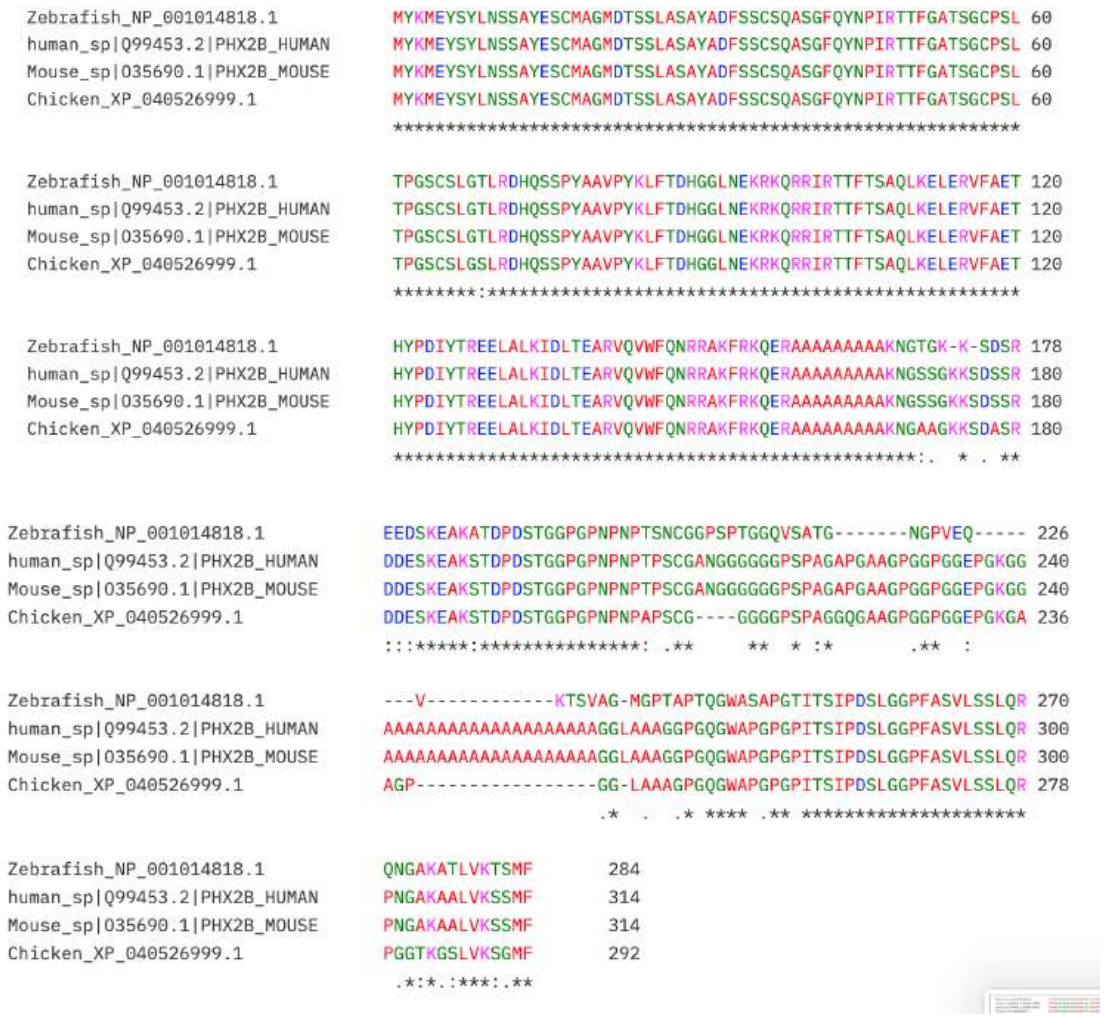

Figure 3. Multiple protein alignment of PHOX2B. The three lines named represent PHOX2B amino acid sequences of three different organisms. Symbols between each line show sequence similarity:* The identical amino acid appears at this position in all three sequences; : amino acids with highly similar chemical features; . amino acids with weakly similar chemical features. In addition, amino acid letters are labelled with different colours based on their chemical properties. For instance, blue indicates hydrophobic amino acids, and pink represents positively charged amino acids. Other colours correspond to negatively charged residues and polar amino acids. Different colours mark groups of amino acids that share similar chemical properties, so viewers can quickly spot conserved protein regions.

3.3 The protein-protein interaction map of PHOX2B

The STRING database stores massive experimental data about protein binding. It collects how the target protein interacts with other proteins. The tool creates a network graph: colored circles represent different proteins, and connecting lines stand for their binding links. In the STRING official website the gene name PHOX2B was selected as input The Homo sapiens (human) was then chosen as a species, t and the analysis was run. The complete colored

interaction graph shown as Figure 4 as a result. This interaction network shows the proteins cooperate with PHOX2B inside human cells. The result helps clarify how PHOX2B regulates the brain-heart axis together with its partner proteins. PHOX2B (the central red node) and RET, GDNF, ASCL1, DBH and HAND2 have close connections. RET is a protein that receives outside signals to control the growth and survival of autonomic nerve cells. GDNF binds to RET and turns on signals which are needed for nerve cells to grow normally. ASCL1 that helps immature precursor cells turn into fully functional nerve cells. DBH can make a kye signalling chemical inside sympathetic nerve cells so nerves can send signals properly. HAND2 works alongside PHOX2B to help autonomic nerve cells develop and keep working.

Most connecting lines between these proteins are thick, which means these protein pairs have stable, reliable binding relationships. All these partner proteins are closely related to nerve cell development and cardiovascular regulation. This network clearly shows the cooperation system of PHOX2B. It proves PHOX2B does not work alone; it relies on these partner proteins to control autonomic neuron growth. If the mutation damages PHOX2B, all these coordinated protein functions will be broken, eventually leading to neurocardiac disorders.

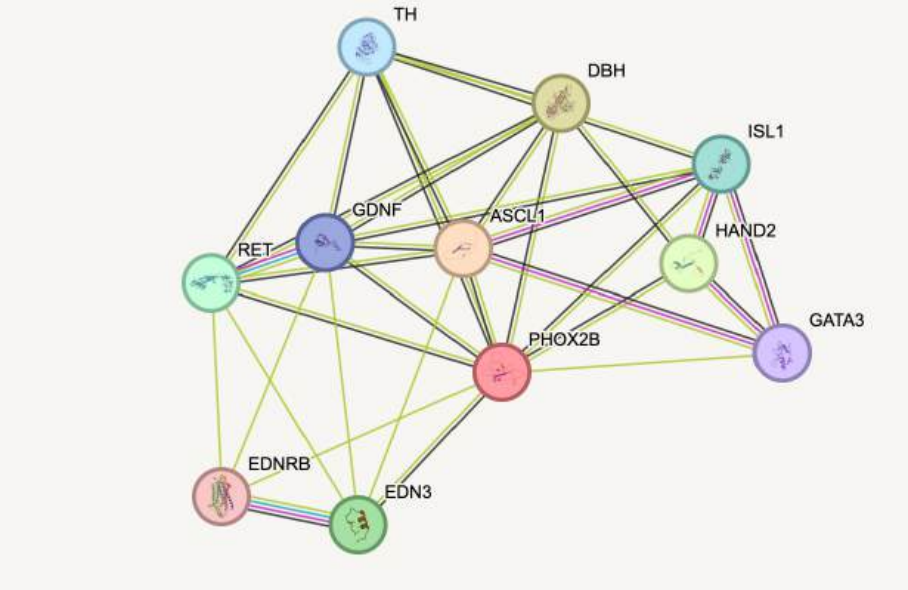

Figure 4. The protein-protein interaction map of PHOX2B. Each colored circular node represents one functional protein in human cells. The central red node represents PHOX2B, other nodes in different colors represent different proteins that bind with PHOX2B. Lines connecting two nodes show the physical or functional binding relationship between each two proteins. All partner proteins such as RET, GDNF, ASCL1 and HAND2 work together with PHOX2B to regulate the growth of autonomic neurons and maintain normal brain-heart physiological balance. The purple line marks experimentally validated protein interactions, while yellow-green lines represent relationships from scientific literature mining.

3.4. CRISPR/Cas9 gene editing strategy for PHOX2B

CRISPR/Cas9 is a popular gene-editing tool, like a pair of molecular scissors. A guide sequence (RNA) finds the target gene, and the Cas9 protein cuts the DNA. One can use this tool to modify the PHOX2B gene (Figure 5) to generate mutations or knock-ins. After breaking the DNA strand cells will apply their repair mechanism which then could lead to sequence alterations such as single nucleotide substitutions, insertions or deletions. Here the aim was designing a strategy to develop mutant variants that can be used in a wet-lab setting to study the role of PHOX2B in the brain-heart axis for future studies.

The two guide RNAs were used to achieve a potential deletion mutation with a higher specificity to generate mutant protein. The guide RNAs were designed via CRISPR Finder database and snapGene application was used to visualise the sequence and the targeted sites with PAM sequences (Figure 6) that will define the cut points for the Cas9 enzyme.

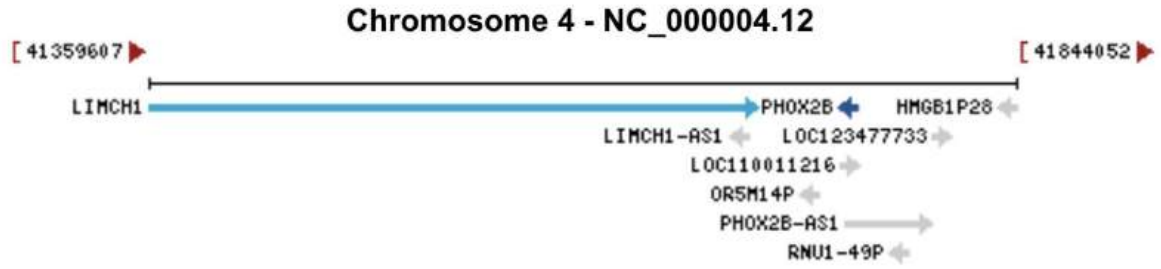

Figure 5. Chromosomal Location of PHOX2B. The long horizontal black line indicates human Chromosome 4 with the reference sequence NC_000004.12. The small dark blue arrow in the middle on the line marks the position of the PHOX2B gene. The light blue arrow is a neighbour gene to PHOX2B. Other short arrows represent neighbouring pseudogenes and RNA coding genes on this chromosome, and each arrow points to the transcription direction of the gene.

Marker Symbol

PHOX2B

Exons for ENST00000226382

1. ENSE00001014991 (length 601)
2. ENSE00003743222 (length 188)
3. ENSE00001014992 (length 2241)

972726230	4:41748360-41748382	AGGTCCTTACCTGCGGCGTACGG	0: 1 1: 0 2: 0 3: 0 4: 29

972726242	4:41748408-41748430	CCCAGGCTGCAGGATCCCGGCGT	0: 1 1: 0 2: 0 3: 9 4: 182

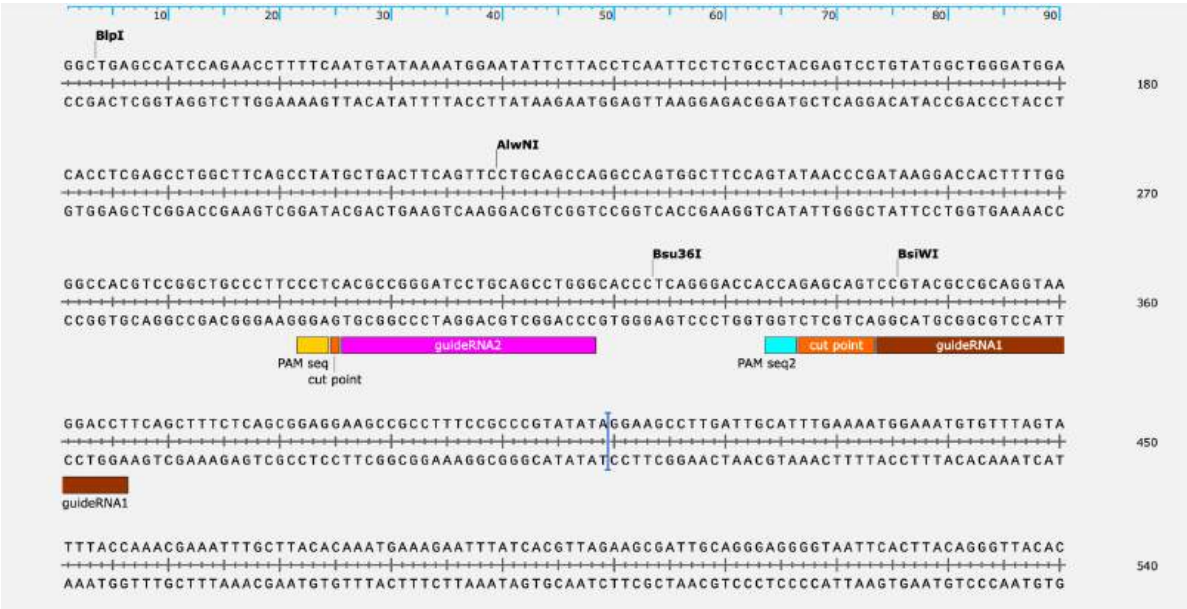

Figure 6. Snapgene view of CRISPR/Cas9 Strategy for PHOX2B. This snapgene view displays the target DNA sequence of the PHOX2B gene. It marks the positions of two CRISPR Finder recommended guide RNAs, their cutting sites and PAM sequences. The guide RNAs were selected based on off-target scores provided by the CRISPR Finder. The scores were aimed at 0=1, 1=0, 2=0 and for 3 and 4 as low as possible.

3.5. Validation techniques can be used to confirm the gene editing outcome

The gene editing outcomes can be validated with the techniques listed below:

3.5.1. DNA isolation and Polymerase Chain Reaction (PCR)

DNA isolation is a standard technique to extract and purify genomic DNA from cells (M G Murray 1980). PCR can produce a large number of copies of the target PHOX2B DNA fragment. One can design a primer pair targeting the edited region and amplify the fragment to use for the further steps such as sequencing.

3.5.2. Sanger Sequencing

Sanger sequencing determines the precise order of DNA nucleotides (Beate M Crossley 2020) .After getting PCR products, one can carry out Sanger sequencing to analyse the PHOX2B fragment. The outcome of the sequencing can be compared with the sequencing data of the wild

type fragment to observe the differences. Insertions or deletions created by CRISPR-Cas9 could be identified directly. This method provides accurate and reliable confirmation of gene mutations after editing.

3.5.2. Western blotting

Western blotting is a technique used to detect specific proteins in a sample (Rasheed Sule 2023). Proteins are first separated by size using gel electrophoresis, then transferred onto a membrane. A primary antibody binds to the target protein, and a secondary antibody makes it visible. Western blotting can be used to check the PHOX2B protein level in edited cells to compare mutant vs wild type. If CRISPR-Cas9 successfully disrupts the PHOX2B gene, the PHOX2B protein will be reduced or absent. By comparing the protein band between edited and normal cells, one could confirm whether gene editing affected protein expression.

4. Discussion

A combination of three bioinformatics approaches successfully reveals the binding features and internal structure of human PHOX2B protein. The TMHMM transmembrane prediction verifies that PHOX2B lacks a transmembrane domain, since an intracellular nuclear transcription factor. Moreover, the multiple sequence alignment run on clustal omega detects long evolution-conserved amino acid regions among different organisms, which proves these unchanged sequences keeps the protein working normally is essential. Furthermore, the string derived protein-protein interaction network can illustrate that PHOX2B forms closely cooperates with many regular proteins to thus control the growth and development of autonomic nerve cells. Previous papers merely confirmed a causal link between PHOX2B pathogenic mutations and neurocardiac disorders related to the brain-heart axis. This study provides detailed evidence to serve as a framework establishment for future studies focusing on the PHOX2B in the brain and the heart axis.

The brain heart axis refers to an essential physiological communication system which can connect brain nerve activities and cardiac regulatory functions (Valenza et al. 2025b). Autonomic nerve cells are the main medium responsible signals between our brain and heart (Alba Simats 2025). PHOX2B dominates the developmental progress of these neurons. PHOX2B master regulator autonomic nervous system development (S et al. 2026). Once PHOX2B loses normal expression structural mutation, inter nerve signals will collapse, resulting in heart rhythm disorders and various neurocardiac diseases (Lee et al. 2024). This research selects PHOX2B as an example protein, which explains a widely recognized rule within neurocardiology.

CRISPR-Cas9 gene editing technology shows the greatest potential for future neurocardiology and gene therapy research. It allows researchers to precisely modify target genes such as PHOX2B and study how specific mutations affect the brain-heart axis. As limitations, all the outcomes in this study come from online databases and prediction-based analysis rather than wet-lab experiments such as cell culture and animal model construction. Therefore, there is still no real experimental data to prove these bioinformatic predictions. Future studies should

prioritise laboratory validation, including building PHOX2B mutant cell lines and animal models, to confirm the effects of gene editing.

This study compares PHOX2B sequences from four kinds of organisms to measure the conservation level of amino acids of different species. Future research can include more species to strengthen the reliability of evolutionary conservation analysis. A broader comparison will help identify which amino acid regions are truly essential for PHOX2B function.

In addition, further studies should focus on how PHOX2B mutations change nerve signal transmission along the brain-heart axis. It is also important to develop safer and more accurate gene therapy methods to correct harmful PHOX2B mutations. These efforts will help us understand neurocardiac diseases better and create new treatments for heart rhythm problems.

5. Conclusion

PHOX2B is irreplaceable to keep the brain-heart axis working normally, its conserved structure and protein partners control autonomic nerve development. PHOX2B is irreplaceable for maintaining normal function of the brain-heart axis. Its highly conserved protein structure and interacting protein partners together regulate the growth and maturation of autonomic neurons. Loss of normal PHOX2B function or harmful structural mutations disrupt brain-heart neural communication and increase the risk of neurocardiac diseases. This research reveals the vital role of PHOX2B in neurocardiology and offers a CRISPR-Cas9-based strategy to guide further experimental exploration.

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Storytelling and Gamification as Alternatives to Traditional Behavior Management in Pediatric Dental Anxiety By Jaeeun Choi

ABSTRACT

Pediatric dental anxiety affects a substantial number of children worldwide, and if unaddressed, could persist into dental phobia that persists into adulthood. Traditional methods to alleviate pediatric dental anxiety, such as the Tell-Show-Do (TSD) method, reduce anxiety during routine care but tend to lose effect during invasive dental procedures. This review examines two types of increasingly studied alternatives: storytelling and gamification. This review also argues that these methods differ from Tell-Show-Do in terms of psychological distance; the two methods provide this distance, while Tell-Show-Do fails to do so since the descriptions are done directly to the child. These two methods are seen not only as single-visit interventions but also as interventions that may interrupt the trajectory towards dental phobia, in the long run. Although there are limitations regarding the current state of studies regarding these two methods such as the lack of long-term analysis and standardization in results, studies conclude that these two approaches are generally more effective in reducing pediatric dental anxiety than traditional approaches such as Tell-Show-Do.

Keywords Pediatric Dental Anxiety, Storytelling, Gamification, Dental Phobia

INTRODUCTION ABOUT PEDIATRIC DENTAL ANXIETY AND TRADITIONAL METHODS AGAINST PEDIATRIC DENTAL ANXIETY

Dental fear and anxiety is one of the most common problems in pediatric dentistry. It affects 23.9% of children and adolescents, with prevalence highest among preschoolers (36.5%) and lowest among adolescents (13.3%) (Grisolia et al.). Although prevalence appears to lower with age, dental anxiety often begins early and can persist. For instance, around half of adults suffering dental anxiety report that their fear started from childhood (Locker et al.).

The reason this issue is so crucial is because fear increases chances of trapping children in a vicious cycle. A child with dental anxiety would avoid dentists, even when they suffer from dental pain. This would cause minor issues such as small cavities going untreated and growing into more serious ones that need more serious and painful treatment, which deepens dental anxiety (Almarzouq et al.). Simply said, avoidance caused by dental anxiety leads to worse oral health, and the treatment to bad oral health causes more fear.

General anesthesia is often used in cases where dental fear is very high. Despite being effective, anesthesia costs average over US$2,000 per use. It also carries a mortality risk, and its temporary nature which requires it to be repeated when new cavities form, makes it a controversial choice (Almarzouq et al.). Therefore, preventing dental fear at early stages brings benefits not only in terms of the child's mental comfort, but also in terms of safety and cost, as well.

Childhood dental fear usually develops through direct conditioning (a painful or negative experience) or vicarious learning (copying an anxious parent) (Milgrom et al, qtd. in Elicherla et al.). A study regarding children with post-traumatic dental anxiety supported this, finding that some of the most common causes were painful restorative treatments, injections, drilling, and the dentist's own behavior (Hussein and Akşit-Bıçak). Most of these triggers are the more invasive and physically intrusive procedures, which suggests that the type of treatment a child undergoes may have an impact on pediatric dental anxiety. Because these early negative experiences later result in lasting fear, the way a child is treated during their early dental visits could help mold their perception toward dental care in the long term.

Because early experiences are one of the most crucial influences on how a child perceives dental care, this review focuses on prevention as well as effectiveness. One limitation should be clear from the start, however. Few studies monitor children long enough to confirm that an intervention actually prevents phobia. Almost all of them measure anxiety within a single visit or a few visits. This review therefore treats a lasting reduction in childhood dental anxiety as a proxy for prevention. This is a reasonable inference, since phobia tends to grow from repeated anxious experiences, but it is not something the current evidence can prove directly.

Storytelling here refers to using a narrative, such as a picture book or an animated story, to prepare a child for treatment, while gamification refers to applying game elements such as rewards, role-play, and interactive challenges to the dental experience; some interventions combine the two as "serious games." The research question is: can storytelling and gamification reduce children's dental anxiety more effectively than traditional methods, and could they help prevent dental phobia? This review will propose that storytelling and gamification differ from Tell-Show-Do in how directly the child encounters the procedure, a difference the discussion develops later.

TELL-SHOW-DO (TSD) METHOD

The Tell-Show-Do (TSD) method is one of the most common methods and the established standard used to manage children’s dental anxiety, alongside other methods such as distraction, modeling, positive reinforcement, relaxation, and hypnosis (Anthonappa et al.). Introduced by pediatric dentist Harold Addelston in 1959, it consists of three main steps. First, the dentist explains the procedure to the child in simple language (tell). Second, the dentist demonstrates the procedure; an instance would be showing the instruments or how they work (show). Third, the dentist proceeds with the treatment as it was previously explained and shown (do). Due to this simple, clear structure and long history, TSD is often the gold standard against which newer methods to alleviate pediatric dental anxiety are compared (Karkoutly et al.). Therefore, TSD serves as the main comparison method against the two methods (storytelling and gamification) discussed within the studies in this review.

The main point of this method is familiarization. By letting the child know what will happen before the actual procedure, it aims to reduce the fear that is derived from facing something unfamiliar and unknown.

Many studies show that TSD is effective in terms of reducing pediatric dental anxiety. For instance, in one randomized controlled trial of 50 children, TSD lowered both self-reported anxiety and heart rate. It performed better than Ask-Tell-Ask (ATA), a newer method where the dentist first asks the child what they know or feel, gives a small piece of information, then asks again to check their response (Elicherla et al.). Since this rests on a single trial, it is better read as supportive than as firm proof.

Although shown as effective, the effects of TSD reduce when invasive dental procedures are involved. For instance, children given TSD before a pulpotomy showed an increase in pulse rate of about 10 beats per minute (Karkoutly et al.). A review of 66 trials regarding various methods found that distraction methods performed better than TSD in terms of anxiety, cooperation, and pulse rate. This advantage, however, disappeared during invasive treatment, where both distraction and TSD approaches performed similarly (Almarzouq et al.). This suggests that invasive dental procedures often cause fear that is harder to alleviate. TSD is generally regarded as an effective method, but it has its limitations, and the possibility of more effective methods exists.

As mentioned previously, TSD is based on reducing the fear of the unknown. It loses effect during invasive procedures, which often frighten children by its nature, in addition to unfamiliarity. Therefore, reducing the fear of the unknown, as TSD does, may be insufficient when fear comes from the procedure itself. This also suggests that other methods that deal with more than the elements of the unknown may show different results.

STORYTELLING METHODS

Storytelling interventions within dental visits are indirectly done. Rather than describing a dental procedure to the child directly prior to the procedure as in TSD, it puts the procedure in a narrative in forms of an illustrated storybook, an audio-visual story, or an outside character who visits the dentist.

Storytelling methods have proven to show effectiveness in different trials. A randomized controlled trial conducted at King Saud University, which involved eighty-eight children aged from six to eight, compared a group of children who were given specially designed dental storybooks and another group without them. Both groups went through screening, examination, cleaning, and restorative treatment. The trial showed that children in the storybook group showed significantly lower anxiety and were more cooperative during treatment. The multivariate analysis also confirmed that the storybook was a significantly independent factor after controlling for several different variables such as sex, previous dental visits, family income, and mother’s education (Alsaadoon et al.). The most notable result of this trial was the direction it showed regarding the effectiveness of storytelling. While the anxiety in the control group rose during the three visits, the anxiety in the storytelling group fell. The fact that this was a consistent factor on different visits suggests the effectiveness of the storybook.

Another trial involving 160 children found that an interactive dental app and a dental song reduced heart rate and facial image anxiety scores, while TSD reduced neither (Abbasi et al.). This suggests that newer, engagement centered methods can outperform TSD.

Other trials also support the effectiveness of storytelling methods, without saying that it is superior to TSD. For instance, an Iranian trial that assigned children ages six to nine to audio-visual storytelling, TSD, or a control group, found that the storytelling and TSD groups showed significantly lower anxiety compared to the control group. Audio-visual storytelling was deemed an effective and applicable method. The study does suggest, however, that the type of story that is chosen and the relationship between the dentist and the child also impact storytelling's effectiveness (Gozin et al.).

Regarding differences within the storytelling methods, a trial that involved 240 children ages five to twelve divided them into three groups: a group given a self-designed storybook along with storytelling, a group that was only given storytelling, and a control group. Both groups that received storytelling methods showed significantly lower anxiety scores and lower pulse rates than the control group. However, the group that received the self-designed storybook as well as storytelling did not score significantly better than the group that was only given storytelling. This suggests that the narrative in storytelling, rather than the methods such as a physical book itself, may be what is impacting lower anxiety (Krishnadasan et al.).

Storytelling by nature puts the child in a different position than regular dental practices. The child watches another character within a dental visit, not themselves. Alsaadoon's storybook is based on Bandura's social learning theory, which suggests that children learn through watching others, and on self-regulation theory as well (Alsaadoon et al.). Through storytelling, a child receives information about what will happen in the dental procedures, and evidence that it could be handled. This differs from verbal explanations before the procedure, which cannot easily provide the latter.

GAMIFICATION METHODS

Gamification is an anxiety alleviation method that applies elements common in games. These elements include rewards, role-play, and interactive challenges. Gamification is used to either distract a child during treatment or to prepare them beforehand. Gamification has a large body of evidence, though there are some inconsistencies.

Regarding the effectiveness of gamification, a meta-analysis of 42 studies that covers around 2,500 children found that digital distraction shows strong support that it significantly reduced dental anxiety, with a standardized mean difference of −0.97. The authors, however, do note that none of the included trials were strongly designed (Haridoss et al.).

Some of the clearest evidence comes from games that let children rehearse the procedure beforehand, playing at being the dentist. Gamification's effectiveness was shown in a triple-blinded trial of 60 children, where a simulation game called Baby Panda Dental Care lowered pulse rate, self-reported anxiety, and observed pain during a pulpotomy compared with Tell-Show-Do (Karkoutly et al.). It is worth noting, however, that neither the gamification group

nor the TSD group's anxiety stayed below baseline during the procedure itself. Although the gamification group performed better than the TSD group, the fear during an invasive treatment remained. A separate trial regarding simulation games reported similar gains in pain, fear, and compliance (Zhang et al.).

One mobile game app called Little Lovely Dentist has been tested across three countries, which gives us a case with replication. Different trials in India (Elicherla et al.), Pakistan (Abbasi et al.), and Iraq (Aziz and Jafar) all concluded that children who used the app were less anxious than those given Tell-Show-Do or no intervention. This replicated result amongst individual research teams especially strongly suggests the effectiveness of gamification regarding dental anxiety.

Gamification methods that involve virtual reality have also been studied. A meta-analysis of 12 randomized trials with 818 children reported large reductions in anxiety and pain, although there was very high heterogeneity between studies (I^2 of 91 to 99 percent) and acknowledged publication bias. This means the true effect may probably be less than the pooled figure suggests (Yan et al.). A separate trial of 80 children aged 5 to 10 found similar benefits (Gómez-Polo et al.), and a study involving 56 children found the possibility of gamification being effective even during invasive procedures by showing that children given gamified VR reported lower pain and heart rate while undergoing molar extractions (Mladenovic et al.).

A network meta-analysis of 61 trials and 6,113 children ranked various intervention methods against one another. It ranked music highest for reducing anxiety, followed by aromatherapy. Games were ranked third. Storytelling was not included as a separate category in this analysis (Kong et al.). This suggests that while gamification may be effective, there possibly may be more effective measures.

An interesting aspect of gamification is that it can help a child outside of the dental chair as well. One review found that gamified oral-health tools raised regular engagement from 48.3 to 72.4 percent and were linked to better brushing and more preventive visits (Chen, J. et al.). This returns to the previously mentioned vicious cycle. Children who engage in more dental care may go through less painful emergency treatments that create fear. As of 2021, this preparation-based use of digital tools was still largely untested in dentistry (Cunningham et al.), but the simulation game trials are starting to fill this gap.

EVIDENCE FROM OTHER MEDICAL SETTINGS

Because direct studies of storytelling and gamification in dentistry are limited, viewing this in terms of other medical situations outside of dentistry, such as surgery and hospital care, is helpful. Although the dental chair may differ from an operating room, the fear involved is similar in kind. There are shared elements such involving anticipated pain, an unfamiliar setting, and a loss of control. Therefore, findings from these non-dental medical settings can reasonably inform what may happen in dentistry, as long as the differences are understood and acknowledged.

The clearest comparison between the two methods this review examines comes from outside dentistry. In a trial with hospitalized children, storytelling and play therapy were tested

against each other, and only storytelling significantly reduced anxiety (Abdi et al.). This study suggests that the element of narrative may do more than simply keeping a child occupied. The same study found the effect was strongest in children aged three to eight and became weaker in older children, which supports that methods that cater to each age group rather than having one approach fit every patient would be effective.

Two studies point to something that the dental trials often miss: what happens after the procedure is over. These studies showed that children who were not prepared beforehand tended to get worse instead of recovering. A trial showed that unprepared children's anxiety was still high after two weeks post-surgery (Mete İzci and Çetinkaya), and in another, the unprepared children's behavioral problems increased ten days afterward surgery (Sekhavatpour et al.). These show that anxiety could outlast the procedure or appointment, which makes these two non-dental studies valuable. One study used digital storytelling, and the other one used a printed, illustrated story. Both studies showed that either way protected children from anxiety. This finding suggests the story itself is helpful, rather than the technology or method the story was given to the children.

Similar pattern in surgical settings regarding gamification. A meta-analysis of 26 studies covering 2,525 children found that children who played a game before surgery had lower anxiety scores than those who did not, at the moment of anesthesia induction (Suleiman-Martos et al.). One comparison compared video games to midazolam, a sedative drug that reduces pre-surgery anxiety, and showed that video games modestly outperformed midazolam regarding anxiety (Luo et al.). A preparation game called CliniPup also lowered preoperative anxiety when children played it before surgery (Buffel et al.), although this was a small pilot involving twenty children, and its developers were involved within the study.

These findings outside of the dental realm suggest that both storytelling and gamification can reduce children's anxiety in medical situations with similar elements to dental treatment. Storytelling especially has the potential to leave children better off after the event, as benefits persist regarding storytelling, not gamification. Whether these findings are applicable within the dental clinic are questions the discussion turns to.

DISCUSSION

The above sections regarding various methods, such as TSD, storytelling, and gamification, show us the different levels of effect and ways in which they alleviate pediatric dental anxiety. The evidence presented so far raises a question: that familiarization is an important aspect, but dealing with it alone does not fully eliminate dental fear. This is shown in the gap regarding Tell-Show-Do, which is itself a familiarization technique. TSD reaches its limit during invasive procedures (Elicherla et al.; Abbasi et al.; Karkoutly et al.). Familiarization, therefore, cannot fully account for the results, and further factors may exist for why gamification and storytelling showed different levels of effectiveness when compared to TSD.

One factor that is suggested by research that may explain this difference is the psychological distance each method provides. When young children approach a demanding or

tempting task from a third-person perspective, they demonstrate greater self-control and lower distress. This effect is shown when children adopt the role of a competent character rather than acting as themselves (White et al.). Another study indicates that this form of self-distancing allows children to process a stressful experience rather than re-experience it, establishing a psychological buffer between the child and the source of distress (Kross et al.). Therefore, psychological distance may affect how a child perceives a stressful experience, with more distance possibly bringing more protection towards fear.

This review proposes that psychological distance is the principal characteristic that may explain the difference between storytelling and gamification from Tell-Show-Do. In TSD, the dentist describes and demonstrates the procedure to the child directly as an event about to be performed upon them. This gives them little distance. Storytelling and gamification, however, present the same information in indirect manners. The child encounters the procedure through a character in a narrative or a role within a game. During this process, they are in the position of an observer rather than that of the patient (Alsaadoon et al.). This proposal is consistent with the findings of a study where app-based and simulation-game interventions tended to outperform TSD (Abbasi et al.; Karkoutly et al.). This is further supported by the single head-to-head trial in which storytelling reduced anxiety while conventional play did not (Abdi et al.). As no dental study in this review tests this mechanism regarding distance directly, this is more of a proposed explanation.

The same idea regarding distance also may explain the conditions under which the discussed methods fail. While psychological distance may be effective while a threat remains anticipated, its effect is likely to diminish once physical sensation and pain occur, since pain cannot readily be observed or felt from an external perspective until the moment when it happens. This corresponds to the invasiveness ceiling evident throughout the evidence: games and applications reduce anxiety effectively before and around routine treatment, but their advantage narrows during painful procedures. No method maintained anxiety below baseline during these painful procedures (Aziz and Jafar; Karkoutly et al.; Almarzouq et al.). Therefore, distance may assist a child in approaching a frightening procedure, but its effect may not last through the procedure itself.

A related distinction can be drawn between active preparation and passive distraction, regarding storytelling and gamification methods. Interventions such as music or video occupy a child's attention during treatment and distract them, whereas storytelling and role-play games require the child to actively participate in and interpret what would happen next (Cunningham et al.). This distinction also addresses an apparent inconsistency. A large network meta-analysis ranked music above games for the reduction of anxiety (Kong et al.), a finding that at first glance appears to weaken the case for gamification. However, the study measured anxiety within single visits. This is a situation where a passive, calming intervention such as music would be expected to perform well, and it did not include storytelling as a distinct category. It therefore cannot capture the intended function of preparation-based methods, which is to influence how a child approaches dental care across repeated visits. In practice, these approaches are not competing

alternatives: a child may be prepared with a narrative at home, engaged by a game in the waiting area, and calmed by music during treatment.

Two further limitations exist throughout the studies. First, the measurement of children's fear is inherently difficult and may be inaccurate. Children may appear calm due to their internal coping strategies although they may be anxious (Versloot et al.). Self-report results and heart rate frequently diverge (Elicherla et al.; Karkoutly et al.). Physiological measures, though objective, are not precise enough (Almarzouq et al.). Second, parental influence remains underexamined. Parental anxiety, education, and attachment style are each associated with how children experience dental care (Alsaadoon et al.; Hussein and Akşit-Bıçak; Beaven et al.), and dental fear is frequently acquired from an anxious parent in the first instance (Milgrom et al, qtd. in Elicherla et al.). Finally, the same techniques used to prevent fear appear capable of reducing it once established, as demonstrated by a study in which 75 percent of children referred for general anesthesia were subsequently treated without it (Hussein and Akşit-Bıçak). Prevention and recovery thus appear to rely on a shared set of methods.

LIMITATIONS, CONCLUSIONS, AND SUGGESTIONS

Several limitations exist regarding the studies reviewed. The prevention of dental phobia, which would require a long-term assessment, is not included in the studies reviewed. Most of the studies measure anxiety within a period of a single visit or a small number of visits. The longest follow-up extends to about two weeks (Almarzouq et al.,; Chen et al.,; Mete İzci and Çetinkaya,). Therefore, the claim of reducing anxiety during childhood lowering the likelihood of phobia developing through adulthood is based on inference. Although this may be reasonable, it remains untested.

The evidence is also methodologically limited. An article that reviewed various studies and trials rated the certainty of its evidence as very low (Almarzouq et al.). The virtual-reality meta-analysis study reported extremely high heterogeneity and acknowledged publication bias (Yan et al.). The network meta-analysis stated that its rankings require further confirmation (Kong et al.); a review of gamification found that 95 percent of studies carried at least a moderate risk of bias (Chen et al.); and the largest distraction meta-analysis noted that no included trial achieved a low risk of bias (Haridoss et al.). A Cochrane review of these interventions was registered in 2017 but has still not been completed (Anthonappa et al.). These weaknesses share identifiable causes: behavior is often rated by unblinded operators, no standardized set of outcome measures exists across studies, samples are frequently small, and young children's self-reports are difficult to obtain reliably.

Three further limitations should be noted. The invasiveness ceiling indicates that these methods are the most effective when the risk of phobia is lowest, such as during routine care, and least effective during painful procedures, which are likely to cause lasting fear (Almarzouq et al.,; Karkoutly et al.; Aziz and Jafar). The evidence shown is geographically narrow; 44 of the 66 trials in the principal review were conducted in Asia, which limits the generalizability of these

findings (Almarzouq et al.). No study within this review combined storytelling and gamification, which leads to their intersection remaining untested.

Despite these limitations, the evidence generally points consistently in one direction. Across different countries, settings, and research teams, storytelling and gamification reduced children's dental anxiety, often outperforming Tell-Show-Do, particularly during the earlier, anticipatory stages of treatment where TSD is weakest. Evidence further suggests that the two approaches may cater to different age groups of children. While storytelling appears to be most effective for younger children around ages three to ten (Abdi et al.), gamification appears better suited to school-age children and older. However, neither of these methods should be regarded as a direct replacement for established techniques. Instead, research suggests that they should be viewed as supplements that can be combined with existing methods rather than substitutions (Haridoss et al.).

The findings from this research leads to two suggestions. First, a longitudinal study that follows children into adolescence would test whether reduced childhood anxiety translates into genuine dental phobia prevention in the long run. Measuring a child's anxiety through continuous appointments would also be effective. For instance, even a single follow-up visit observed in a storybook trial showed that prepared children became less anxious over successive visits while unprepared children became more so, suggests this (Alsaadoon et al.). Until such studies are conducted, storytelling and gamification would remain plausible but weakly proven means of reducing children's dental anxiety and dental phobia in the long term.

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Evaluating IVF Beyond Biological Success By Sofia Pistol

Abstract

In vitro fertilization (IVF) has become one of the most widely recognized treatments for infertility because of its ability to overcome biological barriers to conception. Advances in pharmacology have improved IVF success rates, however, evaluating treatment effectiveness only through outcomes such as fertilization, pregnancy, and live birth may overlook important limitations. This paper evaluates IVF beyond its biological success. It considers factors such as affordability, accessibility, psychological effects, and the role of alternative approaches to infertility treatment. Particular attention is given to patients with polycystic ovary syndrome (PCOS), for whom lifestyle intervention, ovulation induction, and other conservative treatments may be appropriate alternatives before IVF. Research on fertility treatment, healthcare access, and psychological well-being is used to evaluate how financial and social factors influence the practical effectiveness of IVF. The evidence suggests that IVF can be highly effective biologically but its high cost can restrict access, contribute to socioeconomic and racial disparities, and affect whether patients are able to continue treatment through multiple cycles. IVF may also create significant psychological strain for patients undergoing treatment and those who cannot afford treatment may experience a different form of distress caused by limited access. The findings therefore challenge the idea that IVF should automatically be considered the best approach to infertility. Treatment effectiveness should instead be evaluated through biological success as well as affordability, accessibility, psychological well-being, and the availability of appropriate alternatives.

Introduction

Pharmacological treatments (drugs used to prevent, manage, or reverse a biological dysfunction) no longer serve only to manage chronic disease. These treatments are used to produce biological outcomes that may not occur without medical intervention. Assisted reproductive technology, and in vitro fertilization in particular, shows this shift. IVF does more than treat conditions that can cause infertility, such as anovulation, blocked fallopian tubes, or polycystic ovary syndrome (PCOS). It uses hormonal medications and laboratory techniques to help achieve a pregnancy when conception may not otherwise occur. The World Health Organization estimates that one in six adults worldwide experience infertility, which is reason enough to take seriously how that condition gets treated (World Health Organization 25).

Pharmacology is not, however, the only route back to fertility. Ovulation can be restored through lifestyle change, conception can sometimes be timed without medication, tubal blockages can be corrected surgically, and counseling can address the distress that often accompanies infertility independent of its biological cause. Medicine has increasingly positioned pharmacological treatment as the standard response to infertility, and society often reinforces that view. As a result, many people may be more familiar with medical intervention than with other available approaches. Infertility treatment is often evaluated primarily through biological

outcomes such as ovulation, fertilization, and pregnancy rates. However, a treatment's effectiveness means little if patients cannot realistically access it or if pursuing it creates significant physical, financial, and psychological strain. This is especially important in PCOS-related infertility, where treatment can range from lifestyle intervention, ovulation induction, and surgical correction to ovarian stimulation and in vitro fertilization (IVF). Although IVF can offer high rates of biological success, its cost, limited accessibility, physical demands, and psychological burden challenge the idea that it is the "best" approach. Considering these factors alongside clinical outcomes shows that IVF may not be the most suitable approach for every person experiencing infertility.

The Pharmacological Pathway: Ovarian Stimulation and IVF

Pharmacology has improved IVF's biological effectiveness. Controlled ovarian stimulation uses hormonal medication to help the ovaries mature multiple eggs within a single cycle. Drugs such as gonadotropins and GnRH analogues are used to regulate the timing of this process so that eggs can be retrieved for fertilization (Shrestha et al. 137). Refinements to these protocols have since produced approaches that shorten treatment time without compromising outcomes (Kadoura et al. 18). IVF can be highly effective for individuals with anovulation, PCOS, or blocked fallopian tubes because it can overcome biological barriers to conception. However, clinical success alone does not determine whether IVF is the most appropriate treatment option.

The Non-Pharmacological Pathway: Lifestyle Intervention and Conservative Treatment

Non-pharmacological treatments are not suitable for every cause of infertility, but they can be effective for certain patients, particularly those with PCOS. PCOS-related infertility is often caused by hormonal and metabolic factors, including insulin resistance and excess androgen production, rather than a physical blockage. In some anovulatory women with PCOS, a 5-10% reduction in body weight can improve ovulation and may allow pregnancy to occur without hormonal treatment (Wang et al. 1079). A large Dutch study examined 577 women with obesity and infertility, comparing those who completed a six-month lifestyle program before fertility treatment with those who began treatment immediately. Women in the lifestyle group had more spontaneous pregnancies and required less medical intervention, but their vaginal birth of a healthy singleton at term rate within 24 months was lower than that of women who began treatment immediately (27.1% compared with 35.2%) (Mutsaerts et al. 6). Lifestyle intervention is most useful when infertility is connected to specific metabolic or hormonal factors. Even when it is successful, however, it often takes longer to produce results than medication. Ovulation induction with medications such as letrozole and surgical correction of a blocked fallopian tube offer additional treatment options. These approaches are generally less costly and more limited in scope than IVF, which is one reason they are often considered earlier in treatment. These alternatives are not intended to replace IVF in all cases. The concern is that they receive less

attention in both clinical discussion and public awareness, meaning some patients may never have the opportunity to compare them with pharmacological treatment.

Cost as a Gatekeeper

Although IVF may be medically appropriate, its high cost can determine who is actually able to access treatment. IVF is expensive and rarely covered by insurance in full, so access tends to track income rather than clinical need. A cycle in the United States is often quoted at $12,400, and separate figures show that fertility preservation for cancer patients (oocyte cryopreservation ranging from $10,000 to $13,000, and embryo cryopreservation from $13,000 to $16,000) carries its own steep costs, with insurance coverage remaining extremely limited in most states (Insogna and Ginsburg 1153, 1155, 1156). As of 2026, twenty-five states and Washington, D.C. require some form of fertility-related insurance coverage. Even so, differences in eligibility requirements and coverage limits leave access to IVF uneven across the United States (MultiState). Access to advanced reproductive services is often limited by socioeconomic disadvantage, lower income, and inadequate insurance coverage (Mackay et al. 17), and these inequities sharpen further at the intersection of race and ethnicity. Black and Hispanic women in the United States report significantly more difficulty paying for fertility treatment than white women, and they report attempting to conceive for 1.5 years longer before seeking care (Missmer et al.). Access to IVF as a path to biological parenthood is closely tied to financial resources. Because wealth is unevenly distributed across racial groups, these financial barriers can also contribute to unequal access to treatment. IVF protocols are not inherently race-based, but the high cost of treatment can contribute to racial disparities in access in a society where income and wealth are already unequally distributed. In 2022, the median White household held roughly six times as much wealth as the median Black household and five times as much as the median Hispanic household (Aladangady et al.). These wealth disparities help explain why the high cost of IVF can contribute to racial inequalities in access.

Cost does not make IVF less biologically effective, but it limits who can access it. As a result, reported IVF success rates may therefore represent a more financially advantaged group of patients rather than the full population experiencing infertility.

How Cost Shapes What "Effective" Means

IVF is priced per cycle, and because a single cycle may not result in a live birth, patients who can afford multiple attempts have more opportunities to achieve success. Cumulative success can depend partly on whether a patient is able to continue paying for treatment. Minimal-stimulation IVF illustrates this trade-off: although it reduces medication use, clinic visits, and cost, one randomized trial found a lower live birth rate per treatment course than conventional IVF (49% versus 63%), largely because fewer eggs were retrieved (Zhang et al.). Clinics offering this lower-dose protocol point to lower cost as one of its advantages, alongside fewer injections and reduced risk of ovarian hyperstimulation, making it an option some patients turn to when they cannot afford full-dose IVF, even as it comes with a lower per-cycle success

rate (SART). Patients who can afford multiple IVF cycles have more opportunities to achieve a live birth, meaning cumulative success rates are shaped by more than biological effectiveness alone. They are also influenced by whether patients can afford to continue treatment after an unsuccessful cycle. As a result, IVF cannot be evaluated solely by whether it works biologically; access and financial resources also shape who is able to remain in treatment long enough to be represented in its success rates.

The Psychological Cost of Being Priced Out

Cost-prohibitiveness limits who receives treatment, and it also reshapes how infertility is experienced by those who cannot obtain it. When an effective treatment exists but remains financially out of reach, infertility may take on an additional psychological burden. Patients may experience inadequacy, self-blame, or a reduced sense of control when access to treatment depends more on financial circumstances than on medical possibility. Patients in this position report a sense of inadequacy, self-blame, and an erosion of felt control, because the line between what is medically possible and what is personally possible has been drawn by a price tag rather than by biology (Rooney and Domar 41). In a study of 250 women with PCOS, anxiety symptoms were present in 40 percent of the sample overall, but in 67 percent of those in the lowest income bracket and 45 percent of unemployed women, compared with 31 percent of employed women, a gap that tracked income and employment status more closely than any single physical symptom of the condition (Almeshari et al. 1). A pooled analysis of PCOS patients across low and middle-income countries, where limited healthcare access and financial constraint are common, found depression in just over half the sample and anxiety in nearly half, both higher than comparable estimates from wealthier countries (Atinga et al. 22). Across these studies, psychological distress appears to be strongly associated with financial strain and limited access to treatment, rather than with PCOS alone. This suggests that some of the emotional burden associated with IVF may result from the cost and accessibility of treatment rather than the treatment itself.

The Psychological Cost of Undergoing Treatment

Patients who are able to access IVF face a different set of challenges. The treatment itself can involve substantial emotional and physical demands. A systematic review of IVF's psychological effects found that anxiety, depression, and general psychological distress are elevated in both men and women during treatment, regardless of whether cost was a barrier to reaching it (Grammenou et al. 2). Some of this distress has specific, identifiable causes. Repeated IVF cycles can create repeated periods of hope and disappointment. Over time, unsuccessful attempts may contribute to a continuing sense of grief rather than a single experience of loss. Sexual dysfunction was significantly more common among women than men in one Tunisian sample of infertile couples (28% versus 5%), with women reporting higher rates of arousal and orgasmic disorders specifically; desire disorders were the most frequently reported dysfunction for both partners (Mahfoudh et al. 1140). Infertility itself is associated with an increased risk of

mood and anxiety disorders. This supports the need to identify and treat these conditions directly rather than allowing them to remain unaddressed during fertility care (Becker et al. 765). Infertility can disrupt a person's sense of identity and self-esteem, and repeated failed cycles may intensify this distress. In addition, the hormonal medications used during treatment can directly affect mood, meaning that emotional changes may result from both the treatment itself and the psychological stress surrounding it (Becker et al. 765). Whether infertility-related distress negatively affects relationships remains unclear, with research showing mixed results. One study from Tunisia attributes more than a fifth of divorces among infertile couples to relationship strain connected to infertility (Mahfoudh et al. 1140), while a Danish national registry study following more than 40,000 women found that undergoing fertility treatment did not, on its own, increase the risk of divorce, and some couples described treatment as having forced them into better communication and coping strategies (European Society of Human Reproduction and Embryology). The psychological strain of infertility treatment may be similar to that experienced with other chronic and uncertain medical conditions (European Society of Human Reproduction and Embryology). However, this burden is different from the distress caused by being unable to access treatment in the first place. The two problems therefore require different responses. Improving affordability and access can address financial barriers, while integrating psychological support into standard fertility care can help patients manage the emotional demands of treatment.

Whose Interest Does the Model Serve?

IVF research has primarily focused on improving success rates and refining stimulation protocols. Affordability and equitable access have received comparatively less attention. Private pharmaceutical incentives can help shape which fertility drugs and treatment protocols receive greater investment in research and development. Clinical trials and regulatory approval tend to focus on measurable outcomes such as live birth, hormone levels, and implantation rates. As a result, changes that improve treatment success are more likely to be prioritized than changes that mainly make treatment more affordable (Kyle). Patent protection and market exclusivity play a major role in directing private-sector pharmaceutical research. These systems tend to reward innovations that can generate financial returns, which may place less emphasis on improvements designed primarily to expand access (Selker and Pardes 267).

The strongest counterpoint to this argument comes from mainstream health economics, where patent protection functions less as an extraction mechanism than as the standard justification for how biopharmaceutical innovation gets funded at all. Developing a new drug or protocol can require hundreds of millions of dollars and more than a decade of research, testing, and regulatory review. Once a pharmaceutical product has been developed, however, it may be relatively inexpensive for competitors to reproduce. Market exclusivity gives companies a period in which they can recover the costs and risks associated with development. From this perspective, reducing patent protection could lower the financial incentive for pharmaceutical research rather than simply redirecting innovation toward more affordable treatments (Grabowski et al. 1).

Patent-funded research has clearly contributed to advances in IVF. The issue is whether the incentives created by this model place greater value on innovations that improve clinical outcomes and generate revenue than on those intended primarily to make treatment more affordable and accessible. History offers a case that tests this distinction directly. When Frederick Banting, Charles Best, and James Collip discovered insulin in 1921, they deliberately declined to profit from the patent, selling the rights to the University of Toronto for one dollar each specifically so the drug could remain affordable to everyone who needed it (Diem et al. 4). That equitable-access model did not survive contact with a commercialized pharmaceutical market: a century later, insulin, produced for a few dollars a vial, is sold in the United States at prices that have pushed patients to ration a drug they will die without (Fralick and Kesselheim 1). The insulin case does not challenge the need to fund and recover the costs of medical research. Instead, it shows that recovering development costs and keeping treatments affordable are separate issues. A system may successfully fund innovation without ensuring that the resulting treatment remains accessible to the patients who need it. Innovation happens; whether it happens in a form most patients can afford is left largely to chance. A treatment developed within a system shaped by financial incentives should not automatically be considered the best approach. Its effectiveness should be evaluated alongside the potential influence of those incentives on how the treatment is developed, priced, and made accessible.

Toward a More Equitable Model

If cost is the primary barrier standing between most patients and a working treatment, it is worth naming specific changes that would actually address it rather than gesturing at the injustice of the price tag.

Insurance coverage for fertility treatment should be expanded and standardized, as current policies leave many patients without adequate coverage. The state mandates described above vary enough that many exclude single patients or LGBTQ+ couples, or cap coverage at a dollar amount a single complicated cycle can exceed (MultiState; The Ethics Committee of the American Society for Reproductive Medicine). Broader fertility coverage can increase access to treatment. Denmark's public health system funds multiple IVF cycles for eligible patients, with coverage expanded in 2024. Roughly one in eight births in Denmark now involve assisted reproduction, illustrating how publicly funded treatment can support much greater use than a predominantly self-pay system (Adamson et al. 10). The Danish model is not without limitations, since eligibility can still depend on factors such as age and other requirements. However, it demonstrates that access to fertility treatment can be shaped substantially by healthcare policy. A federal minimum standard, based on the more comprehensive state mandates already in place, could reduce differences in fertility coverage across the United States.

Lower-cost pharmacological protocols deserve to become a default offering rather than a niche option patients must seek out themselves. Minimal-stimulation IVF costs far less per cycle and, per guidance from the American Society for Reproductive Medicine, is already recommended as a first approach for patients classified as poor responders. Providing patients

with an estimate of cumulative treatment costs could make these choices clearer. Rather than seeing only the price of a single cycle, patients would be able to compare the cost of a lower-dose approach with a more expensive protocol that may provide a greater number of eggs. For appropriate patients, non-pharmacological interventions should be considered before pharmacological treatment. For anovulatory patients with PCOS, a structured lifestyle program can be less expensive than hormonal treatment and may restore ovulation without medication. The Dutch trial discussed earlier also shows that this approach has limitations, particularly for patients who require faster treatment, so it is better considered as an additional option rather than a replacement for IVF in every case.

None of these changes requires abandoning IVF or denying its biological effectiveness. It does require greater attention to the way pharmaceutical research is funded and prioritized. Research could continue to support advances in treatment success while also placing greater emphasis on approaches that make effective treatment accessible to more patients. That was the model Banting, Best, and Collip chose for insulin, before the market reshaped it.

Conclusion

IVF should not be judged solely by whether it can produce a pregnancy. A complete evaluation should also consider whether patients can realistically access IVF and the psychological effects associated with both undergoing treatment and being unable to afford it. IVF is biologically effective, but its high cost and limited accessibility prevent many people from benefiting from that effectiveness. At the same time, patients who do undergo IVF may experience significant emotional strain throughout treatment. For these reasons, pharmacological treatment should not automatically be considered the best approach to infertility. Both pharmacological and non-pharmacological treatments should therefore be assessed using factors such as biological effectiveness, accessibility, affordability, and psychological impact. Ultimately, a treatment cannot be considered fully effective if many of the patients who need it are unable to access it.

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The Effects of Music and Dog Therapy on Stress and Burnout in Nurses
By Marcus Aou

Abstract

Nursing continues to be one of the most emotionally and physically burdening professions, and the resulting toll on staff wellbeing has ripple effects that impact far beyond the individual nurse i.e., influencing patient outcomes, workforce retention, and the overall functioning of healthcare systems. Music therapy and dog-assisted therapy (both nonpharmacological approaches), have each gathered support as ways to reduce occupational strain, yet the research supporting both interventions rarely isolates nurses from the wider healthcare workforce, giving rise to open questions about how well each approach serves the specific population of nurses. This review brings together the available evidence on both interventions as they relate to nurse-specific stress and burnout. The literature points to dog-assisted therapy producing physiological calming effects (e.g., lower cortisol, eased anxiety, improved mood) after both short and repeated encounters with therapy animals, while music-based approaches, through passive listening or active engagement, corresponds with drops in stress hormones, heart rate, and emotional exhaustion. Practical application also seems plausible for both approaches, given their relatively low cost and infrastructure needs compared to medication-based options, although what each requires to run day-to-day differs. Because burnout is the outcome of a variety of workplace conditions such as personal circumstances, and systemic demands, this review does not promote music or dog-assisted therapy as standalone fixes; rather, it clarifies how each intervention may correlate with reduced stress and burnout in nurses, could be considered by healthcare organizations as part of a broader workplace wellness efforts.

1. Introduction

Stress and burnout are common issues across many industries and professions, and one of the leading causes of mental health which affects millions of people throughout the world (Raittila et al.). Prominently in today's world, burnout is classified as a state of physical and psychological stress due to high loads and stressors from work (Ardani et al.). Heavy workloads, long working hours, intense working environment, shortage of staffing and a poor work-life balance all can contribute to stress and eventually burnout. The effects of workplace-related stress extend beyond the workplace and can negatively influence many aspects of everyday life, including physical health, family relationships, and social interactions (Ratnaningsih and Idris). These negative impacts raise concerns due to their impact on both employees and organizations.

Stress and burnout are especially prevalent in the healthcare system as constant high-pressure environments promote higher levels of burnout. Its impact on workers and patients can be seen in past studies which show that higher levels of burnout in nurses correlate to medical errors, decreased patient care, and higher rates of patient mortality (Woo et al.). This shows how burnout among nurses is of great concern because it may compromise patient safety.

A study in 2018 showed that 31.5% of nurses reported leaving because of burnout (Shah et al.). As staff shortages are becoming an increasing problem in the healthcare services, it is important that a solution be found. Recognizing the causes and consequences of workplace-related stress and implementing effective strategies to manage stress is essential in reducing burnout, improving employee health and well-being, keeping steady employment levels and providing an overall better patient's care. The existing literature highlights the effects that both music therapy and dog-assisted therapy have as effective nonpharmacological interventions for reducing stress and improving psychological well-being. Most studies only focused on the general healthcare workforce and, as a result, do not show the true effectiveness both of these interventions have on nurses specifically. This gap is important because nursing is a job characterized by demanding workloads, emotional exhaustion, high pressure environments and long shifts, all of which contribute to increased levels of stress and burnout. Burnout among nurses has been associated with decreased job satisfaction, increased turnover, poorer mental health, and reduced quality of patient care. Although previous research supports the benefits of music therapy and dog-assisted therapy individually, it is still unclear the impacts dog and music therapy have on nurses. A direct comparison of these interventions would expand the current, limited body of knowledge by clarifying how music and dog-assisted therapy each affect stress and burnout specifically in nurses, rather than the healthcare workforce as a whole. This information could help healthcare organizations make better decisions when developing workplace wellness interventions and better implement safe, effective, evidence-based stress management programs.

This literature review will add to existing knowledge by explaining the effects of both music therapy and dog therapy on nurses' stress and burnout. By combining all previous research from these nonpharmacological interventions, this review will give a better understanding of how these two therapies may support nurses' psychological well being. The findings from this review may provide concrete, scientifically backed ways to incorporate music and dog therapy into healthcare settings as stress management tools and potentially guide future research studies to identify gaps in current data.

2.1 Stress and Burnout in the Workplace

Stress is the psychological or physiological response to internal or external situations (American Psychological Association) and can occur when workplace demands become difficult to manage. While short-term stress can be manageable for many, prolonged workplace stress can contribute to burnout. As a result of chronic workplace stress, burnout is characterized by emotional exhaustion, increased disinterest or negativity toward work, and a diminished sense of professional accomplishment (World Health Organization). This illustrates the relationship between stress and burnout, as exposure to stressful working conditions that can result in physical and emotional exhaustion, leading to burnout. This is particularly concerning in healthcare, where professionals often experience heavy workloads, staff shortages, long hours, and high levels of responsibility (Sipos et al.). When experienced over a long period of time, these challenging conditions can increase workplace stress and may contribute to the

development of burnout. Understanding this relationship is important because reducing the factors that cause chronic workplace stress may also help lower the likelihood of burnout.

2.2 Dog-Assisted Therapy as an Effective Therapeutic Intervention for Workplace Stress

Dog-assisted therapy, a type of animal-assisted therapy (AAT), involves interaction with a trained therapy dog to improve individual's psychological or physiological well-being. As organizations look for non-pharmaceutical ways to support staff well being, the incorporation of dog therapy programs in healthcare settings has grown substantially in recent years (Acquadro Maran et al.; Beetz et al.). Researchers believe that the stress-reducing effects of human-dog interaction are driven by the activation of the oxytocinergic system, which regulates bonding, social behavior, and the physiological stress response (Beetz et al.). A systematic review of the psychophysiological literature similarly found that human-dog interaction is consistently associated with increased oxytocin, increased heart rate variability, and decreased cortisol, indicating activation of the parasympathetic nervous system (Teo et al.). This mechanism provides scientifically proven physiological reasons for why even brief interactions with a therapy dog may be correlated with reductions in stress among individuals: such as nurses working in high-pressure environments.

Several studies have applied this specifically to nurses and have found physiological and psychological evidence of stress reduction. When researchers measured nurses' salivary cortisol levels during three different work conditions—a normal work period without a break, a regular break without animal-assisted therapy (AAT), and a break that included AAT—they found a significant reduction in cortisol during the AAT condition was particularly significant among working nurses (Machová et al.). Rather than simply taking a break, findings suggest that the presence of the therapy dog may have contributed to the physiological reduction in stress observed among these nurses. A correlational study was similarly conducted to determine the effect of pet-assisted therapy on nurse work-related anxiety during shifts, using the Beck Anxiety Inventory to assess nurses before and after a 10 minute interaction with a therapy dog. The intervention demonstrated a significant reduction in anxiety scores, and a negative correlation between the therapy dog interaction and levels of anxiety. This finding indicates that nurses who spent more time with the dogs tended to show reduced levels of anxiety (Coto et al.). These physiological results are also supported by qualitative studies: in a study of nurses who scheduled weekly visits with a therapy dog during their shifts, participants said that it felt forced but still valued breaks as they claimed that it helped them reset emotionally and return to patient care with a clearer mind (Esposito Kubanick and Scharfman). These studies highlight the meaningful impacts on stress that dog-assisted therapy can have on nurses even if the interaction are only short periods of time during a demanding work shift.

Research on sustained dog-assisted programs indicates that repeated exposure may not only impact momentary stress but also affect broader indicators of burnout. A 5-minute interaction with a therapy dog has also been associated with reduced stress by the end of a shift among emergency medicine providers, including nurses (Kline et al.). In a study conducted at

three high-acuity oncology units between November 2020 and July 2021, a 20-minute interaction with a full-time facility dog was found to produce statistically significant improvements across all measures of a validated mood. Additionally, calmness scores nearly doubled ($M = 6.2$ to $M = 11.0$, $p < .001$), while tension scores dropped by more than half ($M = 5.1$ to $M = 1.9$, $p < .001$) (Craig et al.). Participants also reported reductions in physical symptoms of stress including elevated heart rate and muscle tension, even though the staff had an 80% baseline burnout rate. Together, these findings highlight the benefits of dog-assisted therapy and its potential for impact beyond the immediate interaction.

2.3 Music Therapy as an Effective Therapeutic Intervention for Workplace Stress

Music therapy involves structured listening or active musical participation to influence physiological arousal and emotional state. Researchers propose two reasons behind its effects: activation of the mesolimbic reward pathway, associated with dopamine and pleasure, and parasympathetic activation, which slows heart rate and lowers cortisol levels (De Witte et al.). Across 104 randomized controlled trials involving 9,617 participants, de Witte and his team found that music had its strongest effect on heart rate ($d = .456$), supporting the psychological pathway.

In another study, 54 first-line nurses were randomly assigned to alternating music and chair-rest conditions, with 30-minute sessions of self-selected soothing music (Lai and Li). Compared to chair rest alone, the music condition resulted in significantly lower perceived stress, cortisol, heart rate, and mean arterial pressure, and higher finger temperature. The extent of stress reduction was also correlated to how much participants liked the music. Therefore, the intervention's success may be less reliant on music itself and more on whether nurses get to choose what they listen to (Lai and Li). (Matthew et al.) tested the effectiveness of personalized music listening on emotional exhaustion and burnout among nurses. The study involved 42 nurses and randomly split them into two equal groups: 21 nurses received the music intervention, and 21 formed a control group which received no intervention. The participants listened to music of their own choosing for 30 minutes per session, three times per week, over five weeks, while the control group received no music intervention. Moreover, the nurses in the music group started the study with more emotional exhaustion and greater depression than the control group at baseline; however, over the course of the experiment, the music group showed significantly less emotional exhaustion than the control group. Because emotional exhaustion is one of the three core dimensions of burnout on the Maslach Burnout Inventory, this study can clearly show the correlation between burnout and music therapy.

2.4 Implementation of Nonpharmacological Interventions in Healthcare Settings

(Etingen et al.) ran an animal-assisted support program at a multidisciplinary VA healthcare clinic that included a concrete implementation model. The program included 20, one-hour sessions over three months, with dog handlers, signs placed in high-visibility areas of the clinic, advance email reminders before each dog visit, and a staff escort from the hospital

lobby to the session location. About 51% of clinic employees (n = 39) participated in each session with an attending average of 9 of the 20 sessions offered. Mood ratings which were collected immediately before and after each session rose from an average of 2.9 to 4.5 on a 5-point scale ($p < .001$). Employees also reported a drop in patient-related burnout, from an average score of 40.0 before the program to 18.0 after ($p = .002$).

Music-based interventions were implemented differently in workplace environments than Dog Assisted Therapy. Different studies implemented Music based interventions differently, such as (Matthew et al.) who ran a personalized music intervention that had no dedicated facility, handler, or scheduled group session: nurses listened to self-selected music for 30 minutes, three times per week, over five weeks, using their own device and playlist. (Ozgundondu and Gok Metin) however, used a more involved approach by relying on a trained facilitator to lead sessions and combining progressive muscle relaxation with music twice per week over eight weeks. Both different types of implementation of this methodology required none of the specialized equipment, animal handling, or facility coordination that the AAI programs relied on.

Despite these differences in methods, both interventions do not require costly equipment, pharmaceutical resources, or specialized medical staff making them both realistic options for resource-limited healthcare settings. Animal-assisted and music-based interventions have also been characterized in articles as low-barrier options specifically because they can be adapted to fit existing routines rather than requiring a structural change in schedule. Across multiple studies, AAI programs were consistently rated as feasible specifically because they could be carried out in normal clinic operations without disrupting patient care (Acquadro Maran et al.). Music-based interventions show a similar adaptability. Overall, music represents a low-cost, feasible way to address stress among nurses, requiring no ongoing purchase of equipment or consumables beyond the initial session setup (Ploukou and Panagopoulou). This suggests that the usage of both interventions may also rely on how easily they can be integrated into the day-to-day realities of nursing work.

2.5 Limitation of current research

Nevertheless, current research on burnout and music therapy as a nonpharmaceutical stress-prevention strategy has its limitations. A limited number of studies combined with their small sample sizes make existing findings that show music therapy can help reduce stress, anxiety and emotional exhaustion, difficult to generalize. Comparing results across studies is also difficult because of variation in the type of music used, the duration of therapy, and the frequency of sessions. In much of the healthcare-focused literature, nurses are grouped together with other job sectors or other healthcare professionals, making it difficult to isolate the specific impact of music and dog therapy on nurses alone. Additionally, many studies only focus on reducing stress after it has already developed, rather than determining whether music therapy can be used to prevent burnout from developing in the first place. Studies with longer durations and larger, more targeted nurse populations may be needed to fully determine whether or not music

therapy can serve as an effective and sustainable strategy for preventing stress that lead to burnout.

2.6 Need for Integrated Approaches to Nurse Wellness (one solution doesn't fix all)

Since burnout is not caused by one issue, no single therapeutic intervention is able to completely address nurse burnout on its own. Factors such as work conditions, personal struggles and family circumstances help shape burnout alongside other contributors, showing that burnout tends to build up from several issues at once rather than a single factor (Galanis et al.). These findings have direct implications for how music and dog-assisted therapy should be implemented within broader wellness strategies. If burnout is caused by a multitude of different reasons, then a therapeutic intervention that only targets a person's short-term stress is only treating a small puzzle piece of a larger picture of burnout. Instead, a more integrated approach might be more beneficial by combining these interventions with structural changes such as adjustments to staffing ratios, scheduling, or workload distribution with specific therapy programs that target multiple specific problems that drive burnout .

3 Conclusion

Burnout among nurses still remains a pressing concern, not only for the wellbeing of individual nurses but for the safety and quality of patient care. This review's purpose is to address a specific gap in the literature: while music therapy and dog-assisted therapy have each been studied as nonpharmacological stress interventions, most existing research treats healthcare workers as a group of professions rather than examining specific jobs that make up the group (e.g., nurse profession). By drawing together the evidence available specifically on nurses, this review found that both interventions may be linked to meaningful reductions in both physical and psychological signs of stress and lower levels of emotional exhaustion. While music therapy may provide a more flexible, lower-infrastructure intervention, dog-assisted therapy through studies has shown strong physiological evidence. Neither intervention, however, addresses all the factors that drive burnout- ensuing from a combination of workload, staffing, and personal factors that no single therapeutic tool can prevent on its own. Rather than comparing music therapy and dog assisted therapy, this review suggests that their main impact may lie in how they can be incorporated with structural changes in the healthcare department. Still, the evidence that supports both interventions has its shortcomings. For example, the current studies have limited sample size, inconsistent designs, and do not track results long enough to determine whether or not short-term stress relief can be a legitimate intervention against burnout over time. Addressing these gaps through larger, more nurse-specific, and longer-duration research may allow healthcare organizations progress from only treating music and dog-assisted therapy as simply notional, and towards implementing them as practical components of a sustainable nurse wellness programs.

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The Effect of Morning Caffeine Consumption on Reaction Time Throughout the Day
By Alexander Shu

Abstract

Caffeine is the most widely used drug in the world and is taken every day by more than 80% of the global population in order to increase alertness and lessen fatigue. This study examined whether drinking one can of Red Bull (250 ml) in the morning was linked to changes in reaction time over the course of the day. Reaction time was assessed using the Human Benchmark reaction-time test at six different times each day: at 8:00 a.m., 11:00 a.m., 2:00 p.m., 5:00 p.m., 8:00 p.m., and 11:00 p.m. The measurements were obtained on four consecutive days when no caffeine was consumed (from August 24 to 27, 2026) and on four consecutive days when one can of Red Bull was drunk in the morning (from August 28 to 31, 2026). Altogether, 48 reaction time measurements were made over the 8-day period, the mean reaction time in the no-caffeine situation being 217.25 milliseconds as compared with 210.17 milliseconds in the caffeine condition. Thus, the caffeine condition resulted in an average reaction time that was 7.08 milliseconds quicker than in the no-caffeine condition, corresponding to a decrease in reaction time of about 3.3%. The largest difference was observed at 11:00 a.m., when the mean reaction time in the caffeine condition was 25.25 milliseconds lower. It should be noted, however, that the caffeine condition was a little slower at 2:00 p.m. These results indicate a possible association between consuming caffeine in the morning and having a faster reaction time, even though the small sample size, the fact that the study involved only one participant, the sequential nature of the experimental conditions, and the presence of uncontrolled variables mean that conclusions regarding causation cannot be drawn.

Introduction

Reaction time refers to the time it takes for a person to respond to a stimulus, and it is a significant part of human performance since the ability to quickly react to visual or auditory information is relevant to a wide variety of activities such as sports and gaming, as well as driving and other everyday tasks. Variation in reaction time can be caused by factors including alertness, fatigue, attention, sleep, and the consumption of stimulants.

Caffeine is a stimulant that can be found in a number of drinks commonly consumed by people. It is usual for people to take in caffeine in the morning since they believe it helps to boost their alertness and energy. One source of caffeine is energy drinks, which are often advertised as products capable of enhancing energy and concentration.

It is especially interesting to consider the possible connection between caffeine and reaction time since caffeine might have different effects on alertness at various times of the day. A measurement obtained a short time after having consumed caffeine will yield a different result from one taken many hours later. Hence, it is better to measure reaction time several times during the day than to do so only once.

The study looked into whether drinking one can of Red Bull in the morning was associated with changes in reaction time over the course of the day. Reaction time was measured six times each day by means of the Human Benchmark reaction-time test, and the results from four days on which no caffeine was consumed were compared with those from four days when one can of Red Bull was taken in the morning.

The research question was:

Does consuming a single can of Red Bull in the morning relate to faster reaction times throughout the day compared with days without any caffeine intake?

The hypothesis was that reaction times would be faster after consuming caffeine, with the greatest improvement occurring during the late morning after the morning caffeine consumption.

Methods

Participant

The experiment was carried out by one person (myself) who was 15 years old, and the participant stated that he usually slept about eight hours each night during the experiment.

The fact that the study consisted of only one participant means that the results refer to that person only and cannot and ought not to be applied to a wider population.

Experimental Design

The experiment employed a within-subjects design with reaction time being measured under two conditions: one with no caffeine and one with caffeine.

The caffeine-free period lasted for four consecutive days, from August 24 to August 27, 2026, and during this time caffeine was not consumed.

The caffeine trial lasted for four consecutive days, from August 28 to August 31, 2026, and on each of those days the participant drank one can of Red Bull in the morning.

The reaction time was recorded six times each day, at about 8:00 a.m., 11:00 a.m., 2:00 p.m., 5:00 p.m., 8:00 p.m., and 11:00 p.m.

For the caffeine-free condition there were 24 measurements and for the caffeine condition there were 24 measurements, making a total of 48 reaction-time observations.

Reaction-Time Measurement

The reaction time was measured by means of the **Human Benchmark Reaction Time Test**, an online test which is designed to assess how quickly a user responds to a visual stimulus.

The reaction time was measured in milliseconds (ms) and a smaller value indicated a faster response. The same testing platform was used during the entire experiment in order to ensure consistency in the method of measurement.

Caffeine Condition

In the caffeine condition the participant drank a can of Red Bull in the morning, while in the no-caffeine condition no caffeine was consumed.

Instead of comparing the effects of different amounts or types of caffeine, the experiment looked at the reaction-time measurements obtained under the two conditions.

Data Analysis

The mean reaction time was calculated separately for both the no-caffeine and caffeine conditions, and means were also determined for each of the six measurement times in order to see whether the difference between the conditions changed throughout the day.

In the case of the no-caffeine condition the average reaction time was 217.25 ms, and in the caffeine condition it was 210.17 ms.

The mean reaction time in the case of the caffeine condition was lower than in the other case by 7.08 ms and therefore was faster.

Results

Overall Results

48 reaction-time measurements were obtained. Twenty-four of these were recorded in the no-caffeine condition and twenty-four in the caffeine condition.

Condition	Number of Measurements	Mean Reaction Time
No caffeine	24	217.25 ms
Caffeine	24	210.17 ms

The mean reaction time during the caffeine condition was **7.08 ms faster** than during the no-caffeine condition. This represents an approximately **3.3% decrease** in average reaction time.
Results by Time of Day

The difference between the caffeine and no-caffeine conditions varied throughout the day.

Time	No Caffeine	Caffeine	Difference
8:00 a.m.	217.75 ms	204.50 ms	−13.25 ms
11:00 a.m.	222.25 ms	197.00 ms	−25.25 ms
2:00 p.m.	214.00 ms	217.25 ms	+3.25 ms
5:00 p.m.	228.00 ms	223.50 ms	−4.50 ms
8:00 p.m.	216.75 ms	215.50 ms	−1.25 ms
11:00 p.m.	204.75 ms	203.25 ms	−1.50 ms

The largest difference occurred at **11:00 a.m.** During the caffeine condition, the mean reaction time was 197.00 ms, compared with 222.25 ms during the no-caffeine condition. The caffeine condition was therefore 25.25 ms faster at this time.

At 8:00 a.m., the caffeine condition was also faster, with a difference of 13.25 ms. Smaller differences were observed at 5:00 p.m., 8:00 p.m., and 11:00 p.m.

At 2:00 p.m., the pattern was reversed. The caffeine condition had a mean reaction time of 217.25 ms compared with 214.00 ms during the no-caffeine condition, making the caffeine condition 3.25 ms slower.

Discussion

The findings offer initial evidence that drinking caffeine in the morning might be linked to quicker reaction times; in the 48 cases examined, the average reaction time fell from 217.25 ms in the no-caffeine situation to 210.17 ms in the caffeine situation.

The biggest difference was observed at 11:00 a.m., the caffeine-condition mean being 25.25 ms quicker than the appropriate no-caffeine mean. This finding is in agreement with the hypothesis that caffeine consumed in the morning might be linked to better reaction-time performance in the late morning.

The results didn't indicate an improvement at all time points. At 2 p.m. reaction time was a bit slower in the caffeine condition and in the evening the differences between the two conditions remained relatively small.

The data therefore show that caffeine does not always lead to faster reaction times over the whole day; rather, the results indicate that any possible link between caffeine and reaction time might depend on the time at which performance is assessed.

There are a number of reasons which could account for the differences in the results. Even though the participant stated that they usually got about eight hours' sleep each night, the length of their sleep and the quality of their sleep were not separately recorded for each day of measurement. It is therefore possible that variations in their sleep had an effect on their reaction time.

A further important limitation concerns the order in which the experiment was carried out. The four days on which caffeine was not taken came before the four days on which caffeine was taken. Since the participant carried out the same reaction-time test over and over again, practice or becoming familiar with the test could have affected their performance. It is possible that the participant became more familiar with the Human Benchmark test as time went on, and this might have caused some of the improvement seen during the second four-day period to occur regardless of caffeine intake.

The study included only a single participant and there were eight experimental days in all. Since there are large individual differences in reaction time, it cannot be assumed that the results will apply to other people. Evidence that is more reliable could only be obtained in a larger study with multiple participants.

The Human Benchmark test could also cause variation. Reaction-time tests may be influenced by attention, anticipation, distractions, the performance of the device, the internet or browser conditions, and other environmental factors. Even if the same platform is used throughout the experiment, this does not remove these sources of variation.

It does not follow from the study that caffeine was the cause of the improvement observed. The results show only an association between the caffeine condition and a smaller average reaction time in this experiment. To find out whether caffeine actually causes the effect observed, it would be necessary to carry out a randomized experiment with a larger number of participants and with more controlled conditions.

Conclusion

The study looked into whether drinking one can of Red Bull in the morning was linked to changes in reaction time during the day, and reaction time was measured six times a day by means of the Human Benchmark reaction-time test on four days when no caffeine was taken and on four days when caffeine was taken.

In the case of the no-caffeine condition the overall mean reaction time was 217.25 ms and in the caffeine condition it was 210.17 ms; reaction time was therefore on average about 7.08 ms, or 3.3 per cent, faster in the caffeine condition.

The biggest difference was observed at 11:00 a.m., when the caffeine condition was 25.25 ms quicker than the no-caffeine condition. It was, however, a bit slower at 2:00 p.m. and the differences in the evening were fairly small.

In fact, the results offer initial evidence suggesting that drinking caffeine in the morning might be linked to a slight enhancement of reaction time, especially in the late morning. Yet, since the study was based on a single participant, had a small number of observations, involved sequential conditions, and could not control for various factors, it is not possible for the findings to show a causal effect.

Further research might build on this experiment by taking a larger number of participants, extending the number of experimental days, alternating or randomly assigning the caffeine and no-caffeine conditions, and recording various factors such as the exact length of sleep, the quality of sleep, the level of physical activity, and other possible influences on reaction time; such modifications would allow it to be more reliably established whether it is caffeine itself that causes the changes in reaction-time performance.

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Exploring the functions of C8ORF37 By Chester Wang

Abstract

C8ORF37(or officially named CFAP418) is a 207-amino-acid protein whose disruption is linked to autosomal recessive retinitis pigmentosa, and Bardet-Biedl syndrome. Yet its function is still not fully uncovered. This study uses publicly available genomic and protein data sets –including AlphaFold, Human Protein Atlas, and BioGrid— to create a hypothetical function. Three interacting proteins with C8ORF37 is studied for further support of the hypothesis: KATNB1, a gene that regulates the microtubules and essential for cilia development; HSPA8, a molecular chaperone implicated in protein quality control; and ATG16L1, a microtubule regulator that supports the photoreceptors. Evidence of these proteins combined with the main expression areas of C8ORF37 in the brain and the cilia supports the hypothesis that C8ORF37 helps keep the photoreceptors stable, as well as making sure the outer segments of cilia remain safe with homeostasis. Because these conclusions rely on computational predictions rather than actual lab experiments, further studies would be required in order for the hypothesis to become viable.

Introduction

C8ORF37, officially named CFAP418 (cilia and flagella associated protein 418), has a length of 207 amino acids. The C8ORF37 protein plays an important role in the human physiology and when disrupted, it has been linked to various diseases such as autosomal recessive retinitis pigmentosa (RP) or even Autosomal Recessive cone-rod dystrophy (CRD); one disruption in the C8ORF37 protein can cause progressive blindness, and in some patients, a full on syndromic disease. The protein is expressed throughout our body in different tissues; however, it is enriched in places such as the cilia, the brain and the heart. To this day, scientists are still debating amongst each other how this protein functions at the molecular level—some sources argue that it is an important aspect in melanosome transport, other sources argue that it plays a major role in acting as a stabilizer for cone and rod cells.

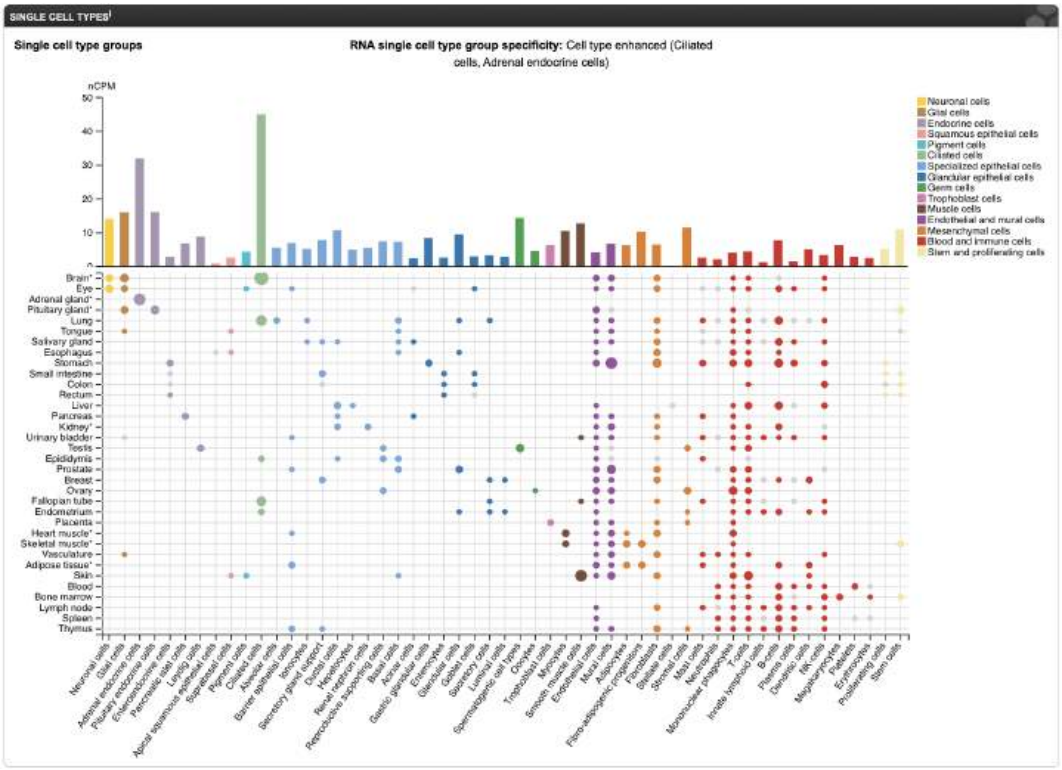


fig 1. Expressions of C8ORF37 in the Human Body (Human Protein Atlas)

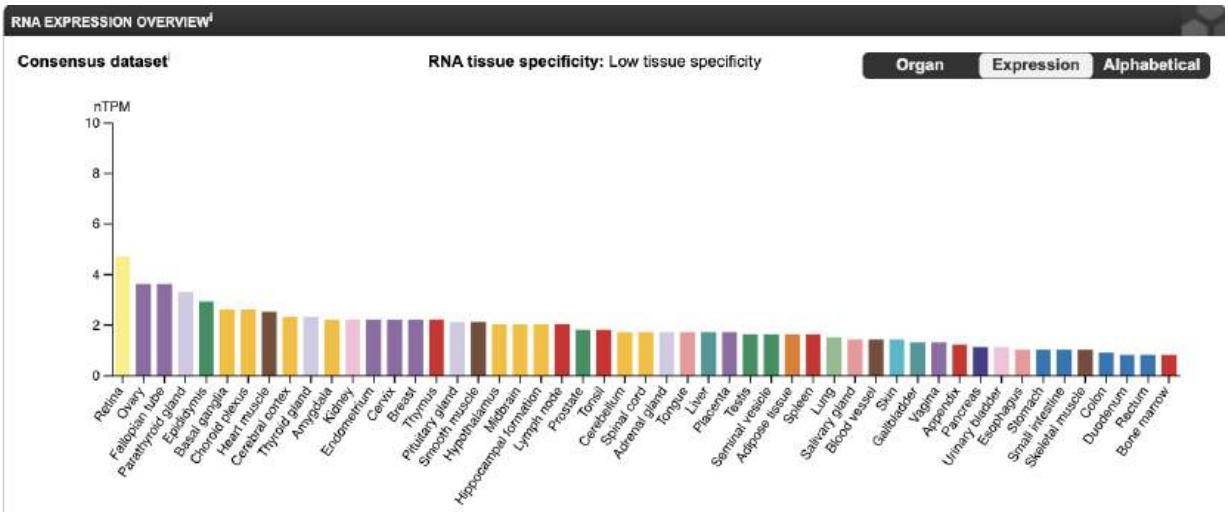


fig 2. C8ORF37 tissue specificity in the Human Body (Human Protein Atlas)

Methods

To investigate the potential functions of C8ORF37, publicly accessible online genomic databases can be used to analyze its structure, function and related protein. Alphafold was used to predict its protein structure based on the available data, the Human Protein Atlas shows us the main activation areas and the group specificity of C8ORF37. BioGrid was used to analyze

protein interactions and related proteins specifically to C8ORF37. Three proteins were selected for further investigation because of their strong correlation with C8ORF37 along with the human retina and cilia. After investigating the relative protein, public studies based on the different proteins were used to strengthen the potential function of C8ORF37.

Structural and Functional Predictions

Despite its small size, C8ORF37 has been a protein that has been tough to classify as. Alphafold modeling predicts that C8ORF37 is a compact protein structure, the current prediction of the protein C8ORF37 has the majority of the protein at a very high confidence in Alphafold. Although we can see that Alphafold has large confidence in the protein structure, one source alone is not viable as the answer to all. Other sources such as Pfam have noted this as an absence in notable function. However, with the help of TheBioGrid, we can find a variety of protein interactions with C8ORF37; BioGrid gave us a large amount of proteins to work with, among them I picked three proteins that could help us find a solution.

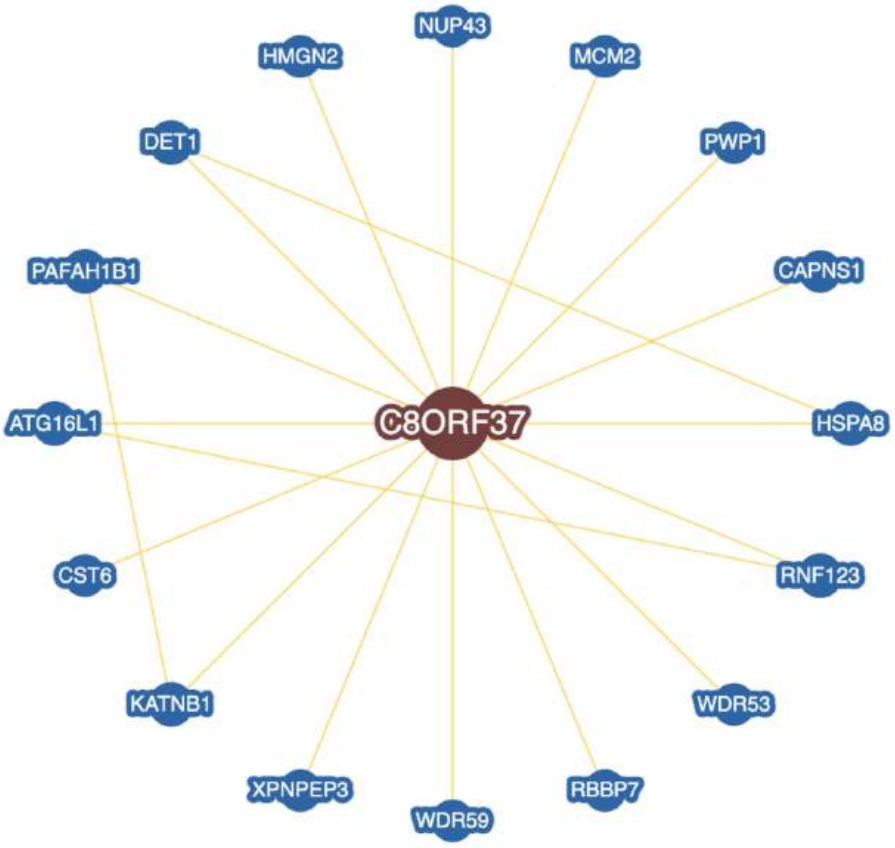


Fig 3. Known Interactions with C8ORF37 (BioGrid)

Among the proteins that interact with C8ORF37, I looked for proteins that have strong correlations and direct interactions with C8ORF37, I picked: KATNB1, a protein known for acting as a microtubule-severing protein, important for cilia and photoreceptor structure; HSPA8, a protein acting as a chaperone involved in protein quality control and retinal stress responses and at last ATG16L1, a Autophagy regulator required for photoreceptor survival. Currently, with the sources we have, scientists are highly confident that C8ORF37 is likely serving a function in defects and melanosome transport responsible for rod and cone photoreceptors.

Hypothesis

Given the resources we have, I propose that C8ORF37 is required for homeostasis of outer-segment membrane proteins at the photoreceptor connecting cilium; its loss disrupts disc morphogenesis and causes retinal degeneration; C8ORF37 is a structural stabilizer for photoreceptors and plays a big role in development of photoreceptors "quality control" modulator in photoreceptors.

Protein Interaction Analysis: KATNB1

The first protein I analyzed was KATNB1; KATNB1 is a gene that regulates the microtubule by severing the enzyme Katanin. In an experiment done by Hu Et al. The scientists are experimenting with KATNB1 through a process named "Knocking Out". Essentially, knock out is a process that works firstly by picking out a specific gene that the scientists want to target, secondly, the scientists use gene editing tools such as CRISPR-CAS9 to introduce a change in the DNA that prevents the corresponding gene product from functioning properly, after that, the scientists graft the edited cells on the organism. The modified cells are allowed to divide, or the edited embryo develops into an organism. Lastly, the scientists compared the control with the knock-out. In this case, the gene KATNB1 is knocked out in an experimental mouse for the experiment. In the research, not only did scientists find out that Katanin (p60 + p80/KATNB1) actively cuts microtubules, which is essential for reorganizing the microtubule network in neurons, the scientists also discovered various phenotypes from the knocked out mice, more notably: severe defects in brain development, dramatically reduced cortical progenitor cells, almost complete loss of cortical neurons and embryonic lethality. It is confident to say that disrupting Katanin leads to negative growth of axon. Working with C8ORF37 may be used for photoreceptors and cilia when it comes to microtubule remodeling.

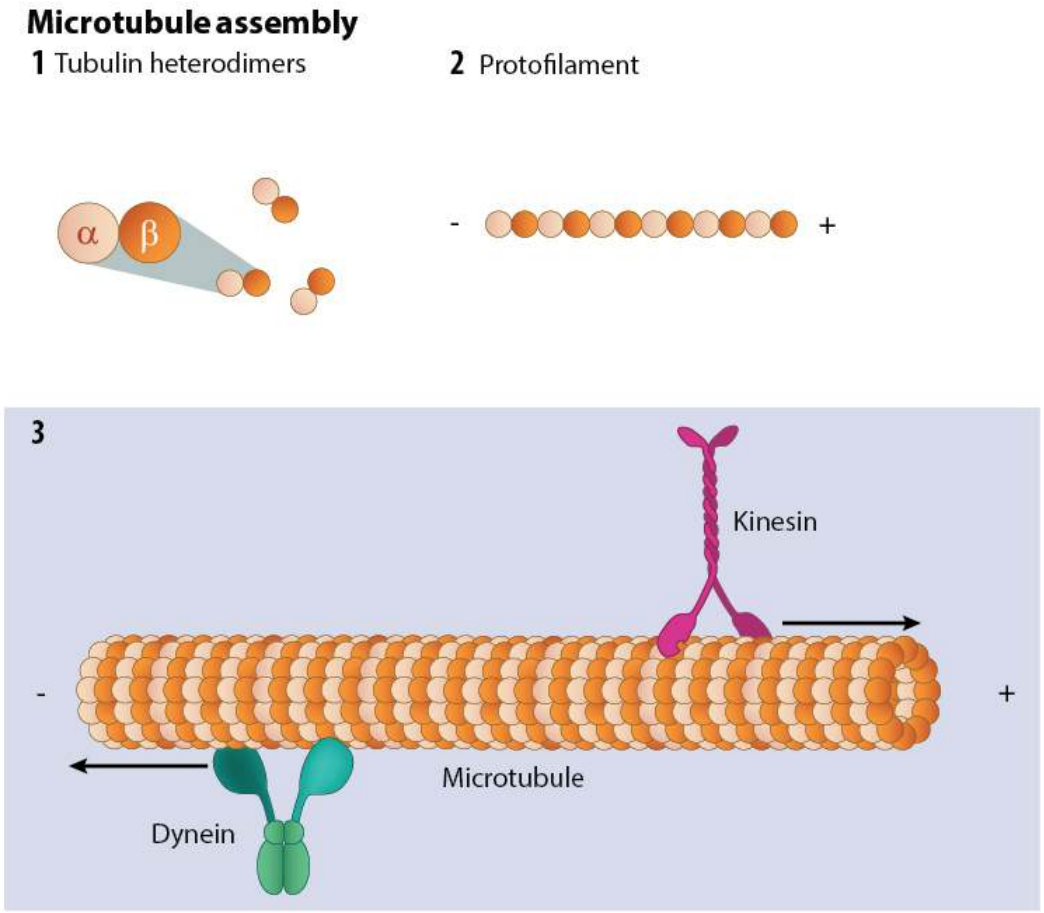


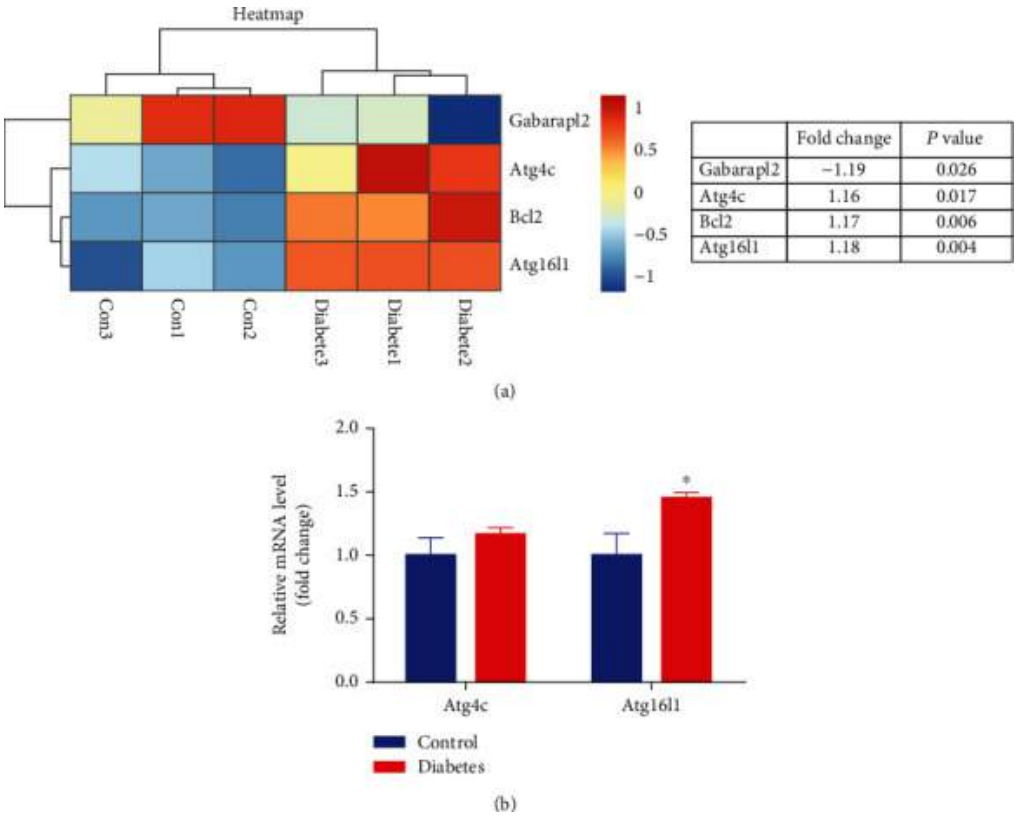
Fig 4. Model of Microtubules in the Human Body (Mechanobiology Institute, National University of Singapore)

Protein Interaction Analysis: HSPA8

The second protein I analyzed was HSPA8. HSPA8 is a gene that acts as a chaperone that is involved in protein quality control and retinal stress responses. A research done by Seese Et Al., describes how HSPA8 works with MAB21L2, MAB21L2 is known to be responsible for several eye diseases, such as Microphthalmia or Aniridia-like defects. The researchers approached the questions through different complementary ways such as utilizing Zebrafish embryos to test whether the interacting proteins are actually required for normal eye development or even cell culture experiments to verify these interactions. The main finding of the research was that HSPA8 directly binds to MAB21L2, the interaction suggests that HSPA8 may stabilize MAB21L2, makes sure that MAB21L2 folds correctly and works together to maintain eye development to become successful. HSPA8 may relate to C8ORF37 by acting as a "quality control" when it comes to eye development. Both of the genes would help out and complement each other's functions.

Protein Interaction Analysis: ATG16L1

The third protein I analyzed was ATG16L1. ATG16L1 is an autophagy regulator required for photoreceptor survival. An autophagy gene can be defined as an agent that helps cells remove damaged proteins. ATG16L1is also highly expressed in the photoreceptors, similar to C8ORF37. In the research done by Gao Et Al. ATG16L1 is proposed as a biomarker, potentially helping the human body identify or monitor retinopathy. In a diabetic cell, the orders usually go from: High Glucose → Cell Stress → Autophagy changes → Retinal Damage. During the process, researchers found that ATG16L1 expression was associated with diabetic retinal disease. Since the photoreceptor cells are constantly renewed in order to repair photo-oxidative damage and maintain vision, with ATG16L1 replacements are possible from autophagy. In a case without ATG16L1, however, outer segments of the photoreceptors would break down completely, leading to degeneration of the photoreceptors. Interactions with C8ORF37 may imply that ATG16L1 enables autophagy repairs, regulating mutations of C8ORF37.



	Fold change	*P* value
Gabarapl2	−1.19	0.026
Atg4c	1.16	0.017
Bcl2	1.17	0.006
Atg16l1	1.18	0.004

Fig 5. Representation of ATG16L1 in Diabetic Retinopathy(Gao, X Et.Al)

Conclusion/Discussion

Based on the sources available, C8ORF37 likely plays a role in photoreceptor maintenance. Evidence drawn from disease-causing mutations in C8ORF37 suggest the protein is essential for photoreceptor survival factor. C8ORF37 may also contribute to cilia's structural support, transport, and structural maintenance. Overall, C8ORF37 likely functions in ciliary

transport, outer segment stability, or proteostasis which are all important to photoreceptor survival.

Limitations

This study has several limitations. First, the proposed function of C8ORF37 is based on online databases, protein-protein interactions, and previously published studies on the related protein rather than actual hands-on experiment in a lab. Although Alphafold shows us a predicted structure, the structure alone does not fully explain the potential protein function. Additionally, the interactions shown through BioGrid doesn't necessarily show us if the direct protein interactions are guaranteed. The roles of KATNB1, HSPA8, and ATG16L1 in C8ORF37 interactions are based on previously published studies meaning that the function still remains unconfirmed.

Future Steps

The future research of C8ORF37 should focus on the precise cellular location and how its location can further impact its function or how large of a contribution it is to the human eye. Further studies are needed to evaluate whether C8ORF37 plays a role in protein homeostasis (proteostasis) and cellular maintenance pathways. Identifying C8ORF37's interaction partners would reveal the role that C8ORF37 plays in retinal development and degeneration. Ultimately, combining these findings would allow researchers to build a better and a more complete model of C8ORF37

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ZNF12 in Glioblastoma: Current Evidence and a Multi-Omics Framework for Investigation By Rishabh Jaiganesh

Abstract

Glioblastoma (GBM) is an aggressive brain tumor characterized by high genetic, transcriptional and cellular heterogeneity. Heterogeneity of the disease makes finding conserved regulatory mechanisms behind tumor biology and prognosis challenging. Zinc finger protein 12 (ZNF12), also known as ZNF325, is a transcriptional regulator which was not extensively studied in the scope of glioblastoma. Earlier studies have shown that ZNF325 inhibits transcription via AP-1 and serum response elements and some public cancer data repositories refer to the expression of ZNF12 in glioblastoma and even its potential prognostic value. However, the above observations do not mean any causal participation of ZNF12 in GBM. The current review gives an account of the available information on ZNF12 and develops hypothesis-driven multi-omic strategy for investigating the possible role of ZNF12 in glioblastoma. The strategy will use gene-expression, genomic, clinical, pathway, protein-protein interaction and single-cell data from publicly available databases, including TCGA and Chinese Glioma Genome Atlas. The key points include independent verification, time-to-event analysis, multiple testing adjustment and careful interpretation of correlations. Reproducible interactions between ZNF12 and molecular pathways will give reasons for conducting functional experiments, whereas insignificant or uncertain correlations will point out to the irrelevance of ZNF12 studies.

1. Introduction

Among the most aggressive primary brain tumors, glioblastoma belongs to the group of tumors of the adult central nervous system. According to the 2021 World Health Organization classification, adult-type glioblastoma is a subtype of IDH-wildtype diffuse astrocytic glioma with histologic or molecular characteristics (Louis et al.). The challenges of the disease do not lie in the rapid growth of the tumor. GBM cells migrate into the surrounding brain tissue, and the molecular variability of tumors is high, not only between different patients but also within individual tumors.

It has been shown on the large-scale genetic study that glioblastoma is not a molecularly homogeneous disease. Recurrent mutations in genes involved in receptor tyrosine kinase signaling, PI3K pathway, p53 pathway, and RB-dependent cell cycle regulation were found in The Cancer Genome Atlas studies (Brennan et al.). In earlier molecular classifications, expression-based subtypes with changes in PDGFRA, EGFR, and NF1 genes have been identified (Verhaak et al.). However, given the changing diagnostic criteria, recent analysis should align historical cases with current WHO classifications in order to draw a conclusion about modern glioblastoma.

The studies at the single cell or single nucleus level bring another layer of complexity. Within individual tumors, there are multiple transcriptional states that are malignant as well as

non-malignant microenvironmental cells comprising the immune system, blood vessels, and stroma (Patel et al.; Neftel et al.). Hence, a transcript level observed within the bulk tumor may represent either a particular transcriptional state that is malignant or a non-malignant microenvironmental population or combination of both. The complexity is even more critical in assessing the transcriptional regulator like ZNF12 which is not well characterized.

2. ZNF12: Molecular Background

ZNF12 is a protein coding gene found on chromosome 7. Alternative names for this gene include ZNF325, KOX3, HZF11, and GIOT-3 according to the National Center for Biotechnology Information. This gene also belongs to the family of C2H2-type zinc finger proteins (National Center for Biotechnology Information). While this information suggests that this gene has transcriptional regulatory function, it does not indicate the manner in which ZNF12 affects glioblastoma.

One important experimental investigation of the protein named ZNF325, conducted by Yulian Zhao et al., revealed that over-expression of ZNF325 inhibited AP-1 and SRE-mediated transcription, confirming that ZNF325 is a transcriptional repressor (Y. Zhao et al.). It is essential to note that this investigation was not performed on glioblastoma models; thus, one can use the results of the experiment only to confirm the biochemical capacity of the protein. Since a transcriptional regulator may have various effects on cellular mechanisms depending on multiple conditions (type of cell, state of chromatin, protein partners, and signaling environment), there is biological plausibility for further investigation of ZNF12.

3. Current Evidence Linking ZNF12 to Glioblastoma

Public cancer resources offer early evidence that ZNF12 is significant enough to study in GBM. In the Human Protein Atlas, there is RNA expression data on ZNF12 in glioma samples, and ZNF12 expression is currently considered high in glioblastomas (Human Protein Atlas). This finding could be useful, yet it should not be considered as evidence that ZNF12 is a driver in cancer progression or serves as a predictor of patient prognosis.

Survival data from a portal may be influenced by many factors, such as differences in cohort composition, diagnoses, treatment, and the way patients have been grouped depending on their expression levels. Best expression cutoff in the Human Protein Atlas survival tool was chosen based on maximizing survival separation, thus making replication even more valuable (Human Protein Atlas). A better way to test ZNF12 expression is to use it as a predictor in Cox proportional hazards model and check if its effect remains significant after controlling for other covariates.

These sources lack experimental data demonstrating the effects of perturbation of ZNF12 on the behavior, treatment response, invasiveness, and survival of glioblastomas. Thus, the available evidence is only enough to suggest ZNF12 as a potential target for further study, rather than a validated driver of GBM. In essence, the gap is the following: does ZNF12 have a reproducible link to clinical/biological features of GBMs?

4. Research Question and Hypothesis

Research question:

Is the expression of ZNF12 reproducibly correlated with molecular characteristics, cell states, biological processes, and clinical endpoints associated with glioblastoma?

Research hypothesis:

ZNF12 expression is correlated with specific transcriptional signatures of GBM, and some of these correlations are likely to be consistent across independent datasets. Should ZNF12 have any value for prognosis, it is likely to show this correlation even when analyzed as a continuous variable.

Null hypothesis:

These associations will turn out to be relatively small, dependent on the dataset being used or otherwise accounted for by other biological/clinical factors.

5. A Multi-Omics Framework for Investigation

5.1 Cohort Selection and Diagnostic Harmonization

The first step of the analysis may use a well-annotated cohort of glioblastomas from The Cancer Genome Atlas, followed by validation of results using an external source, like the Chinese Glioma Genome Atlas. CGGA includes genomic, transcriptomic, and clinical data about a large number of glioma patients from China, and is thus helpful in replicating the expression, survival, and correlation data (Z. Zhao et al.). Since the older data sources might include tumors classified using earlier nomenclature, the samples must be re-annotated or filtered to make sure that the analysis cohort represents the modern IDH-wildtype glioblastoma.

While choosing datasets, one must record the type of samples, whether they are primary or recurrent, the sequencing platform used, the normalization technique, and the availability of clinical data. Datasets must usually be analyzed separately rather than combined since technical differences between studies can lead to batch effects that are similar to biological differences.

5.2 ZNF12 Expression and Genomic Context

The first stage of the analysis will involve describing the pattern of ZNF12 expression in the GBM samples and relevant molecular classes. This would be done by considering the relationship between the expression levels and common genomic alterations in GBM, including pathway changes around EGFR, TP53, and other known GBM regulators. In analyzing data from tumor samples vs reference non-tumor samples in different studies, care needs to be taken when comparing the data since any large expression difference may be as a result of differences in protocols used in collecting and processing the samples.

Differential-expression methods, like DESeq2, that can calculate fold changes adjusting for dispersion in RNA-seq count data are available (Love et al.). Effect sizes need to be estimated along with the p-values, and thousands of gene tests require control of the false discovery rate and not significance tests.

5.3 Survival Analysis

Due to the implications that the availability of public data might carry, survival analysis would have to be considered a crucial aspect of the experiment, albeit needing to be well-controlled. While a Kaplan-Meier curve might serve as a graphic representation of the data, the statistical analysis must refrain from choosing a particular cutoff point just because it provides the lowest p-value. ZNF12 as a continuous variable may be easier to work with and less susceptible to subjective dichotomization.

The Cox proportional hazards model could be used to estimate the relationship between the expression of ZNF12 and overall survival, controlling for all the factors that the particular dataset allows. The covariates would be specific for each database used, but a failure to replicate the results in the CGGA database, as well as the disappearance of the results after adjustment, would make the ZNF12 a much weaker independent factor.

5.4 Co-Expression and Pathway Analysis

Correlation between ZNF12 and other genes in expression can be used to find transcriptional programs linked to the gene. Rather than relying on individual correlation, the study will determine whether groups of such correlated genes have reproducible biological processes. The gene ontology and pathway analyses can help test the overrepresentation of biological processes such as cell cycle, DNA repair, signaling, transcription, metabolism, immune function, migration among others without having the preconceived idea about which pathway could be involved (Gene Ontology Consortium).

The best evidence in relation to pathway analysis is recurrence in an independent cohort. In case genes correlated with ZNF12 are found to enrich for a particular biological process in both TCGA and CGGA, this would be more meaningful than a pathway discovered in one particular cohort alone. Correction for multiple testing will still be required due to simultaneous comparison in pathway analysis (Benjamini and Hochberg).

5.5 Protein Associations and Single-Cell Resolution

Using tools such as STRING, one can look into the proteins that are experimentally validated, curated, computationally predicted, or in other ways related to ZNF12 (Szklarczyk et al.). Such edges in networks must not be automatically assumed to represent physical protein-protein interactions. Rather, information about the type and confidence of evidence should be provided to distinguish between experimentally validated relationships and predictions.

Single-cell/single-nuclei data sets can answer a question that bulk RNA sequencing data cannot: which cell populations contribute the most to the expression of ZNF12? Earlier GBM studies have found several malignant cellular states and various types of non-malignant cells to exist within GBM tumors (Patel et al.; Neftel et al.). A finding that ZNF12 is enriched in one of the malignant states would indicate an entirely different biological picture than being enriched in immune, endothelial, or other microenvironmental cells. One should keep in mind that there

could be several cells from the same patient; each cell cannot be treated as an independent biological replicate since the patient is the sampling unit.

5.6 Reproducibility and Independent Validation

Independent reproducibility must be an essential characteristic of the framework. Important results obtained in one group need to be replicated in another group when such data are available. These results include expression profiles, survival correlations, highly co-expressed genes, enriched pathways, and cell-specific expression. The analysis pipeline, software versions used, filtering criteria applied, datasets used, and other relevant statistical parameters must be described in such a way that the process can be independently reproduced.

6. Interpreting Possible Outcomes

There can be several kinds of findings which can have interesting implications for science. First, the most important finding in this study will be a reproducible association between ZNF12 expression, a set of molecular alterations and clinical outcomes in independent studies. Although this will not prove the druggability of ZNF12, this will certainly justify the further investigation of ZNF12 in experimental models of GBM.

On the other hand, it is also possible that ZNF12 is not a driver gene per se, but a biomarker of a certain cell population or tumor phenotype. For example, the correlation between bulk tumor expression and survival rates may occur because of the overrepresentation of ZNF12 in a cell population associated with aggressive phenotype of tumors. In this case, single-cell analysis will help to distinguish markers of cell populations from drivers.

Lastly, correlations between ZNF12 expression and outcomes can prove to be not sufficiently strong. In this case, it will also be an interesting finding, since it will lower the priority of ZNF12 in future research in the laboratory. Thus, the main objective of this framework is not proving any hypothesis, but verification of reproducibility of signals.

7. Limitations

However, there are some major weaknesses in this approach. First of all, public databases are different regarding patient population, clinical annotation, sample preparation, sequencing technology, and data analysis pipeline. The fact is that bulk RNA-seq averages out the signal from many cells and single-cell RNA-seq has its own sampling and technical biases. In addition, the abundance of RNA does not imply the abundance or the activity of protein. Protein networks are often based on computational predictions and survival observations do not imply causality.

Given these weak points, it is crucial to reach convergence of independent lines of evidence. In the case of ZNF12, an explanation supported by multiple types of analyses (expression, clinical, pathway, protein, and cellular) is very strong. On the contrary, discrepancies should be mentioned explicitly.

8. Conclusion

ZNF12 is an appropriate target for investigation in glioblastomas, but its characteristics are not well understood. ZNF12 is a transcription factor, a previous study using its alternative name, ZNF325, has revealed the ability to repress transcription by AP-1 and SRE, and there is information about ZNF12 expression and potential role in glioma prognosis from public databases for gliomas (Y. Zhao et al.; Human Protein Atlas). However, none of these facts is able to prove that ZNF12 can be involved in glioblastoma progression.

Therefore, the next step is a thorough omics-based analysis of different datasets instead of any assumptions about the function of the gene. If ZNF12 demonstrates reproducibility of associations with survival, molecular signatures, genomic features, and specific cellular conditions, it will be a more appropriate candidate for further experiments on manipulation in GBM cells. In case the relationships are not confirmed in other datasets, the evidence will suggest that the gene cannot be prioritized.

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Gun-Law Strength, Firearm Ownership, and Firearm Death Rates Across The U.S.
A Correlation-Based Comparison of Ten States **By Yeojoon Kim**

Research Topic: Mathematics, Public Policy, and Firearm-Related Mortality

Abstract

In this study, I examined U.S. gun policy from a mathematical perspective rather than through a political lens. I created a 100-point index across five policy categories to measure gun-law strength, and I compared these scores against firearm death rates across ten states. To gain a fuller picture, I also compared state firearm death rates with household gun ownership levels and violent crime rates.

Using Pearson correlation coefficients, I found a moderate negative relationship between gun-law strength and firearm mortality rates ($r \approx -0.697$). In contrast, I observed a stronger positive correlation between household firearm ownership and firearm death rates ($r \approx 0.870$), along with a weaker positive link to violent crime ($r \approx 0.368$). I identified New Mexico as a significant outlier, as its data deviated noticeably from the overall policy trend.

Because my sample size was limited to ten states and my scoring system involved subjective criteria, I cannot draw direct causal conclusions from these findings. However, my analysis suggests that state-level firearm mortality is best understood by evaluating multiple intersecting factors rather than relying on a single policy score.

Keywords gun policy; firearm mortality; gun ownership; violent crime rates; Pearson correlation; public policy; state-level analysis

1. Introduction

There are many different opinions about gun issues in the United States. Some people argue that gun laws should be stronger, while others argue that making new laws may not have much effect on people who already use firearms illegally.

I thought numbers might make this issue a little clearer.

My first plan was to divide states into those with strong gun laws and those with weak gun laws, and then compare their actual firearm death rates. If states with stronger laws consistently had lower death rates, I thought I might be able to find a meaningful relationship. I chose California, Illinois, New York, Washington, and New Jersey as states with stronger gun regulations.

On the other side, I included Texas, Kentucky, New Mexico, Alaska, and Mississippi. Once I started collecting data, however, the two groups did not separate as neatly as I expected. New Mexico was especially interesting.

Using the gun-law strength system I created, New Mexico received a score of 83, which is fairly high. However, its firearm death rate was 26.6 deaths per 100,000 people. Texas, on the other hand, had a law-strength score of only 27, but its firearm death rate was 13.9.
This was the opposite of what I expected in that comparison.

At that point, it seemed that simply comparing whether a state's laws were strong or weak would not be enough.

Other factors could matter too, such as the percentage of households that own firearms, the overall violent crime rate, and how closely gun laws are actually followed.

In this research, I first turn the strength of each state's gun laws into a numerical score and compare that score with the firearm death rate. I then look at other variables, including household firearm ownership and violent crime.

If enough consistent data can be found, I also want to separate P, the strength of the law itself, from C, the level of actual compliance with that law.

The purpose of this research is not simply to decide which side of the gun debate is correct. Instead, I want to see whether a mathematical relationship appears between the strength of gun laws and firearm-related deaths, and, when a state does not fit the general pattern, to investigate why.

2. How Can Gun-Law Strength Be Turned into a Number?

The first problem was deciding how to represent the idea that a state has “strong” gun laws with a number.

One possibility would be to count the number of gun laws in each state.
But that would treat a small regulation as equal to a much broader policy such as a background-check requirement. I did not think that would be a good way to measure policy strength.

Instead, I divided gun policy into several major categories and gave each category a weight based on how important I thought it was.
The total possible score is 100 points.

The three categories I considered most important receive 25 points each.

The Big Three

Background Checks: 25 points

Child Access Prevention and Safe Storage: 25 points

Extreme Risk Intervention (Red Flag Laws): 25 points

Together, these three categories make up 75 points.

The remaining 25 points are divided into:

Restrictions on Specific Weapons: 10 points

Public Carry: 15 points

Therefore, the gun-law score P for a state can be written as

$P = B + S + E + H + C$□

Here, B represents Background Checks, S represents Safe Storage, E represents Extreme Risk Intervention, H represents Hardware Restrictions, and C□ represents Public Carry Regulation.

3. The Big Three

3.1 Background Checks

I gave 25 points to background checks.

The main reason I considered this policy important is that it affects the process of obtaining a firearm, rather than only regulating what a person does after already owning one.

In my first notes, I wrote a simple sentence:

“Background checks are the baseline.”

I still think this is the simplest way to explain why I gave this category so much weight.

California, Illinois, New York, Washington, and New Jersey had them, and so did New Mexico.

Texas, Kentucky, Alaska, and Mississippi didn’t.

Because this category is worth 25 points, the presence or absence of a background-check policy changes a state's overall score quite a lot.

An image of a gun store, with three columns of guns: 1st Column: Unknown AR-15 style rifle, SIG MCX SPEAR, Unknown AK-100 series carbine, 2 B&T APC 223 rifles, either a Saiga 9 imported from Russia, or a US made KP9, Unknown thing at the bottom left. 2nd Column, 3 varying sizes of the Q Mini FIX, 3 Unknown AR-15 style rifles, 2 SIG M400 SDI X rifles. 3rd

Column: 7 AAC/Q Honey Badger rifles/pistols (maybe an SBR?)

3.2 Child Access Prevention and Safe Storage

The second 25-point category is child access prevention and safe storage.

I saw this as a somewhat different issue from background checks.

Even when a firearm is legally owned, the way it is stored can determine whether another person can easily access it.

This may be especially important when considering access by children or actions that happen impulsively.

California, Illinois, New York, Washington, and New Jersey had mandatory laws concerning safe storage, Texas and New Mexico likewise.

Kentucky, Alaska, and Mississippi did not.

I was a little surprised that Texas, which I had generally thought of as a state with looser gun regulations, still had a policy in this category.

This was one reason I did not want to divide every state too simply into only “strict” or “loose.”

An example of a safely stored firearm, under a biometric lock, a Beretta M9 (of unknown variant), unloaded and kept safe:

3.3 Extreme Risk Intervention

The third category is Extreme Risk Intervention, often called Red Flag Laws.

I also assigned 25 points to this category.

A background check mainly operates when a firearm is being obtained, but a person's circumstances can change after the purchase.

For that reason, I thought there should be a separate category for policies that allow intervention when a person's level of risk becomes high.

California, Illinois, New York, Washington, New Jersey, and New Mexico had this type of policy.

Together, these three major categories account for 75 points.

This means that most of my scoring system focuses not on a particular type of firearm, but on who can obtain a firearm, how it is stored, and whether intervention is possible when a dangerous situation develops.

4. Hardware and Public Exposure

4.1 Hardware

I assigned 10 points to restrictions on specific types of weapons.

California, Illinois, New York, Washington, and New Jersey had additional restrictions involving assault weapons or large-capacity magazines.

Texas, Kentucky, New Mexico, Alaska, and Mississippi had the federal minimum.

At first, I wondered whether assault-weapon restrictions should receive a larger weight.

While researching, however, I became less certain that restrictions on a specific type of weapon should count more heavily than the other policies in a study of overall firearm deaths.

For that reason, I called this category Hardware and gave it 10 points.

The difference still matters in the model, but I did not make it the most important part of the 100-point score.

Examples of weapon accessories:

4.2 Public Exposure

The final category is public carry.

I assigned 15 points to this category.

I separated open carry from concealed carry and considered whether a permit is required and whether open carry is restricted or prohibited.

California requires a permit for concealed carry, and open carry was classified as prohibited in my data.

New York and New Jersey also had relatively strong restrictions.

Washington and New Mexico require a permit for concealed carry, but open carry is possible without a permit.

Texas, Kentucky, Alaska, and Mississippi were classified as allowing permitless open and concealed carry.

While looking at this category, I did not think public-carry regulation was exactly the same type of policy as a background check.

It may affect firearm exposure and possible escalation in public places, but it does not by itself stop the illegal acquisition of a firearm.

That is why I gave it 15 points instead of 25.

Examples of carrying a weapon. Left image: Conceal Carry with an unknown pistol (perhaps a SIG P226?), right image: Open Carry with a HS2000/Springfield Armory XD (of unknown variant).

5. Gun-Law Strength and Firearm Death Rate

I used each state's gun-law score as the x-value and firearm deaths per 100,000 people as the y-value and made a scatter plot.

California was (99, 7.0), Illinois was (99, 12.4), New York was (98.5, 4.4), Washington was (94, 11.0), and New Jersey was (98.5, 4.0).

Texas was (27, 13.9), Kentucky was (2, 18.5), New Mexico was (83, 26.6), Alaska was (2, 24.4), and Mississippi was (2, 28.0).

Using these ten points, I calculated the Pearson correlation coefficient.

$r \approx -0.697$

The negative value means that, in this data set, higher gun-law scores generally go with lower firearm death rates. A value of -0.697 is not a weak relationship, but it is also far from a perfect linear relationship.

The scatter plot helps show why.

New York and New Jersey appear toward the lower-right part of the graph. Both have gun-law scores close to 100, while their firearm death rates are 4.4 and 4.0.

Alaska and Mississippi are closer to the upper-left. Both have scores of 2, while their death rates are 24.4 and 28.0.

Looking only at those states, the downward trend is fairly clear.

New Mexico, however, is far from that pattern.

Its gun-law score is 83, but its firearm death rate is 26.6, which is much higher than the rates of the other states with high policy scores.

I recalculated the correlation after excluding New Mexico.

$r \approx -0.883$

This is a much stronger negative correlation than -0.697 for all ten states.

The calculation shows that New Mexico is not only visually unusual on the graph. It also has a substantial effect on the correlation coefficient.

Rather than simply removing New Mexico to get a stronger correlation, I think it is more useful to ask why this state differs so much from the general pattern.

6. Household Firearm Ownership

Next, I used household firearm ownership as the x-variable and compared it with firearm death rate (mostly suicides).

California: 16%

Illinois: 23%

New York: 14%

Washington: 32%

New Jersey: 9%

Texas: 36%

Kentucky: 53%

New Mexico: 36%

Alaska: 57%

Mississippi: 54%

The Pearson correlation coefficient between these ownership percentages and firearm death rates was

$r \approx 0.870$

This relationship was stronger than I expected.

The correlation between gun-law score and firearm death rate was about -0.697, while the correlation between household firearm ownership and firearm death rate was about 0.870.

In these ten states, higher firearm ownership was therefore associated with a fairly strong increase in firearm death rate.

New Jersey had 9% household firearm ownership and a firearm death rate of 4.0.

New York had 14% ownership and a rate of 4.4.

At the other end, Alaska had 57% ownership and a death rate of 24.4. Mississippi had 54% ownership and a rate of 28.0.

A high correlation does not mean that firearm ownership directly causes the deaths. This calculation shows association, not causation.

Still, the result was different from what I expected when I began the research.

I expected the gun-law score to have the strongest relationship. In the actual calculations, the absolute value of the ownership correlation was larger.

$|0.870| > |-0.697|$

That difference seemed important enough to examine further.

7. Looking Again at New Mexico

I analyzed New Mexico as the primary outlier in my dataset when comparing gun-law strength against firearm death rates.

Although the state received a high gun-law score of 83, it recorded a firearm death rate of 26.6 per 100,000 people.

I found that household firearm ownership in New Mexico stood at 36%, which is noticeably lower than states like Alaska (57%) and Mississippi (54%), indicating that ownership rates alone do not account for its elevated mortality rate.

When I examined violent crime, however, I noted that New Mexico registered a rate of 717.1 per 100,000 people—ranking near Alaska (724.1) at the top of my sample.

This combination of strong policy scores, moderate ownership levels, and high violent crime rates makes New Mexico a critical case study, demonstrating that single variables are insufficient to explain firearm mortality patterns.

8. Relationship with Violent Crime Rate

I also calculated the Pearson correlation coefficient between violent crime rate and firearm death rate.

The result was

$r \approx 0.368$

The value is positive, but the relationship is much weaker than the other two.

Putting the three results together:

Gun-Law Strength vs. Firearm Death Rate: $r \approx -0.697$

Household Firearm Ownership vs. Firearm Death Rate: $r \approx 0.870$

Violent Crime Rate vs. Firearm Death Rate: $r \approx 0.368$

This comparison was one of the more interesting parts of the analysis.

It might seem reasonable to expect states with higher violent crime to automatically have much higher firearm death rates, but that relationship was not very strong in my data.

Mississippi is a good example.

Its violent crime rate was 210.5, which is relatively low among these states, but its firearm death rate was 28.0.

New York, by comparison, had a violent crime rate of 380.2 but a firearm death rate of only 4.4.

So violent crime by itself also does not explain the differences in firearm death rates across these states.

9. Comparing the Three Scatter Plots

The three scatter plots have noticeably different shapes.

In the Gun-Law Strength vs. Firearm Death Rate graph, the points generally move downward as the gun-law score increases. New Mexico, however, lies far from this trend because it combines a high gun-law score with a high death rate.

In the Household Firearm Ownership graph, the points are more clearly arranged from the lower-left toward the upper-right. Of the three relationships, this one looks the most linear.
The Violent Crime Rate graph is more scattered. The points do not form as clear a line as they do in the other two graphs.

Looking at the graphs, instead of only the correlation coefficients, also made it easier to see where the outliers were.
New Mexico was especially important in the first graph.

10. Why One Variable Is Not Enough

These results suggest that a simple model such as
$Y = f(P)$
is probably not enough.
Here, P is Gun-Law Strength and Y is Firearm Death Rate.
If O represents household firearm ownership and V represents violent crime rate, a broader model could be written as
$Y = a + bP + cO + dV$
I cannot say from this data alone that this equation is a correct causal model.
What I can say is that the three variables have different levels of correlation with firearm death rate.
In this data set, O had the strongest correlation with Y.
That was not the result I expected when I started the research.

11. Compliance

There is one more variable that I have not yet been able to calculate.
It is compliance, C.

An example of non compliance, rioting.

If P represents the strength of a law, C would represent how closely that law is actually followed.

So far, I have not found enough consistent data to measure C in the same way for all ten states, and I do not want to assign numbers without evidence.

Possible measures include private-party background-check use, safe-storage compliance, reporting of lost or stolen firearms, and the filing and execution of ERPOs.

Picture of the aftermath of an ATF raid in Miami, where they recovered scores of illegal firearms, the image is too blurry to discern most, with some that might be crudely improvised, and some legitimate creations by known companies.

If a reliable value for C can be created, the model could be expanded to

$Y = a + bP + cO + dV + eC$

For now, however, I think it is more reasonable to use only the variables P, O, and V for which I already have actual data.

12. Results So Far

Using the ten states, I calculated three correlation coefficients.

The strongest relationship appeared between household firearm ownership and firearm death rate.

$r \approx 0.870$

Gun-law strength and firearm death rate showed a fairly strong negative relationship.

$r \approx -0.697$

Violent crime rate and firearm death rate had the weakest relationship of the three.

$r \approx 0.368$

When New Mexico was excluded, the correlation between gun-law strength and firearm death rate became

$r \approx -0.883$

I think this is an important result.

New Mexico does not erase the overall trend, but it changes the strength of the relationship by a noticeable amount.

For that reason, simply removing the outlier to produce a better correlation would not be appropriate. It is more meaningful to investigate why New Mexico differs from the other states. Based on the results so far, gun-law strength and firearm death rate do show a negative relationship in this group of states.

But that relationship does not explain every state.

Household firearm ownership also showed a strong relationship, and New Mexico did not fit the simple pattern.

The data therefore points less toward the conclusion that only one variable matters and more toward the idea that several variables should be considered together.

The results were less simple than I expected when I first divided the states into a strong-law group and a weak-law group. That was actually useful, because it made me look at the states that did not fit the pattern instead of only focusing on the states that supported my first idea.

13. Limitations and What I Would Improve

There are several limitations in this research. The biggest one is the sample size. I used only ten states, so the correlations in this paper should not be treated as estimates for the entire United States. I selected states that gave me a range of gun-law scores, ownership levels, and firearm death rates, but a larger data set could produce different correlation coefficients.

A second limitation is the gun-law score itself. I created the 100-point system by deciding which policy categories seemed most important and then assigning weights to them. I tried to make the system consistent across all ten states, but another researcher could reasonably choose different categories or different weights. Because of this, the score P should be viewed as a model, not as an official measurement of gun-law strength.

Another problem is that the firearm death rate includes more than one type of death. Homicide, suicide, accidental deaths, and other firearm deaths may respond differently to policy and ownership. If I continue this research, I would separate firearm homicide and firearm suicide instead of combining them into one outcome.

The data also come from observational state-level comparisons. A correlation can show that two variables move together, but it cannot prove that one variable caused the other. Income, urbanization, poverty, age distribution, mental-health conditions, regional differences, policing, and neighboring-state laws could also affect the results.

Finally, I was interested in compliance, C, but I could not find one consistent measure that could be applied to all ten states. I think this is still an important direction for future research. A law that exists on paper may not have the same effect if compliance or enforcement is very different from one state to another.

For example, here are some states that don’t fit into the model.

Scenario A: Strict regulations, high amounts of violence

Case Study A:
Maryland
One of the strictest gun regulations, with licensing required for handguns, assault weapon bans, universal background checks and red flag laws
Homicide and gun violence rates consistently rank significantly higher than the national average, with Baltimore frequently recording homicide rates between 40-50 per 100k residents.
High amounts of concentrated poverty, historical segregation, active illicit drug markets and the smuggling of weapons through the Interstate I-95 highway's "Iron Pipeline" from nearby states (e.g. Virginia) may be responsible.

Case Study B:
Illinois (Chicago)

High state level policy requirements, Firearm Owner's Identification (FOID) cards, mandatory waiting periods and red flag laws, yet high total counts of firearm homicides and violent crime concentrated in specific urban neighborhoods, exceeding national urban averages from a micro level neighborhood disinvestment, high youth unemployment, and gang conflicts. Over 60% of illegal firearms confiscated in Chicago are traced back to point-of-sale origins in states with looser gun laws (Indiana, Wisconsin) or suburban gun dealers outside municipal jurisdiction.

Scenario B: Loose regulations, low amounts of violence
Case Study C:
New Hampshire and Vermont

Neighboring states and loose in gun control, both states have neither background checks or assault weapon bans, and have constitutional permitless carry for both open and concealed forms of having a firearm on their person. The two states consistently show some of the lowest violent crime and homicide rates, with homicide rates under 2.0 in 100k people. The high median household income, low poverty rates, high rates of secondary school graduation and low population density definitely have a hand in making these states one of the safest.

Case Study D:
Utah and Idaho

Also neighboring states, with permissive carry and stand your ground laws (self defense without the obligation to retreat first, basically allowing the victim to respond to an imminent threat using deadly force), and strong preemption laws against local municipal gun bans.

Violent crime rates remain well below the national average, despite high rates of household firearm ownership. Low urban density, relatively high income stability, and very low rates of gang violence and illicit trade are the primary factors that make these states safer despite loose firearm laws.

14. Conclusion

When I started this project, I expected the states with stronger gun laws to have clearly lower firearm death rates. The data partly supported that idea, but not as neatly as I expected. The correlation between my gun-law strength score and firearm death rate was about -0.697, which shows a meaningful negative relationship in these ten states. However, New Mexico did not fit the pattern well, and removing it changed the correlation to about -0.883.

The result that surprised me more was household firearm ownership. Its correlation with firearm death rate was about 0.870, which was stronger in absolute value than the gun-law correlation. Violent crime had a positive but much weaker correlation of about 0.368. Before doing the calculations, I thought violent crime might explain more of the differences between states, so this result made me reconsider my first assumptions.

I do not think these numbers show that gun laws do not matter, or that firearm ownership by itself explains firearm deaths. Instead, the main thing I learned is that one variable is probably not enough. A state can have strong laws and still have a high firearm death rate, and two states with similar policy scores can have very different outcomes.

If I expanded this study, I would include all fifty states, separate firearm homicide from firearm suicide, test different versions of my policy-scoring system, and use a multiple regression model instead of looking only at one correlation at a time. I would also like to find a reasonable way to measure compliance and enforcement.

The part I found most interesting was New Mexico. At first I saw it as a point that was making my correlation weaker. After looking at it more carefully, I realized that the unusual point was probably more useful than a perfect trend line. It showed me where my simple model was missing something. For me, that became the main lesson of the project: the purpose of the mathematics was not to force the states into one pattern, but to show where the pattern works and where I need to ask another question.

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Autism Spectrum Disorder Effect on Connectivity and Activity in the Social Brain
By Aahana Sachdeva

Abstract

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition that affects social communication and interaction. ASD alters brain activity and connectivity in social processing regions, specifically the amygdala, superior temporal gyrus, and medial prefrontal cortex. A literature review identified 10 relevant research papers. Across both resting-state and task-based fMRI studies, the included studies show consistent abnormalities in activation and connectivity related to social interactions and behavior. Results from included research papers suggest that ASD is associated with altered brain activation and connectivity, including reduced amygdala activation during emotional and social reward processing, decreased superior temporal gyrus activity during social interpretation tasks, and reduced medial prefrontal cortex activation and connectivity during theory-of-mind tasks. These alterations suggest that ASD involves differences in social interactions across the brain. In conclusion, these findings indicate that ASD involves differences in the brain regions responsible for emotions, social thinking, and understanding. These brain differences help explain the social communication challenges seen in individuals with ASD.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition that affects social communication and interaction (Patil et al.). Individuals with ASD often have trouble understanding facial expressions, interpreting social cues, recognizing emotions, and responding in social situations (Patil et al.). These struggles are typically noticed in early childhood, when social development, including language and social skills, falls short of expectations for the developmental age. ASD is currently diagnosed through behavioral assessments, but researchers are exploring neurological aspects of ASD to find measurable changes in the body and brain.

The ability to process social information and interactions is the result of several brain regions working together; these regions together are sometimes referred to as the social brain. The most important sections of the social brain are the amygdala, superior temporal gyrus, and medial prefrontal cortex (Patil et al.). The amygdala's main role is processing emotions, particularly fears, stress, and threats (Patil et al.). The superior temporal gyrus plays a role in interpreting social cues, such as body language and tone of voice (Chung and Son). The medial prefrontal cortex contributes to understanding other people's thoughts, emotions, and intentions, a process known as theory of mind (T. Chen et al.). Given ASD's impact on social behavior and development, these brain regions, which are important for social skills, are critical to assess to gain a better understanding of how ASD impacts the brain and behavior.

Functional magnetic resonance imaging (fMRI) is a neuroimaging technique with high spatial resolution. fMRI has significantly advanced our understanding of many neurological, psychological, and developmental conditions, including ASD. fMRI measures the brain's activity

and connectivity by detecting changes in blood oxygen levels, or BOLD signals. When a brain region becomes active, it requires more oxygen, and fMRI can detect these changes in oxygenation (Patil et al.; Liu et al.). Researchers have begun using this technology to compare brain activity, connectivity patterns, and changes in individuals with and without ASD (Liu et al.; Feng and Xu). Previous studies have used both resting-state and task-based fMRI to examine how brain regions communicate with one another and function independently (Liu et al.; Talesh Jafadideh and Mohammadzadeh Asl). Resting-state fMRI measures BOLD activity at rest and allows for examining neural connectivity, while task-based fMRI measures the BOLD response during task performance and allows for assessing brain function across different task types.

Given the role of ASD on social development and the amygdala, superior temporal gyrus, and medial prefrontal cortex's role in social skills, this literature review sought to answer the research question: how does ASD affect brain activity and connectivity in social processing regions of the brain, such as the amygdala, superior temporal gyrus, and medial prefrontal cortex? It is hypothesized that the literature will indicate that individuals with ASD display altered connectivity and reduced activation in certain regions of the brain compared to typically developing individuals. By combining findings from multiple fMRI studies, this review aims to explore brain differences that may underlie social communication characteristics in individuals with ASD.

Methods

A literature search was conducted to examine how ASD affects brain activity and connectivity in social processing regions - the amygdala, superior temporal gyrus, and medial prefrontal cortex. This review combines findings from previously published fMRI studies to identify consistent patterns in brain activation and connectivity within those regions.

The literature search was conducted on Google Scholar. Search terms included "Autism Spectrum Disorder fMRI studies," "ASD fMRI adult studies," "Autism Spectrum Disorder," and "ASD connectivity and activity."

Research studies were included in this review if they involved participants diagnosed with ASD and used fMRI to measure amygdala, superior temporal gyrus, and/or medial prefrontal cortex brain activity and connectivity. Research studies also needed to have assessed ASD participants with social processing tasks, such as facial recognition and social communication videos, to be included. Research papers that did not focus on the amygdala, superior temporal gyrus, and/or medial prefrontal cortex and/or did not provide information regarding activation and connectivity results were excluded. The inclusion and exclusion criteria and search terms ensured all selected research studies specifically examine how ASD affects brain activity and connectivity between the specified social brain regions.

Results

The literature search identified 237 papers for potential inclusion, which were reviewed for title and abstract. Following title and abstract screening, 42 studies were chosen for full-text review. Of these 42 papers, 9 met the inclusion criteria.

The included studies were conducted in many locations worldwide, including the United States of America and China. This geographic diversity increased the cross-cultural generalizability of the results. Some of the included studies also used larger international datasets comprising participants from several countries and institutions (Chung and Son). This allowed researchers to compare brain activity and connectivity across a wide range of individuals with ASD and typically developing brains.

Across all included papers, the participants' ages ranged from 7 to 40 years. Studies that focused on children with ASD sought to better understand how ASD affects brain development and social processing in the early stages of life, while studies that focused on adult participants examined how brain activity, connectivity, and social processing were different compared to earlier in life. Here, we looked at studies of both children and adults to provide a deeper understanding of how ASD affects the brain's social processing regions across different stages of life.

Furthermore, the number of participants differed across the studies (Add Range). Some neuroimaging research includes relatively small groups of participants because of the cost of running fMRI machinery and its complexity.

The results of the studies identified in this literature review suggest that differences in brain activity and connectivity in the amygdala, superior temporal gyrus, and medial prefrontal cortex were more likely related to ASD than to other behavioral and developmental factors (T. Chen et al.; Talesh Jafadideh and Mohammadzadeh Asl; Kim et al.; Delmonte et al.; K. Chen et al.).

Discussion

Amygdala

Multiple neuroimaging studies suggest that the amygdala plays a major role in the alteration of the social processing region of the brain in individuals with ASD. In one study, researchers found significantly lower amygdala activation in children with ASD when they viewed emotional facial expressions compared to typically developing children. The study included 17 children diagnosed with ASD and 24 typically developing children, between 7 and 17 years of age. The paradigm included showing participants images of happy, neutral, and fearful facial expressions while their brain activity was measured using an fMRI machine. Additionally, the fMRI showed decreased amygdala activity, specifically when the children viewed happy and fearful expressions (Kim et al.). While reduced amygdala activation may suggest that emotional facial expressions elicit weaker emotional responses in children with ASD, it is also possible that these differences reflect atypical neural pathways for emotion recognition rather than a lack of emotional response. It is important to consider that lower

activity may represent distinct cognitive strategies or compensatory mechanisms in individuals with ASD, rather than a straightforward deficit in emotional experience.

Additionally, other researchers have conducted studies of reward processing in the brains of individuals with ASD, which have also identified differences in amygdala activity. One study consisted of 24 participants with ASD and 14 without ASD. This study used fMRI to measure brain activity while participants with 24 and without ASD received social rewards, such as smiling faces and positive feedback (Delmonte et al.). The findings indicated that individuals with ASD showed reduced activation in reward-related brain regions during social reward tasks, including the amygdala. However, individuals with ASD response to monetary reward tasks were less affected compared to social reward tasks. This suggests that individuals with ASD may respond more strongly to non-social rewards and processing motivations than typically developing individuals. These findings reinforce that reduced sensitivity to social rewards may also contribute to social communication difficulties.

Superior Temporal Gyrus

Various research studies show that activity in the superior temporal gyrus is altered in individuals with ASD. One study assessed 24 individuals with ASD and 14 typically developing individuals between 18 and 40 years of age. Participants were asked to watch a short animated video featuring interacting shapes that conveyed social meaning in an fMRI scanner. The fMRI results showed differences in activity in this brain area , which may affect how social information is processed from the outset. Researchers found that individuals with ASD showed decreased activation in the superior temporal gyrus during theory-of-mind tasks that required interpreting others' thoughts or feelings (T. Chen et al.). The decreased activation in the superior temporal gyrus suggests that early social understanding may not be fully developed to support complex social thinking, including the interpretation of intentions, tone, and body language in individuals with ASD.

Additionally, researchers have conducted fMRI studies during rest to examine changes in the superior temporal gyrus in individuals with ASD. One study recruited 47 children with ASD and 40 typically developing children, aged 7 and 17. The resting-state scans showed altered connectivity patterns in the superior temporal gyrus, even when not performing tasks. This connectivity pattern suggests that differences in the superior temporal gyrus may exist at baseline, suggesting that the network might have a different structure before social information and interaction are introduced (Talesh Jafadideh and Mohammadzadeh Asl). These rest-state fMRI results reinforce the idea that differences in the social processing regions of the brain in individuals with ASD reflect differences in network organization and structure of these regions at rest.

Furthermore, another group of researchers performed fMRI on 17 children with ASD and 24 typically developing children, aged 7 to 17, who displayed altered functioning in the superior temporal gyrus. Participants in this study viewed emotional facial expressions during an fMRI scan. The fMRI results showed reduced activation in the superior temporal gyrus and amygdala

(Kim et al.). Results suggest that children with ASD exhibit reduced activity in the superior temporal gyrus during facial expression interpretation compared to typically developing children.

Medial Prefrontal Cortex

Numerous previous studies have identified differences in both activation and connectivity in the medial prefrontal cortex in individuals with ASD. In one study, 24 adults with diagnosed ASD and 14 typically developing adults were recruited. The participants were required to interpret what another person may feel in specific situations. The results show that adults with ASD exhibit reduced activation during theory-of-mind tasks (T. Chen et al.). The reduction in activity in this area suggests it may not engage as well during complex social interactions.

An additional research study examined the social reward in learning in children with ASD among a group of 17 children with ASD and 24 typically developing children. In the study, participants were asked to complete a task in which they received positive social feedback to reinforce the correct responses in the fMRI scanner. The results showed that children with ASD had reduced activation in the medial prefrontal cortex and reduced communication between the medial prefrontal cortex and the amygdala during these tasks compared to typically developing children (Talesh Jafadideh and Mohammadzadeh Asl). These findings suggest that the brain interprets and reacts to social processing reinforcement differently during development for children with ASD compared to typically developing children.

Further neuroimaging research showed weaker activation in the medial prefrontal cortex during social reward processing in individuals with ASD, in a study of 24 adolescents and young adults with ASD and 24 typically developing adolescents and young adults. The study involved participants completing tasks with social rewards, such as positive feedback and smiling faces. The results of the fMRI showed that individuals with ASD displayed reduced activation in the medial prefrontal cortex (Delmonte et al.). The reduced activation suggests that individuals with ASD may have difficulty not only in theory of mind tasks but also in social reward processing.

Clinical Implications

The results of this review suggest a pattern in ASD, particularly altered activation and connectivity in the amygdala, superior temporal gyrus, and medial prefrontal cortex. These findings have important clinical implications, as they support the use of neuroimaging to help clinicians better understand certain differences in brain function and to improve diagnostic accuracy, along with behavioral assessments. Additionally, identifying the brain regions involved in social processing suggests that interventions can be more effective and personalized to each individual, and clinicians can target areas such as emotion recognition, social cues, and social motivations through various therapies. The research displays the importance of earlier detection and intervention of ASD because the brain is more adaptable to change during childhood compared to adulthood, and fMRI scans can help clinicians identify ASD earlier. Ultimately, incorporating a neurological perspective into clinical practice may help clinicians develop more effective, targeted, and individualized interventions.

Limitations

Even with the paper's important results, several limitations should be considered when interpreting findings. For example, several neuroimaging studies involved smaller sample sizes due to the cost of fMRI scanner use, complex equipment, and advanced analysis. Smaller sample sizes can limit the extent to which the findings reflect the larger population of individuals with ASD. Additionally, some studies included participants across a wide age range, from children to adults. Since age can influence brain activation patterns due to developmental differences, it is important to conduct additional studies to determine whether findings persist across development or are specific to a particular developmental period. Furthermore, some studies also reported that participants with ASD were taking medication or receiving therapy. Medication and external therapies may confound interpretation, as they can influence brain activity and connectivity patterns, making treatment an important consideration.

Future Direction

Future research should further explore how the social processing brain regions develop over time in individuals with ASD. For example, future research should consider longitudinal study designs that follow participants with ASD from childhood to adulthood. This approach could enable researchers to better understand how brain activity and connectivity patterns change throughout development. Additionally, future work should focus on recruiting larger sample sizes, which may help strengthen the reliability of future discoveries. Researchers may also benefit from combining neuroimaging results with behavioral tests to better understand how brain activity relates to real-world social behaviors. Furthermore, advancements in machine learning and artificial intelligence may enable researchers to analyze larger databases to identify patterns associated with ASD. These future studies could improve our understanding of ASD and help develop more effective diagnostic interventions and equipment.

Conclusion

In conclusion, this literature review examines and explains how ASD affects brain processing of social information, particularly in the amygdala, superior temporal gyrus, and medial prefrontal cortex. The literature review highlights reductions in brain activity and connectivity within social processing regions, which help explain why individuals with ASD have difficulty interpreting facial expressions, emotions, and social cues (T. Chen et al.; Feng and Xu; Talesh Jafadideh and Mohammadzadeh Asl; Kim et al.; Delmonte et al.). Differences in the amygdala, the brain's emotion center, contribute to challenges in emotional recognition and reduced activation and connectivity. The superior temporal gyrus influences the understanding of speech, social cues, and others' intentions. Altered and reduced activity in the medial prefrontal cortex affects theory of mind, how we interpret others' feelings and intentions in social interactions. In sum, the weakened connectivity and activity between brain regions critical for social processing and skills suggests that ASD involves alterations in the social centers of the brain.

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Circadian Patterns of Pruritus and Sleep Disruption in Atopic Dermatitis: The Role of Photobiomodulation By Sahana Kallur

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by intense pruritus and significant sleep disruption, with symptoms frequently worsening during nighttime hours. Increasing evidence suggests that nocturnal pruritus is associated with circadian changes in skin barrier function and neuroimmune signaling, contributing to disrupted sleep and overall disease burden. Photobiomodulation (PBM) is a non-ultraviolet light-based therapeutic approach that uses wavelengths of visible light, including blue and red light, as well as near-infrared light, to modulate cellular activity. This review examines circadian patterns of pruritus and sleep disturbance in AD and evaluates clinical and mechanistic evidence relevant to the effects of PBM. Available clinical evidence indicates that PBM may reduce pruritus and patient-reported sleeplessness in individuals with AD, while experimental studies demonstrate reductions in scratching behavior and changes in inflammatory signaling. However, whether these effects extend specifically to nocturnal pruritus and associated sleep disruption remains unclear. Future studies incorporating objective sleep measures and time-specific symptom assessments are needed to determine whether PBM may have potential as a chronotherapeutic approach for nocturnal pruritus and sleep disruption in AD.

Introduction

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin disorders, affecting approximately 10% to 30% of children and 2% to 10% of adults worldwide(Kolb et al.). It is clinically characterized by recurrent eczematous lesions, skin barrier dysfunction, and a recurrent sensation of itchiness known as pruritus(Kolb et al.). Pruritus is one of the most burdensome symptoms of AD and frequently intensifies during evening and nighttime hours(Chamlin et al.).

Nocturnal pruritus is closely associated with sleep disturbance in individuals with AD. Poor sleep quality is reported in approximately 60% of children with AD, with nocturnal exacerbation of symptoms leading to impaired sleep, specifically in those with more severe disease(Arif et al.). Persistent sleep disruption can contribute to fragmented sleep, daytime fatigue, impaired cognitive functioning, and reduced quality of life(Mann et al.). Circadian rhythms regulate physiological processes involved in sleep and inflammatory signaling, and disruption of these rhythms may contribute to interactions among nighttime inflammation, sleep disturbance, and AD symptoms(Arif et al.). Cytokines implicated in AD associated pruritus include interleukin-31 (IL-31) and type 2 inflammatory mediators such as IL-4 and IL-13 which are immune signaling factors involved in chronic itch(S. Kim et al.; Oetjen et al.). Skin-barrier function, including transepidermal water loss (TEWL), also displays circadian variation that may elevate nighttime amplification of pruritus(Vaughn et al.).

Photobiomodulation (PBM), also referred to as low-level light therapy, is a

non-ultraviolet therapeutic approach that uses specific wavelengths of light to influence cellular and inflammatory signaling(Woo and Padalhin). PBM has been investigated in inflammatory dermatoses because of its potential to modulate oxidative stress and inflammatory pathways implicated in skin inflammation and pruritus(Woo and Padalhin). Preliminary clinical evidence suggests that PBM may reduce pruritus and overall symptom burden in patients with AD(Becker et al.).

Despite preliminary evidence that PBM may reduce pruritus and modulate inflammatory pathways in AD, its potential effects on the circadian pattern of AD symptoms remain insufficiently explored. Existing studies have primarily evaluated overall symptom severity and patient-reported outcomes, with limited assessment of time-specific itch patterns, objective sleep measures, or circadian biomarkers. Consequently, it remains unclear whether PBM simply reduces overall inflammatory burden or may also influence the biological processes underlying nocturnal symptom amplification.This gap is particularly relevant to the potential use of PBM as a chronotherapeutic intervention, as treatment timing may be an important consideration in addressing nighttime symptom aggravation.

This review aims to evaluate the evidence linking PBM with pruritus and sleep disruption in AD and to examine the inflammatory, neuroimmune, and circadian mechanisms that may underlie these effects. This review aims to evaluate the evidence linking PBM with pruritus and sleep disruption in AD and to examine the inflammatory, neuroimmune, and circadian mechanisms that may underlie these effects. By synthesizing clinical and mechanistic evidence, this review assesses the current evidence for PBM in the context of nocturnal AD symptoms.

Methods and Materials

A literature review was conducted using PubMed and Elicit to identify peer-reviewed studies related to PBM, AD, circadian rhythm, pruritus, and sleep disturbance. Search terms included “photobiomodulation,” “blue light therapy,” “atopic dermatitis,” “nocturnal itch,” “circadian rhythm in AD,” and “sleep disruption.” Experimental studies, clinical studies, and review articles published in or after 2000 were considered.

The initial search identified approximately 50 potentially relevant articles, which were screened based on their relevance to these objectives. Studies published before 2000, focused solely on ultraviolet phototherapy, or lacking clinical or mechanistic relevance to AD, PBM, circadian symptom variation, pruritus, or sleep disruption were excluded. Following screening, 20 articles were included in the final review.

Because direct clinical studies of PBM in AD were limited, animal studies using mouse models of AD and in vitro studies using human skin cells were also included when they examined mechanisms relevant to AD, such as scratching behavior, inflammatory signaling, mast cell activity, or epidermal changes. Studies examining PBM outside AD were included selectively when they provided evidence relevant to PBM mechanisms or treatment timing.These findings were considered supporting mechanistic evidence rather than direct evidence of efficacy in AD.

Circadian Patterns of Pruritus

One early study examined patient-reported evidence of nocturnal pruritus in 102 Chinese patients with AD. A structured questionnaire based on the McGill Pain Questionnaire was administered, with 100 participants completing the assessment. The questionnaire evaluated the frequency, intensity, sensory characteristics, and affective impact of pruritus. Itching occurred most frequently at night in 65% of participants, while 84% experienced difficulty falling asleep(Yosipovitch and Papoiu).

A case control study investigated whether nocturnal movement followed a temporal pattern in children aged 6–17 years with moderate-to-severe AD and age- and sex-matched healthy controls. The study enrolled 20 participants with AD and 20 controls, with moderate-to-severe disease defined by a SCORAD score greater than 25. Nocturnal movement and sleep data were available for 19 participants in each group. These outcomes were assessed over 3–7 nights using sleep diaries and actigraphy, a noninvasive method that uses a wearable motion sensor to record physical activity and estimate patterns of sleep and wakefulness. Activity counts were analyzed in 30-minute intervals and standardized relative to sleep onset. Children with AD had greater nocturnal activity than controls between 1 and 6 hours after sleep onset ($p<0.001$), with movement peaking approximately 3 hours after sleep onset. At this peak, the mean difference in activity counts between the AD and control groups was 1,488 (95% CI, 1,085–1,892). Disease severity was also positively correlated with wake after sleep onset (WASO) ($r=0.61$, $p<0.01$). In a small within-subject analysis of five participants who used antihistamines on some nights, total nocturnal movement did not differ between nights with and without antihistamine use ($p=0.82$)(Fishbein et al.).

A separate study objectively measured nocturnal scratching in 20 Japanese adults with AD and 10 healthy controls using a smartwatch-based accelerometer algorithm. Participants wore the smartwatch while sleeping at home for seven consecutive nights, and AD severity was assessed using the Eczema Area and Severity Index (EASI). Participants also rated itch severity and itch-related daytime and sleep disturbance using a 0–10 numerical scale. Median seven-day nocturnal scratching duration was greater in participants with AD than in healthy controls (151 vs. 7 seconds, 0.72% vs. 0.04% of total sleep duration, $p=0.000184$). Recorded scratching duration was also positively correlated with EASI scores ($r=0.60$, $p=0.00841$). In contrast, self-reported itch severity and itch-related daytime and sleep disturbance were not significantly correlated with either EASI scores or objectively recorded scratching duration (Ikoma et al.).

Neuroimmune and Physiological Mechanisms of Nocturnal Pruritus

A clinical study of 55 patients with AD and 38 healthy non-atopic controls examined the relationship between IL-31 levels, disease severity, and pruritus. Serum IL-31 concentrations were measured using enzyme-linked immunosorbent assay (ELISA), while AD severity was assessed using SCORAD and participants rated pruritus and sleep loss on a 0–10 numerical scale. Mean serum IL-31 concentrations were significantly higher in patients with AD than in healthy controls (43,142.8 ± 66,981.6 vs. 7,881.8 ± 1,842.7 pg/mL; $p<0.05$). Serum IL-31 levels

were also associated with greater disease severity (p=0.003) and subjective itch intensity (p=0.03), but were not significantly associated with reported sleep loss (p=0.13)(S. Kim et al.).

A mechanistic study examined whether the type 2 inflammatory cytokines IL-4 and IL-13 could directly influence sensory neurons involved in chronic itch. Analysis of mouse and human dorsal root ganglion (DRG) neurons identified expression of IL-4Rα and IL-13Rα1, receptor components involved in IL-4 and IL-13 signaling. Using calcium imaging, stimulation with IL-4 and IL-13 produced responses in 4.1% and 3.3% of mouse DRG neurons, respectively, with responses increasing in a dose-dependent manner. Similar experiments using sensory neurons obtained from human cadaveric donors showed that a subset of human neurons also responded directly to IL-4(Oetjen et al.).Further analysis showed that 89% of IL-4-responsive neurons and 75% of IL-13-responsive neurons also responded to established pruritogens such as IL-31, histamine, or both (Oetjen et al.). In an AD-like mouse model, selective deletion of IL-4Rα from sensory neurons significantly reduced scratching behavior, providing additional evidence for the involvement of neuronal IL-4Rα signaling in chronic itch (Oetjen et al.).

A 2024 pilot study investigated circadian changes in skin barrier function in four patients with AD and two age- and sex- matched healthy controls aged 15–25 years. Participants underwent a controlled inpatient protocol in which lighting, temperature, humidity, food intake, and sleep opportunity were standardized. TEWL, a measure of water loss through the epidermis used to assess skin barrier integrity, was measured hourly during the day and every 90 minutes overnight at lesional antecubital skin and generally non-lesional forearm skin. Whole blood samples were also collected every four hours for RNA sequencing. In healthy controls, TEWL decreased several hours before bedtime at both measurement sites, consistent with improved barrier function. In participants with AD, this pre-bedtime improvement was absent, and TEWL instead increased in nonlesional forearm skin. Circadian TEWL patterns were also less synchronized and showed flatter oscillations among participants with AD compared with controls. Whole-blood transcriptomic analysis identified 1,576 differentially expressed genes between the groups, with several neuronal pathways among the most upregulated pathways in AD. Sensory perception pathways were also linked with evening increases in lesional TEWL (Iwanaszko et al.).

Sleep Disturbance as a Core Component of Disease Burden

A pilot study evaluated the relationship between AD severity, scratching, and sleep quality in 20 adults aged 18–65 years with mild-to-moderate AD affecting at least 5% of total body surface area. Sleep efficiency and scratching were measured over two nights using polysomnography,which records physiological signals to characterize sleep stages and disruption, alongside actigraphy and participant self-reports. Levels of tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-10 were also measured in a subset of nine participants. Measurements obtained through actigraphy and polysomnography were strongly associated, while decreased sleep efficiency was associated with greater AD severity, increased scratching, and higher IL-6 levels. In contrast, self-reported sleep quality and quality of life were not

significantly correlated with either objective measure of sleep (Bender et al.).

A large cross-sectional study further evaluated the relationship between AD severity and sleep burden in 1,519 adults recruited from six academic medical centers in the United States. Participants completed an internet-based questionnaire and were categorized as having mild (n = 689) or moderate-to-severe AD (n = 830) using the Patient-Oriented Scoring Atopic Dermatitis (PO-SCORAD). Sleep was assessed using the PO-SCORAD sleep visual analog scale along with measures of nightly sleep duration, sleep latency, frequency of sleep disturbance, and interference with daily functioning. Participants with moderate-to-severe AD reported significantly greater sleep impairment than those with mild AD, including shorter nightly sleep duration (6.2 vs. 6.9 hours), longer sleep latency (38.8 vs. 21.6 minutes), and more nights with disturbed sleep during the previous week (2.6 vs. 0.4 nights; all $p < 0.001$). Sleep problems interfered "much" or "very much" with daily functioning in 24.3% of participants with moderate-to-severe AD compared with 6.8% of those with mild disease ($p < 0.001$)(Simpson et al.).

A longitudinal cohort study examined the association between AD and sleep outcomes in 13,988 children enrolled in the Avon Longitudinal Study of Parents and Children in the United Kingdom. Participants were followed with repeated assessments through 16 years of age, with 4,938 children meeting the study definition of AD between ages 2 and 16. Sleep duration was assessed at multiple time points using reported sleep and wake times, while sleep quality was evaluated using nighttime awakenings, early morning awakenings, difficulty falling asleep, and nightmares. Children with active AD had nearly 50% greater odds of experiencing more sleep-quality disturbances than children who never reported AD (aOR = 1.48; 95% CI, 1.33–1.66), with greater impairment among those with more severe disease. In contrast, nighttime sleep duration did not significantly differ between children with active AD and those without AD, and the estimated difference in total daily sleep duration was only two minutes. Notably, children with mild or inactive AD also had increased odds of impaired sleep quality (Ramirez et al.)**.**

Clinical and Experimental Evidence for PBM

An observational, non interventional study evaluated full-body blue light irradiation in 36 adults with severe, chronic AD that had not achieved long term disease control with topical corticosteroid therapy. Treatment was administered in cycles of five consecutive irradiations using a full body device in which more than 66% of the emission spectrum fell between 400 and 500 nm, delivering 28.9 J/cm^2 within this wavelength range. Each treatment involved 24 minutes of exposure to each side of the body, and an additional five-day cycle was administered when patients experienced disease exacerbations(Becker et al.). Disease severity was assessed using the EASI, while pruritus and sleeplessness were rated by patients on a 0-10 scale at baseline, day 15, and after three and six months of treatment(Becker et al.). EASI scores decreased by 29% at day 15, 41% at three months, and 54% at six months, with the reductions at three and six months reaching statistical significance(Becker et al.).Baseline pruritus severity

averaged 7.0/10 and decreased significantly by day 15, with approximately 40% reductions observed at three and six months. Sleeplessness, which averaged 5.1/10 at baseline, also decreased significantly over the treatment period(Becker et al.). Additionally, 25 out of 28 patients (89%) who completed the relevant follow up assessment reported improvement in itching, with most first noticing reductions after two to three treatment cycles(Becker et al.).

A randomized controlled animal study evaluated low-level laser therapy in a DNCB-induced AD model using BALB/c mice. Following AD induction, mice received 650 nm laser irradiation at energy densities of 2, 4, 6, or 8 J/cm^2 once daily for two weeks, for a total of 14 treatments. Outcomes included clinical skin-severity scores, scratching behavior, serum IgE and IL-4 concentrations, splenic expression of IL-4, IL-6, and TNF-α, epidermal thickness, and mast-cell infiltration and degranulation(Y. Kim et al.). By day 7, clinical severity scores were lower in the 4- and 6-J/cm^2 treatment groups than in untreated AD controls, while scratching behavior was reduced in the 6-J/cm^2 group. By day 14, all irradiated groups showed significantly lower clinical severity scores and scratching behavior compared with untreated AD controls (p<0.001) (Y. Kim et al.). Laser treatment also reduced serum IgE and IL-4 concentrations and decreased IL-6 and TNF-α mRNA expression(Y. Kim et al.). Histological analysis showed reduced epidermal thickness and fewer total and degranulated mast cells in irradiated mice compared with untreated AD controls(Y. Kim et al.).

A mechanistic study examined the anti-inflammatory effects of PBM in primary human keratinocytes exposed to 2,4-dinitrochlorobenzene (DNCB) to induce inflammatory stress. Cells were treated with either 660 nm red or 520 nm green light at an energy density of 3 J/cm^2, and inflammatory cytokine expression and antioxidant signaling were subsequently assessed(Salman et al.).Three hours after irradiation, red light reduced DNCB-induced mRNA expression of TNF-α, IL-6, and IL-8 by 62%, 69%, and 28%, respectively, while green light produced reductions of 49%, 46%, and 18%. These effects were not observed in unstimulated keratinocytes(Salman et al.).PBM also enhanced Nrf2 pathway activity, with red and green light increasing Nrf2 protein accumulation by approximately 30% and 50%, respectively(Salman et al.). When Nrf2 expression was reduced using small interfering RNA (siRNA), red light treatment no longer suppressed TNF-α, IL-6, and IL-8 to the same extent, indicating that the observed anti-inflammatory response was at least partly dependent on Nrf2 signaling (Salman et al.).

Evidence from other biological systems suggests that PBM responses may vary according to treatment timing. A review examining PBM during wakefulness and sleep described experimental studies in which PBM increased mitochondrial function and ATP production more effectively in *Drosophila* when administered in the morning compared with the afternoon or nighttime. Similar time-dependent effects were reported in humans, with PBM producing greater improvements in visual function when administered in the morning rather than later in the day(Moro et al.).

Discussion

The evidence synthesized in this review indicates that nocturnal pruritus and sleep disturbance in AD are closely interconnected and take place within a broader pattern of time-dependent physiological change. Patient-reported assessments have identified nighttime predominance of pruritus, while objective monitoring has demonstrated increased scratching and movement during sleep in individuals with AD (Yosipovitch and Papoiu; Fishbein et al.; Ikoma et al.). In particular, nocturnal movement in children with moderate-to-severe AD was concentrated between one and six hours after sleep onset and peaked approximately three hours after sleep onset(Fishbein et al.). Sleep impairment also appears to increase with AD severity across both objective and patient-reported assessments(Bender et al.; Ramirez et al.; Simpson et al.). The concentration of movement during particular hours after sleep onset is especially notable because it posits that nighttime symptoms follow a measurable sequential pattern instead of worsening uniformly throughout the night.

The nocturnal pattern of AD symptoms is likely influenced by interactions among inflammatory, neural, skin-barrier, and circadian mechanisms. Type 2 cytokines implicated in AD, namely IL-4 and IL-13, can directly activate sensory-neuronal pathways involved in chronic itch, while IL-31 provides an additional connection between immune activation and pruritic signaling(Oetjen et al.; S. Kim et al.). Circadian alterations in skin-barrier function may further contribute to the physiological environment in which these itch pathways operate, as time-dependent changes in TEWL appear disrupted in AD(Iwanaszko et al.). However, current evidence does not establish that itch-associated inflammatory mediators consistently peak during nighttime hours.

The PBM literature provides preliminary evidence that light-based treatment can influence several components of this neuroimmune itch pathway, although the strength of evidence differs substantially across experimental models. In adults with severe chronic AD, blue-light treatment was associated with reductions in disease severity and pruritus as well as patient-reported sleeplessness over the treatment period(Becker et al.). Furthermore, controlled experimental evidence provides additional biological support. In a DNCB-induced mouse model of AD, 650 nm irradiation reduced scratching behavior alongside reductions in IL-4, IL-6, TNF-α, IgE, epidermal thickness, and mast-cell activity(Y. Kim et al.). In DNCB-stimulated human keratinocytes, red-light PBM reduced TNF-α, IL-6, and IL-8 expression, while Nrf2 knockdown weakened this anti-inflammatory response, suggesting that Nrf2 signaling contributed to the observed effects(Salman et al.). These experimental findings provide possible biological explanations for the clinical improvements observed with blue light treatment, although the differences between human, animal, and in vitro models do prevent a direct mechanistic comparison.

One possible connection between these findings involves the inflammatory pathways associated with chronic itch. IL-4 and IL-13 can act directly on sensory neurons involved in pruritic signaling, while IL-31 is also associated with greater itch severity in AD ((Oetjen et al.; S. Kim et al.). In the DNCB-induced AD model, PBM reduced IL-4 alongside scratching behavior and other inflammatory markers(Y. Kim et al.). Although these findings come from

separate experimental models and do not establish that PBM reduces nocturnal itch through cytokine modulation, they raise the possibility that PBM could influence inflammatory processes that induce pruritus. If these effects are reproduced clinically, reducing activity within itch-associated cytokines could potentially lessen nighttime scratching and, indirectly, the sleep disruption associated with AD. This relationship has not yet been tested directly and represents an important question for future PBM studies.

An additional consideration emerging from this evidence is whether treatment timing influences the biological response to PBM. Experimental studies found that PBM increased mitochondrial function and ATP production more effectively in *Drosophila* when administered in the morning than in the afternoon or nighttime, while human visual responses to PBM also differed according to treatment time (Moro et al.).These findings raise the possibility that treatment timing may be an additional PBM parameter worth considering. This may be particularly relevant to AD because pruritus itself varies across the nighttime period. Based on these findings, one hypothesis is that the timing of PBM relative to nocturnal symptom worsening could influence its therapeutic effects. For example, PBM administered before the evening increase in pruritus could potentially modify inflammatory or pruritic activity during the subsequent nocturnal period. However, existing evidence cannot establish whether evening administration would be more effective than morning or daytime treatment.

Several limitations prevent this hypothesis from currently being translated into a clinical recommendation. PBM studies employ substantially different wavelengths, energy densities, treatment durations, and experimental systems, complicating direct comparison of their findings(Woo and Padalhin).Furthermore, PBM should not be assumed to produce uniformly anti-inflammatory responses. In an in vitro inflammatory keratinocyte model not specific to AD, 808 nm irradiation at 5 J/cm^2 increased several inflammatory mediators, including TNF-α, IL-6, IL-8, IL-17A, IL-23, and IL-33, compared with nonirradiated inflammatory controls (Sá et al.). These findings contrast with the reductions in inflammatory mediators observed under other PBM conditions(Y. Kim et al.; Salman et al.), suggesting that wavelength, dose, cellular state, and experimental context may influence the direction and magnitude of PBM responses. Clinical evidence specific to AD is also limited.

A further limitation is the absence of direct circadian assessment in existing PBM studies involving AD. The reviewed AD treatment studies did not evaluate treatment effects using circadian biomarkers or compare otherwise equivalent PBM protocols administered at different times of day (Becker et al.; Y. Kim et al.).The Becker study lacked a randomized control group, and its sleep-related assessment consisted of patient-reported sleeplessness rather than actigraphy, polysomnography, or continuous nocturnal scratching measurements(Becker et al.). Consequently, the existing literature cannot distinguish whether PBM primarily reduces overall AD severity, whether reductions in pruritus subsequently improve sleep, or whether PBM can specifically modify the nocturnal amplification of AD symptoms. This distinction represents a central gap in the current evidence.

Future clinical trials should therefore evaluate PBM as both a dose-dependent and

potentially time-dependent intervention. One approach would be to compare otherwise identical PBM protocols administered during morning and evening periods while incorporating repeated time-specific itch ratings and objective nocturnal scratching measurements. Actigraphy could be used to quantify sleep-wake patterns and wake after sleep onset, while polysomnography could provide more detailed assessment of sleep architecture.Serial measurements of selected inflammatory mediators alongside circadian markers could determine whether changes in nighttime symptoms correspond with biological changes across the treatment period. Because existing studies vary considerably in PBM parameters (Woo and Padalhin), future trials should also consistently report wavelength, irradiance, energy density, treatment duration, frequency, and clock time of administration. Such designs would directly test whether treatment timing contributes to PBM efficacy rather than assuming that responses observed at one time of day generalize across the circadian cycle.

Overall, current evidence supports that nocturnal pruritus and sleep disturbance hold a cause and effect relationship as a component of AD.Preliminary human evidence indicates that blue-light treatment can reduce pruritus alongside patient-reported sleeplessness (Becker et al.) , while experimental studies demonstrate PBM-related changes and mitigation in scratching behavior and inflammatory signaling pathways (Y. Kim et al.; Salman et al.). Direct evidence linking PBM to circadian regulation or specifically to nocturnal improvement remains insufficient. Studies comparing PBM at different times of day may help determine whether treatment timing influences its effects on nocturnal AD symptoms.This could clarify whether PBM has a role not only in reducing overall AD symptoms but also as a potential time-based therapeutic approach .

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CAHS8 Enhances GUV Morphological Recovery after Dehydration and Rehydration

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Summary

Dehydration can damage biological membranes and limit the storage and use of synthetic cell models such as giant unilamellar vesicles (GUVs). Tardigrades can survive severe water loss partly because they produce cytoplasmic abundant heat-soluble (CAHS) proteins, a family of stress-related intrinsically disordered proteins. Here, we tested whether CAHS8 could improve the recovery of GUV morphology after dehydration and rehydration. GUVs were prepared with 0, 0.05, 0.19, or 0.27 mM CAHS8 in the inner aqueous solution and imaged before dehydration, in the dehydrated state, and after rehydration. The shapes of vesicle-like structures after rehydration were quantified using circularity, where values closer to 1 represent more spherical shapes. Dehydration caused substantial deformation and loss of spherical morphology across all groups. After rehydration, samples containing 0.19 or 0.27 mM CAHS8 showed more spherical vesicle-like structures than the CAHS8-free control. Mean circularity increased from about 0.20 in the control to 0.30, 0.54, and 0.58 in the 0.05, 0.19, and 0.27 mM groups, respectively. The small difference between the two highest concentrations suggests that the effect may level off at higher CAHS8 concentrations. In a separate experiment, 100 μM CAHS8 reduced the interfacial tension at a POPC-containing oil–water interface compared with the protein-free control, indicating that CAHS8 exhibits interfacial activity. However, this result does not prove that CAHS8 binds directly to phospholipid bilayers or explain how it improves GUV recovery. The findings suggest that CAHS8 helps GUVs recover a more spherical morphology after dehydration and rehydration, although further experiments are needed to determine whether membrane function is also preserved.

Introduction

Water is essential for maintaining the structure and function of biological molecules (1). During dehydration, water loss alters molecular interactions, increases intracellular crowding, promotes protein unfolding and aggregation, and disrupts lipid-membrane organization (2,3). Most cells cannot tolerate extensive water loss without irreversible damage (4). However, some organisms survive near-complete dehydration by entering an anhydrobiotic state in which metabolism is greatly reduced and biological activity resumes after water becomes available again (5, 6). Understanding these natural protection mechanisms may provide new strategies for stabilizing biological materials and engineering more robust synthetic cells, which are engineered, cell-like structures built from biological parts.

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Tardigrades are microscopic animals known for their remarkable tolerance to dehydration and other environmental stresses (7, 8). Although many desiccation-tolerant organisms rely on protective sugars such as trehalose, several tardigrade species contain little detectable trehalose and instead produce families of tardigrade-specific heat-soluble proteins (7, 8). Among these proteins, cytoplasmic abundant heat-soluble (CAHS) proteins have emerged as important contributors to dehydration tolerance. More broadly, reducing the expression of tardigrade-specific intrinsically disordered proteins decreases tardigrade survival after drying, whereas expressing selected tardigrade proteins in yeast and bacteria improves desiccation tolerance, supporting a direct protective role for these proteins (7).

CAHS proteins are intrinsically disordered under dilute, hydrated conditions and do not adopt a single stable three-dimensional structure (7, 9, 10, 11,12). Their flexibility allows them to respond to changes in hydration, concentration, ionic conditions, and molecular crowding. Early studies proposed that CAHS proteins protect cellular components by forming non-crystalline, glass-like matrices during drying (7, 13). These vitrified states were suggested to reduce molecular mobility, thereby limiting protein unfolding and aggregation under dehydrating conditions. Later work showed that CAHS proteins can also undergo reversible and more organized structural transitions. Under increasing protein concentration, osmotic stress, or dehydration, some CAHS proteins develop α-helical structure and assemble into fibrils, condensates, or gel-like networks (9, 10, 11). For example, CAHS1 remains largely disordered at low concentration but forms reversible fibrous condensates and hydrogels as the surrounding environment becomes more concentrated (9). These observations led to the idea that CAHS proteins behave as stress-responsive biomolecular materials rather than as passive molecular stabilizers.

The formation of CAHS assemblies may also provide mechanical protection at the cellular level (10, 12). Expression of gel-forming CAHS proteins increases cell stiffness during hyperosmotic stress and reduces stress-induced cell shrinkage (14). This behavior suggests that CAHS networks can act as a temporary intracellular scaffold that mechanically supports cells as water is lost. The networks dissolve when the cell is returned to its hydrated state. Thus, CAHS-mediated protection may involve both biochemical stabilization of sensitive molecules and mechanical reinforcement of cellular structure.

Protection by CAHS proteins is unlikely to depend on a single mechanism. Studies of CAHS D showed that the protein interacts strongly with residual water in both hydrated and dehydrated samples but does not simply preserve a large quantity of water (15). Other studies have shown that the protective performance of CAHS proteins depends on concentration, sequence, assembly behavior, and the type of molecule or stress being tested (16). These findings suggest that vitrification, reversible assembly, residual-water interactions, and client-specific stabilization may contribute differently across the CAHS protein family.

Recent work has extended CAHS research from soluble proteins and living cells to artificial membrane compartments (12). Using giant unilamellar vesicles (GUVs) as a minimal syntheticcell model, our lab showed that CAHS12 undergoes reversible structural changes that

protect vesicle architecture during dehydration, elevated temperature, osmotic stress, and long-term storage (12). We found that CAHS12-containing GUVs retained their encapsulated components, recovered their spherical morphology after rehydration, and showed increased resistance to mechanical deformation. Domain-truncation experiments and molecular simulations identified that the N-terminal region plays an important role in protein-network formation, lipid association, and membrane protection. CAHS12 also preserved biochemical activity within synthetic cells. GUVs containing a cell-free transcription–translation system recovered gene expression after dehydration and extended dry storage, and membrane-associated protein function could be restored after rehydration (12). These findings demonstrated that CAHS-mediated protection can extend beyond vesicle morphology to the preservation of encapsulated biochemical systems. They also showed that CAHS proteins can function at several levels, including protein assembly, membrane interactions, mechanical stabilization, and recovery of synthetic cell activity.

Although our prior study has focused on CAHS12, there are numerous isoforms of CAHS proteins. The features of one isoform do not predict the behavior of another. Individual CAHS isoforms differ in amino acid sequence, structural transitions, assembly behavior, localization, and protective performance. CAHS8 has attracted interest because it remains soluble under hydrated conditions but can undergo stress-responsive structural transitions and form fibrous or gel-like assemblies. Previous studies have mainly examined its effects on soluble biomolecules or intracellular organization (17). Whether CAHS8 can improve the recovery of simplified lipidbilayer compartments after dehydration remains less clear.

GUVs provide a useful system for addressing this question because they consist of a single phospholipid bilayer surrounding an aqueous interior and are large enough to be directly examined by fluorescence microscopy (18, 19, 20). Unlike living cells, GUVs lack cytoskeletal support, active repair pathways, and complex stress-response systems. As a result, dehydration-induced changes in membrane shape, integrity, and recovery can be examined without many of the biological processes present in intact cells (21, 22). This simplified system,therefore, allows the effects of CAHS8 on membranes to be studied with fewer confounding variables.

Here, we investigated whether CAHS8 improves the morphological recovery of GUVs following dehydration and rehydration. GUVs were prepared with 0, 0.05, 0.19, or 0.27 mM CAHS8 in the inner aqueous solution and examined before dehydration, in the dehydrated state, and after rehydration. Circularity was used to quantify the morphology of vesicle-like structures after rehydration, while interfacial tension was measured separately in the presence and absence of CAHS8. We hypothesized that increasing CAHS8 concentration would result in more regular vesicle morphology after rehydration.

Results

CAHS8-containing GUVs show improved morphological recovery after rehydration

To determine whether CAHS8 improves GUV recovery following dehydration, we compared vesicles containing Cy5 dye and varying concentrations of CAHS8 (0, 0.05, 0.19, or 0.27 mM) before dehydration, in the dehydrated state, and after rehydration (**Figure 1**). Encapsulated Cy5 dye enables visualization of vesicle morphology. Spherical GUVs were observed under all four conditions prior to dehydration, although vesicle size and density varied among representative fields.

Dehydration caused substantial morphological disruption in all groups. In the absence of CAHS8, few recognizable vesicle-like structures remained, whereas CAHS8-containing samples retained more distinct membrane-associated structures, particularly at 0.19 and 0.27 mM.Because dehydrated GUVs can collapse into flattened membrane structures, morphology in the dried state was not interpreted as evidence of vesicle integrity.

Figure 1. Representative fluorescence microscopy images of GUVs containing different concentrations of CAHS8 before dehydration, in the dehydrated state, and after rehydration. GUVs containing Cy5 dye and 0, 0.05, 0.19, or 0.27 mM CAHS8 were imaged under the original hydrated condition, following dehydration, and after rehydration. Dehydration caused deformation, aggregation, and loss of recognizable spherical morphology in all groups. After rehydration, samples containing 0.19 and 0.27 mM CAHS8 appeared to contain more spherical vesicle-like structures than the CAHS8-free control. Scale bar, 20 μm.

Differences among conditions became clearer after rehydration. A few recognizable vesicle-like structures were observed in the CAHS8-free control. The 0.05 mM condition showed partial recovery, whereas the 0.19 and 0.27 mM conditions exhibited more rounded and clearly distinguishable vesicle-like structures. These images suggested improved morphological recovery in the presence of CAHS8. However, because vesicle size, density, and aggregation varied among representative fields, quantitative analysis was required to determine whether the apparent improvement was reproducible across independent samples.

Higher CAHS8 concentrations are associated with improved GUV circularity after rehydration

To quantify the morphological differences observed by fluorescence microscopy, we measured the circularity of GUVs after dehydration and rehydration (**Figure 2a**). Circularity was calculated from the two-dimensional vesicle outline, with values closer to 1 indicating a more circular and less irregular profile.

The CAHS8-free control exhibited the lowest mean circularity, at approximately 0.20. The mean circularity increased to approximately 0.30 in GUVs containing 0.05 mM CAHS8 and to approximately 0.54 in GUVs containing 0.19 mM CAHS8. GUVs containing 0.27 mM CAHS8 showed a similar mean circularity of approximately 0.58. Thus, the largest increase occurred between 0.05 and 0.19 mM CAHS8, whereas only a small additional increase was observed between 0.19 and 0.27 mM CAHS8.

These measurements were consistent with the qualitative observations in **Figure 1**, in which the higher-concentration CAHS8 groups contained more vesicle-like structures with regular circular outlines after rehydration. However, the relatively large variability within each group and the overlapping error bars, particularly between 0.19 and 0.27 mM, indicate that CAHS8 did not preserve all vesicles equally.

Overall, the circularity measurements support the conclusion that the presence of CAHS8 is associated with improved morphological recovery of GUVs after dehydration and rehydration. The data also suggest that the protective effect may begin to plateau between 0.19 and 0.27 mM under the conditions tested.

Figure 2. Effects of CAHS8 concentration on GUV circularity and oil–water interfacial tension.(a) Circularity of GUVs after dehydration and rehydration as a function of encapsulated CAHS8 concentration. Circularity values closer to 1 indicate a more circular two-dimensional vesicle outline. Data are presented as mean ± SEM from $n > 50$ GUVs. Statistical comparisons were performed using two-tailed unpaired Student's *t*-tests. Circularity was significantly higher at 0.05, 0.19, and 0.27 mM CAHS8 compared with the 0.017 mM CAHS8 ($p < 0.0001$ for all comparisons), whereas no significant difference was observed between 0.19 and 0.27 mM CAHS8 ($p = 0.4$, ns). **(b)** Time-dependent lipid-containing oil–water interfacial tension measured in the absence and presence of 100 μM CAHS8. Data are presented as mean ± SD from three independently formed droplets.

CAHS8 alters the time-dependent oil–water interfacial tension

To investigate whether CAHS8 exhibits interfacial activity, we measured the lipid-containing oil–water interfacial tension in the absence and presence of 100 μM CAHS8 over 50 min (**Figure 2b**). In the absence of CAHS8, the interfacial tension decreased from approximately 36 mN/m at the beginning of the measurement to approximately 19 mN/m after 50 min. In the presence of CAHS8, the initial interfacial tension was lower, at approximately 31 mN/m, and decreased more rapidly during the early stage of the measurement. After 50 min, the interfacial tension reached approximately 12 mN/m.

The CAHS8-containing sample exhibited lower interfacial tension than the protein-free control throughout the measurement. This difference is consistent with CAHS8 accumulating at, or otherwise modifying, the oil–water interface. The more rapid initial decrease may reflect time-dependent transport and adsorption of CAHS8 to the interface, although the present experiment does not directly measure protein adsorption. These results suggest that CAHS8 possesses interfacial activity that may contribute to GUV stabilization. However, because an oil–water interface differs from a lipid-bilayer membrane, additional experiments are needed to determine whether CAHS8 directly associates with GUV membranes and whether this interaction contributes to protection during dehydration.

Discussion

The goal of this study was to determine whether CAHS8 could help GUVs recover from dehydration. Both fluorescence microscopy and circularity measurements showed that GUVs containing CAHS8 generally had more spherical shapes after rehydration than GUVs without CAHS8. This difference was most noticeable at 0.19 and 0.27 mM CAHS8.

Before dehydration, GUVs formed successfully at all CAHS8 concentrations tested. Dehydration caused major changes in membrane morphology across all groups, including deformation, aggregation, and loss of recognizable vesicle shapes. After rehydration, a few vesicle-like structures remained in the CAHS8-free control. In contrast, samples containing CAHS8 showed more rounded membrane structures, especially at the two higher concentrations. CAHS8, therefore, did not prevent all damage during drying, but it appeared to improve morphological recovery after water was added back.

The circularity measurements supported the observations in the microscopy images. Mean circularity increased from about 0.20 without CAHS8 to about 0.54 at 0.19 mM. Increasing the concentration further to 0.27 mM produced only a small additional increase. This suggests that much of the improvement occurred between 0.05 and 0.19 mM CAHS8. The variability within the groups was relatively large, so the current results do not show that 0.27 mM is more effective than 0.19 mM. Statistical analysis showed that circularity differed significantly among the CAHS8 concentration groups, with the largest increase observed between 0.05 and 0.19 mM CAHS8.

CAHS8 also changed the behavior of the lipid-containing oil–water interface. Interfacial tension was lower in the presence of CAHS8 and decreased more quickly during the early part of the measurement. One possible explanation is that CAHS8 accumulates at the interface and changes its physical properties. This could be relevant to the emulsion-transfer process used to prepare the GUVs and may also indicate that CAHS8 has some affinity for interfaces.

The interfacial tension measurement, however, does not show that CAHS8 directly binds to the GUV membrane. The oil–water interface used in this assay is different from a phospholipid bilayer, so these measurements should be viewed as evidence of interfacial activity rather than direct evidence of membrane binding. Experiments using fluorescently labeled CAHS8 or direct lipid-binding assays would be needed to determine CAHS8's localization within the GUVs.

Several limitations should also be considered. Only four concentrations were examined, so the concentration range was too limited to identify an optimal concentration or determine whether the response reaches a true plateau. Circularity also measures only the two-dimensional shape of fluorescent membrane structures. A circular structure may still contain membrane defects or may have lost material from its interior. In addition, individual GUVs were not tracked through the full dehydration and rehydration process, so the experiments cannot directly show that a particular vesicle recovered its original structure.

Future experiments could address these questions by following individual GUVs during dehydration and rehydration and measuring both morphology and membrane integrity.

Cargo-retention or permeability assays would help determine whether vesicles that appear morphologically recovered also remain functionally intact.

Overall, these results show that CAHS8 is associated with improved morphological recovery of GUVs after dehydration and rehydration. The greatest improvement was observed at 0.19 and 0.27 mM CAHS8, while interfacial tension measurements showed that CAHS8 can also modify a lipid-containing oil–water interface. Although the mechanism is not yet clear, the results support further investigation of CAHS8 as a possible way to improve the dehydration tolerance of synthetic cell compartments.

Materials and Methods

Expression and Purification of CAHS8

The gene encoding CAHS8 from the tardigrade *Ramazzottius varieornatus* was cloned into the pET-28b(+) expression vector with an N-terminal His_6-tag. The sequence-verified plasmid was transformed into *Escherichia coli* BL21(DE3) cells (23, 24).

A single bacterial colony was inoculated into 5 mL of lysogeny broth containing 50 μg/mL kanamycin and cultured overnight at 37°C with shaking at 220 rpm. The overnight culture was diluted 1:200 into 1 L of lysogeny broth containing 50 μg/mL kanamycin. Cells were grown at 37°C until the optical density at 600 nm reached approximately 0.5–0.6. The culture was then cooled in an ice-water bath, and protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.2 mM. The induced culture was incubated for 16 h at 24°C with shaking.

Cells were harvested by centrifugation at 6,000 × g for 30 min at 4°C and resuspended in a lysis buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole at pH 8.0. Cells were disrupted by sonication on ice. The lysate was clarified by centrifugation at 25,000 × g for 30 min at 4°C, and the resulting supernatant was passed through a 0.45 μm filter.

The clarified lysate was loaded onto a 1 mL HisTrap FF column using an ÄKTA Start fast protein liquid chromatography system. The column was washed with 20 column volumes of buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 25 mM imidazole at pH 8.0. Bound CAHS8 was eluted using a buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole at pH 8.0. Elution fractions of 1.5 mL were collected at a flow rate of 1 mL/min.

Protein purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Fractions corresponding to the CAHS8 elution peak were combined and dialyzed overnight at 4°C against buffer containing 20 mM Tris-HCl, 25 mM NaCl, and 1 mM ethylenediaminetetraacetic acid at pH 8.0 using a 10 kDa molecular-weight-cutoff dialysis membrane. Purified CAHS8 was divided into aliquots, flash-frozen in liquid nitrogen, and stored at −80°C until use.

Preparation of Giant Unilamellar Vesicles

GUVs were prepared using a modified inverted-emulsion phase-transfer method (25, 26). The lipid mixture consisted of 99.9 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 0.1 mol% rhodamine-labeled phosphatidylethanolamine. The lipids were dissolved in chloroform at a final total lipid concentration of 0.41 mM.

The lipid solution was transferred to a clean glass vial, and the chloroform was evaporated under a stream of argon to form a lipid film. Residual solvent was removed by placing the vial under vacuum for at least 1 h. The dried lipid film was resuspended in 1.1 mL of mineral oil by vortexing for 30 s. The suspension was incubated at 55–75°C for 20 min with intermittent vortexing to promote complete dispersion of the lipids in the oil phase.

To prepare the lipid-coated oil–water interface, 400 μL of outer aqueous solution containing 300 mM glucose was added to a microcentrifuge tube. A 250 μL volume of the lipid-containing mineral oil was gently layered above the glucose solution. The two-phase system was incubated at room temperature for 1 h to allow a lipid monolayer to form at the oil–water interface.

For each experimental condition, 20 μL of inner aqueous solution containing 300 mM sucrose and 0, 0.05, 0.19, or 0.27 mM CAHS8 was placed in a separate microcentrifuge tube. Cy5 was included in the inner solution at 10 μg/mL as an encapsulated aqueous-phase fluorescent marker. A 450 μL volume of lipid-containing mineral oil was added above the inner aqueous solution. The mixture was emulsified by repeatedly moving the tube across a microcentrifuge-tube rack until a visually uniform water-in-oil emulsion was obtained.

The resulting emulsion was gently transferred onto the previously prepared lipid-coated oil–water interface. Samples were centrifuged at 2,500 × g for 10 min at 4°C using a swing-bucket rotor. During centrifugation, the emulsion droplets crossed the lipid monolayer and entered the outer aqueous phase, forming lipid bilayer vesicles.

Following centrifugation, the upper oil layer and visible aggregated material were carefully removed by pipetting without disturbing the GUV-containing lower aqueous phase. GUVs were collected and transferred to optical polymer-bottom imaging plates. Vesicle formation and encapsulation were verified by fluorescence microscopy before dehydration experiments.

The CAHS8 concentrations reported in this study represent the nominal concentrations added to the inner aqueous solution. The actual concentration encapsulated within individual GUVs was not directly measured.

Dehydration and Rehydration of GUVs

For each experimental condition, 5 μL of freshly prepared GUV suspension was transferred to a well of a 96-well glass-bottom plate. Samples were placed in a vacuum desiccator and dehydrated at 22–25°C under a pressure of approximately 31 mbar for at least 12 h.

Images of the samples were collected before dehydration and after the dehydration period. Because dehydrated GUVs can collapse into flattened or irregular membrane structures,

fluorescence patterns observed in the dehydrated state were interpreted as membrane-associated structures rather than as definitive evidence of intact vesicles.

To initiate rehydration, 5 μL of ultrapure deionized water was gently added to each dehydrated sample. Samples were incubated at room temperature for 10 min before fluorescence imaging. All experimental groups were dehydrated and rehydrated using the same volumes, incubation times, temperature, and vacuum conditions.

Fluorescence Microscopy

GUVs were imaged using a confocal fluorescence microscope equipped with a Yokogawa CSU-X1 spinning-disk unit. Images were collected at three experimental stages: before dehydration, after dehydration, and after rehydration. The same objective magnification at 40x and comparable excitation, exposure, gain, and detector settings were used across experimental conditions within each imaging experiment.

Representative images in Figure 1 were selected from three independent GUV preparations. Image fields were selected to illustrate the typical morphology observed under each condition rather than only the most intact vesicles. Scale bars were added using the microscope calibration information.

Circularity Analysis

Fluorescence images collected after rehydration were analyzed using Fiji/ImageJ. Vesicle-like membrane structures were identified using the rhodamine-lipid fluorescence channel. Objects were included in the analysis when they formed a closed, clearly distinguishable membrane-associated outline, were fully contained within the image field, and did not substantially overlap neighboring objects. Structures touching an image boundary or forming unresolved aggregates were excluded.

Eligible structures were manually outlined using the freehand or polygon selection tool. ImageJ was used to calculate the projected area and perimeter of each outline. Circularity was calculated as:

where A is the projected area and P is the perimeter of the outlined structure. A value of 1 represents a perfect two-dimensional circle, whereas lower values indicate a more irregular outline (27).

At least 50 vesicle-like structures were analyzed for each CAHS8 condition across three independently prepared samples. The number of structures analyzed from each independent experiment was recorded separately. Circularity was used as a measure of two-dimensional morphological regularity and was not interpreted as a direct measurement of membrane permeability, cargo retention, or three-dimensional vesicle integrity.

Oil–water interfacial tension measurements

The interfacial activity of CAHS8 was evaluated using pendant-drop tensiometry (28). Measurements were conducted using Krüss DSA100 at 25℃. The aqueous phase contained

either no CAHS8 or 100 μM CAHS8. The surrounding oil phase was mineral oil with 0.41 mM POPC.

An aqueous droplet of approximately 5 μl was formed within the oil phase. The droplet profile was recorded for 50 min, and interfacial tension was calculated by fitting the droplet shape to the Young–Laplace equation using the instrument software. Measurements were performed using three independently formed droplets for each condition.

Interfacial tension values were plotted as a function of time. These measurements evaluated the behavior of CAHS8 at an oil–water interface and were not considered direct measurements of CAHS8 binding to a phospholipid bilayer.

Statistical Analysis

Data are presented as mean ± standard deviation unless otherwise stated. Three independently prepared GUV samples were analyzed for each condition. Circularity values were first averaged within each independent preparation so that independently prepared samples, rather than individual vesicles from the same preparation, served as the experimental replicates for statistical comparisons.

Selected pairwise comparisons between CAHS8 concentration groups were performed using two-tailed unpaired Student's *t*-tests assuming equal variances. Statistical significance was defined as $p < 0.05$.

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Alzheimer's Disease, Air Pollution, and Biomarkers: A Mechanistic Analysis
By Dhiya Prabhuram

Abstract

Air pollution has become a major global health concern with increasing discoveries and evidence linked to neurodegenerative diseases, particularly Alzheimer's disease. While existing research and studies have shown to examine the role of individual pollutants such as PM2.5, NO_2, O_3, and their linked pathways, there are still limited studies specifically comparing these individual pollutants and their influence on distinct biological pathways, while linking those to their corresponding biomarkers for a more precise comparison and outcome. Through a secondary data analysis using peer-reviewed studies, air quality and biomarker databases, the levels of each pollutant were compared with and linked to specific changes in biomarkers such as amyloid pathology (Aβ42), tau pathology (t-tau), axonal injury (NfL), and neuroinflammation (YKL-40 / GFAP). Findings were then organized into two tables, Table 1 recording the pollutant and mechanism relationships, and Table 2 recording the strength of Alzheimer's biomarkers. Results from this study indicate that PM2.5 is associated with decreased CSF Aβ42 levels (β= -0.10), and increased NfL levels. NO_2 showed strong associations with tau pathology (t-tau) with increasing value of (β = 0.34), as well as an increase in NfL (β= 0.36). O_3 data showed strong associations with neuroinflammation (YKL-40 / GFAP), neuronal/axonal injury, and some increased levels of amyloid accumulation. However, the data collected for O_3 did not include any statistical values to show their relationship and there were limited studies that used human CSF data. Overall, all three pollutants were not directly measured with neuroinflammation markers, but were all recorded to be linked with increased neuroinflammation levels. The biomarker data analysis showed tau pathology-related biomarkers and NfL biomarkers had the largest effect sizes, which resulted in a stronger association with Alzheimer's disease development, compared to amyloid alone, where it had the smallest effect size, but one of the strongest associations with Alzheimer's disease development. Ultimately, these findings and results emphasize the importance of linking environmental exposure data with biomarker analysis data, when researching its effects on Alzheimer's disease, as it provides a more precise comparison and measurable outcome.

Literature Review

"Air pollution now ranks as the second leading risk factor for death, accounting for 8.1 million deaths globally," according to HEI's new State of Global Air 2024 Report (Health Effects Institute)**.** Air pollution has become one of most prevalent environmental health concerns in the world, in which it's known for its severe effects on the lungs, heart, brain, and overall life expectancy. Over the past several years, researchers have started to connect long-term exposure to air pollution to neurodegenerative diseases like Alzheimer's disease. Alzheimer's disease, being one of the most common forms of dementia, is caused by the destruction of brain cells ,which can lead to inflammation and cognitive decline. This disease is characterized by cognitive

decline and brain damage. From a biological aspect, Alzheimer's disease is indicated and identified by several key biomarkers. Biomarkers are measurable indicators of a certain condition or disease, in this case, Alzheimer's disease. These markers indicate specific processes or mechanisms that eventually lead to the development of Alzheimer's disease (AD). AD is indicated by several key biomarkers such as amyloid beta plaque accumulation, tau protein phosphorylation, axonal injury (or neuronal injury), and neuroinflammation. Additionally, these biomarkers are often measured in a fluid called cerebrospinal fluid (CSF), which is a fluid that surrounds the brain and spinal cord, providing proper insight into biological and chemical changes and processes that occur in the nervous system. These biomarkers represent the underlying mechanisms that are involved in neurodegeneration and are often utilized to track disease progression, as summarized in a recent peer-reviewed neurological research paper (Lane et al.).

Alzheimer's Biomarkers and Disease Progression

The accumulation of amyloid-beta (Aβ42) is one of the earliest and most common changes in Alzheimer's disease. Amyloid-beta is a protein that accumulates in the brain which forms "amyloid plaques," eventually leading to disruption of communication between neurons. Decreased levels of Ab42 in cerebrospinal fluid are often associated with increased plaque accumulation in brain tissue because most of the amyloid-beta proteins deposit inside the brain tissue, moving away from the cerebrospinal fluid, which also makes this a crucial early indicator of the development of Alzheimer's disease (Abukuri). However, emerging research on neuropathology by Kim and other researchers suggests that tau-related biomarkers show stronger associations with Alzheimer's disease development, compared to amyloid-beta, specifically talking about their roles in neuronal injury and cognitive decline (Kim et al.). Additionally, other research has emphasized the significance of key biomarkers which include neurofilament light chain (NfL), which is a structured protein and when damaged, is released into the CSF, and results as a measurable indicator of disease severity and progression. Increased levels of these biomarkers in CSF further contribute to their role in the development of neurodegenerative diseases (Agah et al.). While markers such as GFAP and YKL-40, which Pelkmans and other researchers explain to be elevated in the dementia phase of AD (Pelkmans et al.), associated with neuroinflammation is referred to as the brain's immune response, where cells release chemicals that can signal and cause swelling and damage to neurons, contributing to neuronal damage (Brockmeyer and D'Angiulli). Ultimately, these different biological mechanisms and biomarkers are shown to be interconnected, yet distinct pathways that all lead to the development of Alzheimer's disease at different levels.

Association with Air Pollutants

Air pollution has appeared to be a significant environmental factor contributing to neurodegenerative diseases, which shifts from traditional age or genetically related factors that are normally seen behind the diagnosis of Alzheimer's. Key air pollutants such as particulate

matter 2.5 (PM2.5), nitrogen dioxide (NO_2), and ozone (O_3), compared to other pollutants, are well known for inducing neuroinflammation and oxidative stress, as explained by Brockmeyer and other researchers in a peer-reviewed study on the role of neuroinflammation in air pollution. Additionally, Brockmeyer explains the process by which pollutants enter the brain by entering the lungs, crossing and disrupting the protective blood-brain barrier, and eventually leading to overall neuronal damage. (Brockmeyer and D'Angiulli).

Among these air pollutants, PM2.5 has been shown to be the most extensively studied by most environmental science researchers. A large population-based cohort study discovered that increased exposure to PM2.5 may be associated with changes in amyloid-beta related biomarkers (Casey et al.). However, this study reported less consistent findings and results when examining the association between other biomarkers, like tau, which shows that the effects PM2.5 has on Alzheimer's disease development is not uniform across each and every aspect of the disease progression. These findings suggest that PM2.5 effects across different biological pathways are unclear and have not been researched enough. Moreover, nitrogen dioxide, a traffic pollutant, has been examined for its particular role and effects on neuronal damage. Specifically, some studies have shown that NO_2 exposure is possibly associated with neuronal/axonal injury and NfL-related biomarkers (Kim et al.). Similarly, to PM2.5, these findings are not uniform across different biological processes and vary based on the type of biomarker, which is crucial to note here because there is a lack of studies that conduct a comparison between the different effects of these individual pollutants through linking them to their corresponding biomarkers. Lastly, ozone is examined as one of the main environmental factors that contribute to neurodegeneration, although it is less frequently researched in human populations in some published studies. Most research involving ozone and air pollution suggests its effects on oxidative stress and neuroinflammation. However, this pollutant presents a more complex and less consistent association with Alzheimer's disease specifically, compared to the other two pollutants. Many published studies focus on ozone's relationship with dementia or neurodegenerative diseases in a general aspect. Across many studies, this pollutant has been shown to have strong and consistent associations with two key biological pathways, neuroinflammation and oxidative stress. A study on the mechanisms of ozone-induced neurotoxicity specifically shows how this happens, by generating ROS, which stands for reactive oxygen species, which can eventually lead to damage to neuronal cells by disrupting mitochondrial function and lipid peroxidation (Marin-Castaneda et al.). A very recent study, which examines the associations between air pollutants and the markers of neuroinflammation, found no significant association between ozone and core AD biomarkers. However, numerous other studies have shown that ozone contributes to some amyloid beta accumulation, but absolutely no associations with the biomarker tau.

Moving on from the individual effects of these air pollutants, more studies have constantly emphasized the role of neuroinflammation and oxidative stress as they are key pathways that are linked with Alzheimer's disease. Leading researchers in neuropathology and neurotoxicity departments explain that individuals who are exposed to high concentrations of air pollution show early signs of Alzheimer's pathology. Moreover, in Lane's study, they explain

that through sustained inflammation and oxidative stress, air pollution remains to be a major contributor to these neurological processes, reinforcing the idea that air pollution cannot be affected through one biological pathway, but rather multiple interactions with biological processes.

Despite the growing body of research in this field, several limitations remain. Many of these studies focus on an individual pollutant or group of pollutants without comparing their effects on different biological processes in one study. All biological processes are indicated by biomarkers, which are crucial in my study as it provides measurable evidence and a more precise comparison between these air pollutants and biological processes. There are limited studies that focus on and compare the strengths of these pollutants and biological effect relationships, while linking those effects with measurable evidence of their corresponding biomarkers. Therefore, a clear gap exists on comparing how each pollutant influences those key biological pathways from a quantitative data analysis. This leads me to my research question: To what extent do specific air pollutants influence the biological mechanisms associated with the development of Alzheimer's disease as measured through related biomarkers?

Before diving into my methodology, from identifying the gaps in existing literature and forming a research question, I hypothesized that specific air pollutants will differentially influence biological mechanisms associated with Alzheimer's disease while being reflected through its corresponding biomarkers. Specifically, I believe that PM2.5 and NO_2 will have the strongest associations with mechanisms with the strongest correlation with Alzheimer's development, resulting in those pollutants having the most harmful effects compared to O_3.

Methodology

This project uses a quantitative correlational design and secondary data analysis to explore and analyze the relationships between 3 types of air pollutants, PM2.5, NO_2, O_3, and key Alzheimer's biological pathways while linking them to their corresponding biomarkers for a more precise comparison. Rather than collecting primary data, this research design approach is aimed to analyze existing and published studies and databases to properly evaluate how each air pollutant influences these biological processes linked to measurable indicators of Alzheimer's disease.

The primary sources used for analysis and data collection came from academic databases such as PubMed, ScienceDirect, the EPA Air Quality database, the Alzbiomarker database, and academic journals. The independent variables in this research are the long-term exposures to each of the three air pollutants as well as the air pollutants themselves. This exposure data was collected from the EPA (United States Environmental Protection Agency) air quality database. This database collects and monitors exposure data from across the country. The exposure data values were collected to mirror realistic environmental conditions, rather than extreme experimental conditions. The dependent variables involved in my research are the Alzheimer's biomarkers such as Aβ42 (amyloid pathology), tau-p181 (tau pathology), NFL (neurofilament light chain or neuronal injury), and YKL-40 / GFAP (neuroinflammation). These specific

biomarkers were chosen in my study because they correspond to the key biological processes researched in Alzheimer's disease and serve as measurable indicators for these processes, providing a precise comparison for the pollutant and mechanism relationships.

Quantitative data were collected from peer-reviewed studies and academic journals that reported the associations between pollutant exposure and mechanism levels, using statistical values. The data for PM2.5 were collected from large-scale human cohort studies from ScienceDirect by Alemany and other researchers, which specifically looked at the statistical relationships between PM2.5 and NfL and Aβ42. The data for NO_2 was collected from those studies as well, except those studies reported to have associations with NO_2 exposure and tau pathology, using statistical values. The specific biological mechanism data collected for ozone came from studies that used animal experimental models by Zhang and other researchers. Data were also collected from general peer-reviewed studies focusing on the association between ozone and neuroinflammation. However, this pollutant had some limitations due to the limited availability of studies that recorded mechanism and pollutant exposure data from human CSF, as well as specific statistical values to support the strength of the associations.

Biomarker data was collected to assess the strength of its association with Alzheimer's disease. The biomarker data was collected from the Alzbiomarker database which organizes decades on fluid (CSF) biomarkers for Alzheimer's disease. The specific data recorded for each biomarker was compiled of statistical significance (p-values), effect sizes, and the direction of AD.

To properly analyze these findings, they were organized into two tables. Table 1 to record and summarize all of the pollutant-mechanism relationships, including any statistical values that came from the study. While Table 2 further supported those findings by recording all of the appropriate effect sizes and statistical values for each biomarker that was researched in my study and were shown to have an association with the air pollutants. Additionally, this research approach was informed by epidemiological studies and papers, but was instead adapted and refined into a secondary data analysis approach to perform a comparative analysis study. The purpose of creating these two tables is to properly compare the different influences air pollutants can have on Alzheimer's related mechanisms and linking it to how strongly it is associated with the development of Alzheimer's disease.

Results

Table 1: Mechanism Association Between Air Pollutants and Alzheimer's Biological Pathways

Pollutant	**Amyloid Pathology (Aβ42)**	**Tau Pathology (tau-p181)**	**Axonal Injury (NFL)**	**Neuroinflammation (YKL-40 / GFAP)**
PM2.5	↓ CSF Aβ42 (β= -0.10)	**Not reported**	↑ NFL (β= 0.31-0.38)	↑ Proposed mechanism (not directly measured)
NO_2	**Not reported**	CSF t-tau (β =	↑ NFL (β= 0.36)	↑ Proposed mechanism (not

		0.34)		directly measured)
O_3	↑Aβ accumulation (animal model)	**Not reported**	↑ Hippocampal neuronal damage (animal model)	↑ TNF-α, ↑IL-6

* Arrows (↓↑) indicate increased or decreased levels.

Table 2: Strength of Alzheimer's biomarkers (AD vs. CTRL)

Biomarker	**Pathway**	**Effect Size**	**p-value**	**Direction of AD**
Aβ42 (CSF)	Amyloid plaque accumulation	0.552	p<0.0001	↓
tau-p181 (CSF)	Tau phosphorylation/ neurofibrillary tangle formation	1.867	p<0.0001	↑
tau-total (CSF)	Overall neurodegeneration and neuronal damage	2.477	p<0.0001	↑
NFL (CSF)	Axonal injury/neuronal structural damage	1.983	p<0.0001	↑
YKL-40 (CSF)	Neuroinflammation/microglial activation	1.362	p<0.0001	↑
GFAP (Plasma & Serum)	Astrocyte activation/inflammatory response	1.928	p<0.0001	↑

Overview and Analysis

This study examined the extent to which three different air pollutants: PM2.5 (Particulate Matter), NO_2 (Nitrogen Dioxide), and O_3 (Ozone), are all associated with biological mechanisms in Alzheimer's disease. The independent variables in this study included long-term exposure levels (national level) for each air pollutant. The exposure data was collected from the EPA (Environmental Protection Agency) Air Quality database. These typical exposure concentrations were 8-9 ug/m^3 for PM2.5, 40-45 ppb for NO_2, and 0.07 ppm for O_3. Different concentrations included different units based on the physical properties of each pollutant. PM2.5 is a solid particulate matter, so it is measured in micrograms per cubic meter, which represents its mass concentration in the atmosphere. On the other hand, NO_2 and O_3 are gases, so they are measured in parts per billion (ppb) or parts per million (ppm). The dependent variables in this study included amyloid pathology (Aβ42), tau pathology (tau-p181), axonal Injury (NFL), and neuroinflammation (YKL-40 / GFAP).

PM2.5's results showed the strongest correlation with amyloid pathology in human cohorts. At the typical national exposure level of 8-9 ug/m^3, the data reported that higher long-term exposure to PM2.5 resulted in a 0.10 standard deviation (β= -0.10) decrease in CSF

Aβ42 levels. Since the decreased CSF Aβ42 levels resulted in amyloid plaque accumulation in the brain, this finding shows that environmentally relevant exposure levels can clearly contribute to signs of amyloid plaque accumulation. The data reported also that PM2.5 was associated with increased levels of NfL (axonal injury) by 0.31-0.38 standard deviations (β= 0.31-0.38). NFL stands for neurofilament light chain, which is a structural protein that is released into the bloodstream or CSF (cerebrospinal fluid) when neuronal axons are damaged, resulting in structural axonal injury. While neuroinflammation (YKL-40 / GFAP) was proposed as a biological mechanism in the literature and peer-reviewed studies, inflammatory biomarkers were not directly qualified in the studies as well as direct measurements of the neuroinflammation levels. However, the Alzbiomarker database lists YKL-40 / GFAP as one of the biomarkers, which is strongly associated with neuroinflammation. Overall, PM2.5 shows to heavily influence amyloid pathology and axonal injury pathways at its typical exposure levels.

The results for nitrogen dioxide (NO_2) showed to have significant associations with increased levels of tau pathology and axonal injury. At the typical national exposure level of 40-45 ppb, the data reported that NO_2 was positively associated with a 0.34 standard deviation (β = 0.34) increase in CSF t-tau levels. Additionally, NO_2 was associated with increased levels of NFL (axonal injury) by 0.36 standard deviations (β= 0.36). From the reported data in the literature, NO_2 was not shown to have as strong of an association with increased Aβ42 levels as PM2.5 did. As with PM2.5, neuroinflammation was a proposed biological mechanism but not directly measured in the human cohort.

The results for ozone O_3 showed strong associations with amyloid pathology, axonal injury, and neuroinflammation. The data reported for O_3 differed from PM2.5 and NO_2, resulting in some possible limitations in this study. The data collected for this air pollutant were derived from a peer-reviewed study that involved using animal experimental models, rather than human CSF studies. The typical national exposure level was 0.07 ppm, but most of the mechanistic data collected from the animal experimental model study used higher exposure levels, which included 0.25 ppm and 1 ppm. At 0.25 ppm, the data reported increased levels of hippocampal neuronal damage in Table 1. This connects to axonal injury as structural neuronal injury mirrors similar processes as biomarkers such as NFL. At 1 ppm, increased TNF-α and IL-6 levels were observed. TNF-α and IL-6 are proinflammatory cytokines which are established indicators of neuroinflammation, aligning with the neuroinflammation column in Table 1. Additionally, significant increases were observed in Aβ42 levels, resulting in Aβ accumulation in brain tissue. Despite the fact that the animal experimental study collected data in a setting using higher O_3 exposure levels (0.25 and 1 ppm), which are significantly higher than the typical exposure levels, 0.07 ppm, it concluded that O_3 levels are directly proportional to amyloid accumulation, axonal injury, and neuroinflammation. Most importantly, data collected directly from human CSF biomarker-associated studies were not reported or found for O_3 in the peer-reviewed literature. Hence, O_3's influential data is supported by animal experimental mechanistic data instead of larger-scale human biomarker data.

The data observed from Table 2 were collected from the Alzbiomarker database. The data from this table quantifies how strongly each biomarker is associated with Alzheimer's disease by comparing individuals who are diagnosed with Alzheimer's disease and those who are not (healthy/control). The effect size column represents the magnitude of that difference. A greater effect size shows that the biomarker is strongly a characteristic of Alzheimer's pathology. Out of all the biomarkers observed in Table 2, total-tau indicates the greatest effect size, 2.477, showing that the overall neurodegeneration is one of the most prominent biological mechanisms associated with Alzheimer's. Moreover, NfL and tau-p181 also show large effect sizes. NFL's effect size, 1.983, and tau-p181's effect size 1.867, represent significant tau phosphorylation/tangle formations and axonal injury in individuals with Alzheimer's disease. The two biomarkers strongly associated with neuroinflammation had effect sizes such as GFAP as 1.928 and YKL -40 as 1.362. However, Aβ42 (CSF) shows a moderate effect size, 0.552, and is decreased in Alzheimer's. This decrease still means that Aβ42 still has a strong and harmful association with Alzheimer's, indicating that amyloid-beta becomes deposited into plaques in the brain tissue instead of staying in CSF, lowering the Aβ42 (CSF) levels.

Even though amyloid pathology is widely known to be central to the development of Alzheimer's disease, the smaller effect size of Aβ42 (CSF) does not necessarily mean that it is irrelevant. It suggests that amyloid accumulation possibly occurs during the early stages of Alzheimer's, even years prior to diagnosis. By then, individuals are already diagnosed with Alzheimer's disease and biomarkers such as tau-related neurodegeneration and axonal injury might show significantly greater effect sizes between AD patients and the healthy individuals. All the biomarkers listed in Table 2 show a p-value column, where it is observed that they all share the same p-value ($p<0.0001$). This simply indicates that differences between Alzheimer's disease and healthy control groups are statistically reliable. The "Direction of AD" shows whether each biomarker increases or decreases in Alzheimer's disease compared to the control group. This column is relevant in my findings because it helps determine whether air pollution changes biomarkers in the same direction as Alzheimer's disease. Ultimately, this table serves as a comparison between Table 1, to indicate the extent to which air pollutants influence these biomarkers, which differ in association with Alzheimer's disease.

The air pollutants, PM2.5 and NO_2, show strong associations with tau-phosphorylation and NFL, which are the biomarkers observed to have the largest effect sizes in Alzheimer's disease, which possibly indicates an alignment with high-impact neurodegenerative pathways. O_3 shows the activation of inflammatory and amyloid accumulation primarily through animal experimental models, and does not show any association with tau-pathology, which includes biomarkers with the largest effect sizes. Overall, these findings support my hypothesis that individual air pollutants, particularly PM2.5 and NO_2 will differentially influence biological mechanisms and pathways that are strongly associated with the development of Alzheimer's disease.

Limitations

While this research recognizes the significant correlations between particular air pollutants and Alzheimer ’s-related biological mechanisms, there are several limitations that should be considered after analyzing the findings. First, the data collection relies on a secondary data analysis method, utilizing published peer-reviewed papers and publicly available online databases such as the EPA Air Quality Index and Alzbiomarker database, rather than collecting data directly from experimental methods, which might show more accuracy. As a result, this study does not aim to directly identify the cause of air pollution on Alzheimer’s disease. Instead, the analyses are established from observed correlations between air pollutants with biological mechanisms/pathways and their associated Alzheimer’s biomarkers. While the exposure results that were recorded in this paper show the typical exposure levels nationally, they provide a broader and general reference for air pollution exposure. It is important to consider that this air quality database does not show individual exposure levels, which are reliant on geography, environmental conditions, and other factors. Additionally, a limitation that should be considered is that in some scenarios, it is not clear whether an individual has already experienced symptoms or is in the early stages of Alzheimer’s, since Alzheimer’s gradually develops over the span of a few years. Other factors such as age, medical history, and genetics, were not taken into account and would make it more difficult to determine how strongly pollutant exposure alone can influence these biological stages. Another limitation that should be considered is that some of the mechanism findings, specifically for the ozone pollutant, come from an animal experimental model study. Even though these animal models show proper insights and findings focusing on biological pathways like neuroinflammatory pathways and oxidative stress, these findings may not align with human-related results. Regardless of these limitations, this paper still provides a useful and insightful understanding of the correlation between pollutants' association with biological mechanisms and the development of Alzheimer’s.

Summary and Research Implications

Regardless of these constraints, these findings and results contribute to a growing body of research that focuses on and observes the effects of environmental conditions on the development of neurodegenerative diseases. This study aims to provide an additional understanding of the comparison of different air pollutants and their influence on crucial neurodegenerative pathways. The findings from my data collection, such as PM2.5 and NO_2 which align with neurodegenerative pathways, axonal injury and tau-phosphorylysation, the pathways that are associated with the biomarkers, tau-p181 (CSF), tau-total (CSF), and NFL (CSF), which have the largest effect sizes compared to the other existing biomarkers, meaning that they are strongly associated with the development of Alzheimer’s disease. These comparisons serve as a foundation for evaluating and analyzing which environmental exposures may have the greatest biological impact related to Alzheimer’s pathology. Public health policymakers could potentially use this research to enforce stricter and safer health policies as well as testing different methods to reduce pollution in areas where exposure levels are highly toxic and with higher rates of neurodegenerative diseases in individuals. Moreover, urban

planners and environmental officials could focus on reducing emissions that come from traffic and industrial sources that release or produce these toxic pollutants into the atmosphere, especially PM2.5 and NO_2, according to my findings. For researchers particularly specializing in environmental science and neurobiology, these findings emphasize the significance of focusing on and combining air pollution exposure data with mechanism and biomarker-related research, which not a lot of researchers tend to focus on when researching neurodegenerative diseases. Future research could build off of this, while using more accurate methods, by recording individuals' air pollution exposure over time and measuring and observing changes in Alzheimer 's-related biomarkers in those same individuals. Ultimately, these findings reveal public health implications, such as recognizing specific environmental factors that contribute to neurodegenerative diseases, which could potentially support efforts to reduce harmful exposure and to take proper precautions when living in geographic areas with higher levels of exposure, as well as educating different strategies and approaches aimed to protect long-term brain health and prevent early stages or symptoms of neurodegenerative diseases.

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Trade-Offs Between Protein Response and Solution Persistence Across Ethanol Concentrations: An Independent-Sample Proxy Study By Juha Kim

Abstract

Ethanol concentration affects several properties relevant to antiseptic formulations, but concentration alone does not establish antimicrobial efficacy. This study independently quantified two accessible proxy outcomes across 0%, 35%, 50%, 70%, 83%, and 99.9% ethanol: visible egg-albumen protein response and solution persistence on stainless steel. For Experiment A, ten independent albumen samples per concentration were assigned a predefined ordinal coagulation score from 0 to 4. For Experiment B, ten independent 1.0 mL samples per concentration were weighed at 0–60 min, and each curve was summarized by the normalized area under the remaining-mass curve (AUC) and the 60-min remaining fraction (P60). Protein response scores differed across concentrations (Kruskal-Wallis $H = 57.47$, $p < 0.001$), with median scores of 3 at 70% and 83% and 4 at 99.9%. Normalized AUC and P60 also differed ($H = 56.14$ and 55.24, respectively; both $p < 0.001$) and were lower at 83% and 99.9% than at 70%. Four exploratory geometric balance indices were then calculated from concentration-level summary estimates. A linear protein score combined with AUC ranked 99.9% highest, a plateau-transformed protein score combined with AUC ranked 83% highest, and both P60-based indices ranked 70% highest. Thus, increasing ethanol concentration increased the visible protein response score but decreased solution persistence. The integrated ranking depended on the protein-score transformation and persistence endpoint, supporting a concentration-dependent trade-off rather than a single universally optimal concentration.

Keywords

Ethanol; Ethanol concentration; Antiseptic formulations; Egg albumen; Protein response; Ordinal coagulation score; Solution persistence; Area under the curve; 60-min remaining fraction; Geometric balance index; Concentration-dependent trade-off.

1. Introduction

Ethanol is widely used in antiseptic and disinfectant formulations because it can act rapidly against many microorganisms. Its activity is not determined by concentration alone. Alcohols act at several cellular targets, including proteins and membranes, and their performance varies with organism, formulation, exposure conditions, and the presence of water or other ingredients (McDonnell and Russell; Maillard and Pascoe). The United States Centers for Disease Control and Prevention describes protein denaturation as a principal explanation for alcohol activity and reports that aqueous ethanol and isopropanol commonly show their strongest bactericidal activity in the 60–90% volume/volume range (Centers for Disease Control and Prevention). The World Health Organization has likewise recommended alcohol-based hand rubs and has published a local-production formulation containing 80% volume/volume ethanol (World Health Organization, *WHO Guidelines*; World Health Organization, *Guide to Local*

Production). These recommendations were developed from microbiological evidence and standardized use conditions, not from the assumption that a single concentration is optimal for every organism or application.

The biological response to ethanol is multidimensional. Ethanol can disrupt protein conformation and promote denaturation or aggregation, but the extent and morphology of the response can vary with protein identity, water content, ionic environment, temperature, and time (McDonnell and Russell; Maillard and Pascoe). Viral susceptibility also differs substantially among virus types, and a concentration effective against one virus may not provide the same reduction against another (Kampf). Standardized hand-hygiene studies further show that alcohol concentration interacts with application time, volume, and formulation. For example, WHO-recommended formulations and modified formulations have shown different performance depending on duration and test standard (Suchomel et al.), while dry time has been found to depend strongly on application volume and to decrease as alcohol concentration increases (Macinga et al.). Therefore, concentration should be interpreted as one variable within a larger physicochemical and microbiological system.

These considerations raise a practical question: why are intermediate aqueous ethanol concentrations commonly used instead of nearly pure ethanol, even though higher concentrations may produce stronger effects on some biological materials? Directly answering this question through antimicrobial efficacy testing would require controlled microbial cultures, validated test organisms, and standardized efficacy protocols. Such comprehensive testing is neither feasible nor appropriate within the practical and safety constraints of a high school laboratory. This study therefore aimed to examine a narrower and experimentally accessible aspect of the question by evaluating two independently measurable properties of aqueous ethanol: an alcohol-induced protein response and solution persistence over time.

To represent the first property, visible coagulation of egg albumen was used as a proxy for an alcohol-induced protein response. Egg white is a protein-rich mixture in which ovalbumin is the most abundant protein, together with proteins such as ovotransferrin, ovomucoid, ovomucin, and lysozyme (Abeyrathne et al.). Studies of purified egg-white proteins have demonstrated that aqueous ethanol can alter protein conformation and aggregation behavior (Tanaka et al.), while work on ovalbumin has shown that denaturation and aggregation are related but distinct processes whose observed structures depend on experimental conditions (Weijers et al.). Accordingly, the albumen assay used in this study did not measure antimicrobial efficacy or microbial protein denaturation. Instead, it measured a visible albumen response that could be classified using a predefined ordinal scoring procedure applied consistently across the concentration groups.

To represent the second property, mass loss from ethanol-water solutions was used as a proxy for solution persistence. Evaporation of ethanol-water mixtures is composition-dependent and can involve changes in liquid composition, surface behavior, and preferential loss of the more volatile component (Sefiane et al.; O'Hare et al.; Pandey and Chandra). Consequently, the total mass remaining at a given time does not directly indicate either the remaining ethanol

concentration or the duration of effective antimicrobial contact. Nevertheless, a normalized mass time series can provide an operational measure of how much of a fixed initial solution remains over time under a predefined experimental setup. In this study, the area under the remaining-mass curve (AUC) summarized the complete 60-min trajectory, whereas the remaining fraction at 60 min (P60) represented a single final-time endpoint. The use of both measures also allowed the influence of persistence-endpoint selection on the integrated analysis to be examined.

These properties were used as experimental proxies rather than as direct measures of microbial killing. This study first evaluated how each property varied across ethanol concentrations and then combined the two outcomes into exploratory balance indices to examine which concentrations appeared to provide the most favorable overall combination of visible protein response and solution persistence.

2. Materials and Methods

2.1 Study design

This study was designed to address three related questions. First, how does ethanol concentration affect the ordinal visible protein response? Second, how does ethanol concentration affect normalized solution persistence over 60 min, as summarized by AUC and P60? Third, after the protein response and persistence outcomes have been evaluated separately, which concentrations appear to provide the most favorable overall balance when the two outcomes are combined? The first two questions were addressed through primary analyses in which ten samples within each concentration group were treated as independent observations. The third question was examined through an explicitly exploratory integrated analysis using four geometric balance indices: a linear protein component combined with AUC, a plateau-transformed protein component combined with AUC, a linear protein component combined with P60, and a plateau-transformed protein component combined with P60. Comparing these four indices also allowed this study to assess whether the resulting concentration ranking depended on how the protein response and solution persistence were represented. It was hypothesized that the visible protein response would increase with ethanol concentration, solution persistence would decrease at higher concentrations, and the concentration ranked most favorably in the integrated analysis would vary according to the selected protein transformation and persistence endpoint.

The two independent experiments were conducted using six nominal ethanol concentrations: 0%, 35%, 50%, 70%, 83%, and 99.9% volume/volume. Experiment A evaluated visible egg-albumen protein response, and Experiment B evaluated mass-based solution persistence. Each experiment included ten independent measurements per concentration (n = 10). Replicate numbers were not matched across concentrations and were not matched between Experiments A and B.

2.2 Materials and ethanol solutions

Materials included fresh raw chicken egg albumen, distilled water (JW Pharmaceutical, Gwacheon-si, Republic of Korea), 70%, 83%, and 99.9% ethanol stock solutions (70% and 83%, Green Pharmaceutical Co., Seoul, Republic of Korea; 99.9%, The Modern, Bucheon-si, Republic of Korea), 5 mL test tubes, 50 mL preparation tubes, syringes, stainless-steel condiment cups (base diameter, 35mm), a timer, and a precision digital balance (CPLB, Seoul, Republic of Korea).

Distilled water served as the 0% control. The 35% solution was prepared by mixing equal volumes of 70% ethanol and distilled water. The nominal 50% solution was prepared by mixing equal volumes of 99.9% ethanol and distilled water, producing approximately 49.95% ethanol and reported as 50%. The 70%, 83%, and 99.9% concentrations were used from their respective stock solutions. Concentrations were reported as nominal volume/volume percentages.

2.3 Experiment A: visible albumen protein response

For each sample, 1.0 mL of raw egg albumen was transferred to a test tube. A 3.0 mL aliquot of the assigned ethanol solution was dispensed from slightly above the albumen surface. This dispensing method had been selected during pilot testing because dispensing down the tube wall produced incomplete mixing, while inserting the syringe tip into albumen exposed to 99.9% ethanol caused immediate clogging. After 1 min at room temperature, the visible response was scored using the following predefined ordinal scale: 0, clear with no visible change; 1, slight loss of transparency; 2, an apparent diffuse white cloud; 3, extensive coagulation distributed throughout the tube; and 4, sharply localized opaque coagulation with transparent surrounding liquid. Ten independently prepared tubes were evaluated for each concentration. To minimize potential assessment bias, the scores were assigned by a single evaluator who was blinded to the ethanol concentration used in each test tube.

Because score 4 described a distinct morphology rather than a guaranteed proportional increase beyond score 3, the primary analysis treated the outcome as ordinal. The exploratory integrated analysis examined both a linear transformation, which assigned additional weight to score 4, and a plateau transformation, which treated scores 3 and 4 as equivalent adequate responses.

2.4 Experiment B: solution mass persistence

Each clean, dry stainless-steel cup was weighed before solution addition. A 1.0 mL aliquot of the assigned solution was dispensed into the cup, and the timer was started immediately. The total cup-plus-solution mass was measured at nominal elapsed times of 0, 10, 20, 30, 40, 50, and 60 min. The empty-cup mass was subtracted from each measurement to obtain the net solution mass. Ten independent measurements were evaluated for each concentration. A new cup and solution aliquot were used for every sample.

The 60-min period had been selected during pilot testing to produce measurable mass changes across the full concentration range. It was not intended to reproduce the much shorter

application time of clinical hand rubs. The stainless-steel cup was used as a standardized experimental surface and was not considered a model of skin.

2.5 Persistence calculations

For each independently measured cup, the remaining fraction at time t was calculated by dividing the net mass at time t (M_t) by the initial net mass (M_0):

$$P_t = M_t / M_0 \quad \text{(eq.1)}$$

The AUC of P_t from 0 to 60 min was calculated using the trapezoidal rule across the six 10-min intervals:

$$AUC = sum[(P_i + P_(i+1))/2 \times (t_(i+1) - t_i)],\ i = 0,...,5 \quad \text{(eq.2)}$$

The AUC was divided by 60 min to produce a dimensionless normalized AUC:

$$Normalized\ AUC = AUC / 60\ min \quad \text{(eq.3)}$$

The normalized AUC can be interpreted as the mean fraction of initial solution mass remaining over the full 60-min observation period. P60 was defined as M_60/M_0 and represented only the final observed remaining fraction. Neither measure identified the chemical composition of the remaining liquid.

2.6 Exploratory integrated analysis

Concentration-level protein components were calculated from the ten independent Experiment A scores. The linear component was:

$$D_linear = mean(score) / 4 \quad \text{(eq.4)}$$

The plateau component treated scores 3 and 4 as equivalent and was calculated before averaging:

$$D_plateau = mean[min(score / 3, 1)] \quad \text{(eq.5)}$$

For each concentration, a geometric balance index combined one protein component D with one persistence summary P:

$$Balance\ index = sqrt(D \times P) \quad \text{(eq.6)}$$

Four prespecified combinations were reported: D_linear with normalized AUC (reference model), D_plateau with normalized AUC, D_linear with P60, and D_plateau with P60. These indices ranged from 0 to 1. They were exploratory proxy measures rather than validated estimates of disinfectant efficacy.

2.7 Statistical analysis

All samples were analyzed as independent observations. Overall differences among the six concentration groups were evaluated separately for the protein response score, normalized AUC, and P60 using the Kruskal-Wallis test. When the global test was significant, all 15 pairwise concentration comparisons were evaluated using two-sided Mann-Whitney U tests, and p values were adjusted within each outcome using the Holm procedure. Epsilon-squared was reported as an effect-size estimate for each Kruskal-Wallis test. Statistical significance was defined as a two-sided Holm-adjusted $p < 0.05$.

Uncertainty in the exploratory balance indices was evaluated using 20,000 independent bootstrap iterations with replacement (Efron and Tibshirani). Within each concentration, ten Experiment A scores and ten Experiment B observations were resampled separately; no A observation was paired with a B observation. At every iteration, all four concentration-level indices were recalculated. Percentile 95% confidence intervals, the proportion of iterations in which each concentration ranked first, and bootstrap confidence intervals for selected differences among 70%, 83%, and 99.9% were calculated. No p values were assigned to the exploratory indices because they represented alternative transformations of the same underlying proxy outcomes. Analyses were performed in Python using NumPy 2.3.5 and SciPy 1.17.0 (Harris et al.; Virtanen et al.). A fixed pseudorandom seed was used for the bootstrap analyses to ensure reproducibility.

2.8 Ethics statement and use of artificial intelligence

The study did not involve human participants, live vertebrate animals, pathogenic microorganisms, or personal data; institutional ethics review was therefore not applicable. The grammar and style of the English in the manuscript were improved with the assistance of ChatGPT (OpenAI, San Francisco, California, USA). The author reviewed and edited the AI-generated suggestions to ensure the accuracy and integrity of the content and take full responsibility for the final version of the manuscript.

3. Results

3.1 Experiment A: protein response scores

Protein response scores increased across the concentration groups (Figures 1, 2 and Table 1). Median scores were 0, 1, 2, 3, 3, and 4 for 0%, 35%, 50%, 70%, 83%, and 99.9%, respectively. Eight of ten 70% samples and all 83% and 99.9% samples had scores of at least 3. The global concentration effect was significant (Kruskal-Wallis $H = 57.47$, $df = 5$, $p = 4.05 \times 10^{-11}$; epsilon-squared = 0.972). The adjusted comparison between 70% and 83% was not significant ($U = 40$, Holm-adjusted $p = 0.167$), whereas 99.9% had higher ranks than both 70% and 83% (both Holm-adjusted $p = 0.000239$; Table 2).

3.2 Experiment B: solution persistence

The normalized mass curves separated progressively over the 60-min observation period (Figure 3). Mean normalized AUC values were 0.962, 0.913, 0.920, 0.882, 0.828, and 0.722 from 0% through 99.9% ethanol (Figure 4 and Table 1). Mean P60 values were 0.925, 0.847, 0.832, 0.765, 0.676, and 0.488, respectively. Concentration groups differed for normalized AUC ($H = 56.14$, $df = 5$, $p = 7.62 \times 10^{-11}$; epsilon-squared = 0.947) and P60 ($H = 55.24$, $df = 5$, $p = 1.16 \times 10^{-10}$; epsilon-squared = 0.930). Among the three higher concentrations, normalized AUC and P60 were higher at 70% than at 83% and 99.9%, and higher at 83% than at 99.9% (all Holm-adjusted $p \leq 0.00271$; Table 2).

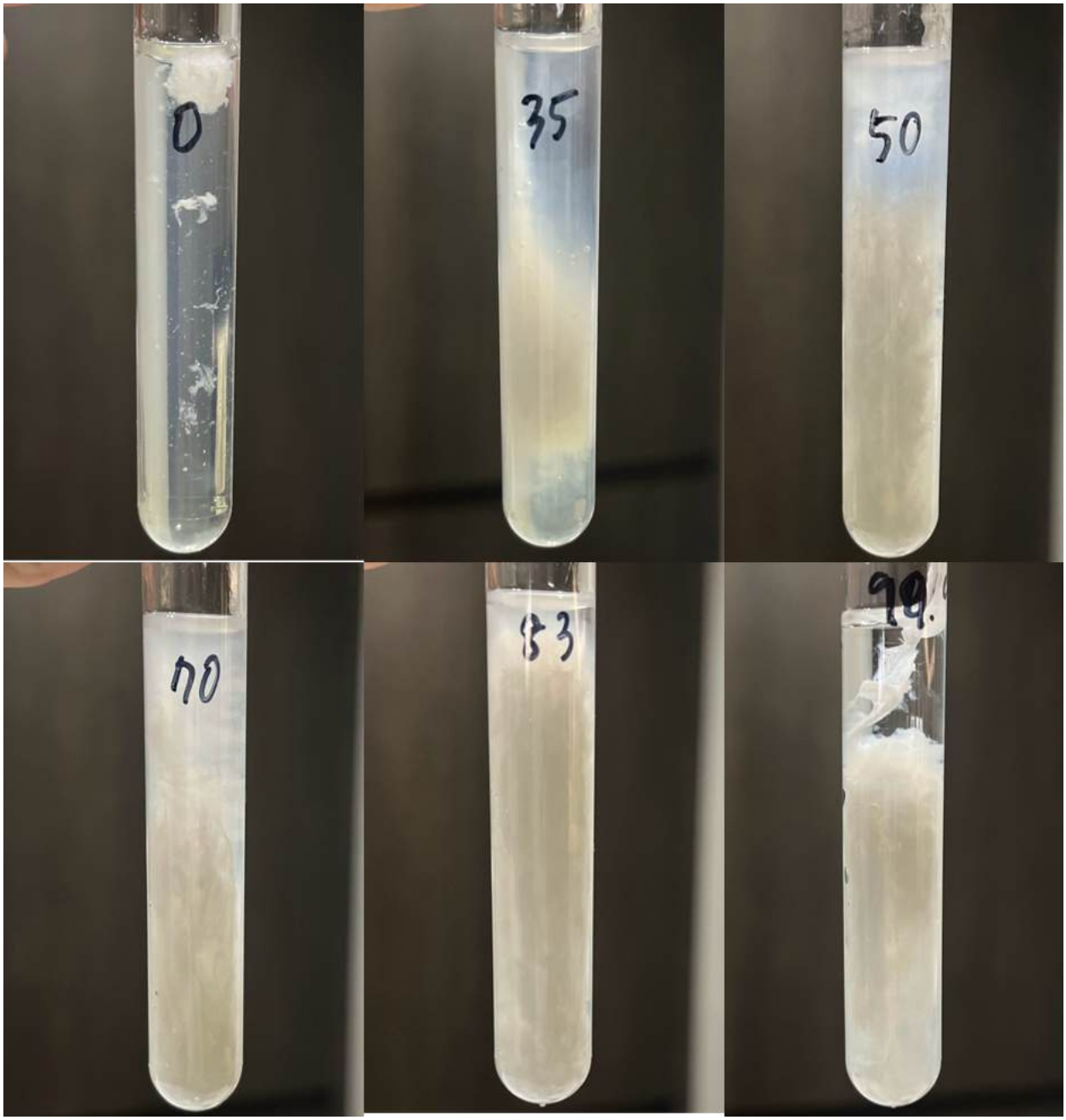

Fig 1: Representative visible albumen responses after 1 min of exposure to the indicated ethanol concentrations. The images illustrate the predefined protein response scale from 0 to 4.

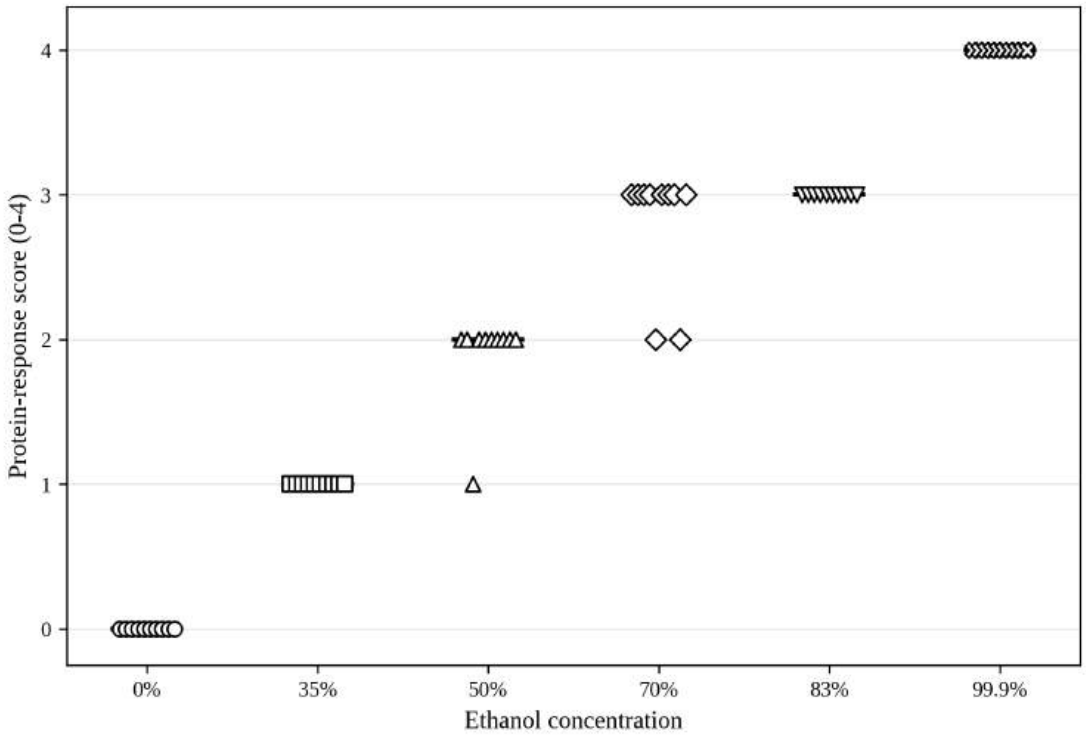

Fig 2: Protein response scores across ethanol concentrations. Each symbol represents one independent sample (n = 10 per concentration).

Table 1. Primary outcome summaries by ethanol concentration.

Ethanol	Protein score, median (IQR)	Score ≥ 3	Normalized AUC, mean ± SD	P60, mean ± SD
0%	0 (0–0)	0/10	0.962 ± 0.008	0.925 ± 0.012
35%	1 (1–1)	0/10	0.913 ± 0.009	0.847 ± 0.028
50%	2 (2–2)	0/10	0.920 ± 0.005	0.832 ± 0.029
70%	3 (3–3)	8/10	0.882 ± 0.011	0.765 ± 0.033
83%	3 (3–3)	10/10	0.828 ± 0.011	0.676 ± 0.033
99.9%	4 (4–4)	10/10	0.722 ± 0.010	0.488 ± 0.038

Abbreviations: IQR, interquartile range; AUC, area under the remaining-mass curve; SD, standard deviation; P60, remaining fraction at 60 min.

Table 2. Primary inferential statistics and selected pairwise comparisons.

Outcome	Test/comparison	Statistic	Raw p	Holm p	Epsilon-squared
Protein score	All groups	H = 57.466	4.05×10^{-11}	-	0.972
Normalized AUC	All groups	H = 56.136	7.62×10^{-11}	-	0.947
P60	All groups	H = 55.241	1.16×10^{-10}	-	0.930
Protein score	70% vs 83%	U = 40	0.167489	0.167489	-
Protein score	70% vs 99.9%	U = 0	0.000033	0.000239	-
Protein score	83% vs 99.9%	U = 0	0.000016	0.000239	-
Normalized AUC	70% vs 83%	U = 100	0.000182	0.002710	-
Normalized AUC	70% vs 99.9%	U = 100	0.000182	0.002710	-
Normalized AUC	83% vs 99.9%	U = 100	0.000183	0.002710	-
P60	70% vs 83%	U = 97	0.000433	0.002605	-
P60	70% vs 99.9%	U = 100	0.000180	0.002605	-
P60	83% vs 99.9%	U = 100	0.000181	0.002605	-

Note: Global rows report the Kruskal-Wallis H test. Pairwise rows report two-sided Mann-Whitney U tests. Holm p values were adjusted across all 15 pairwise comparisons within each outcome.

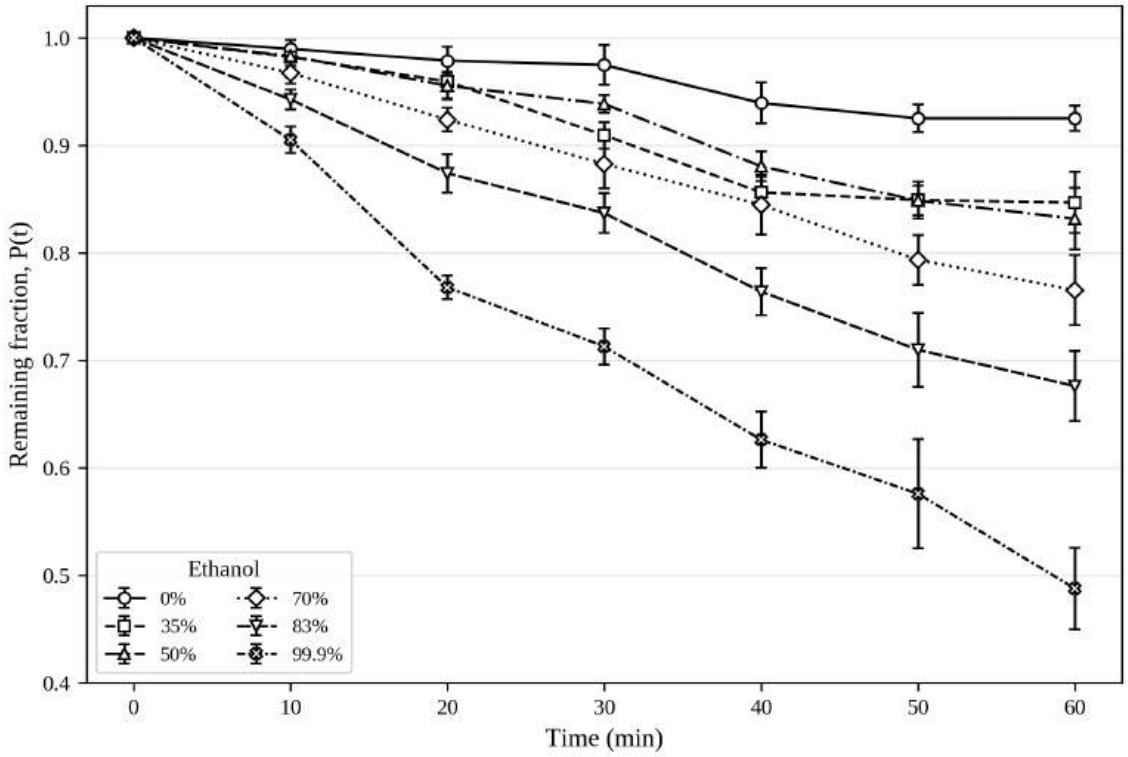

Fig 3: Normalized solution-mass persistence over 60 min. Points and error bars show mean ± standard deviation for ten independent samples per concentration.

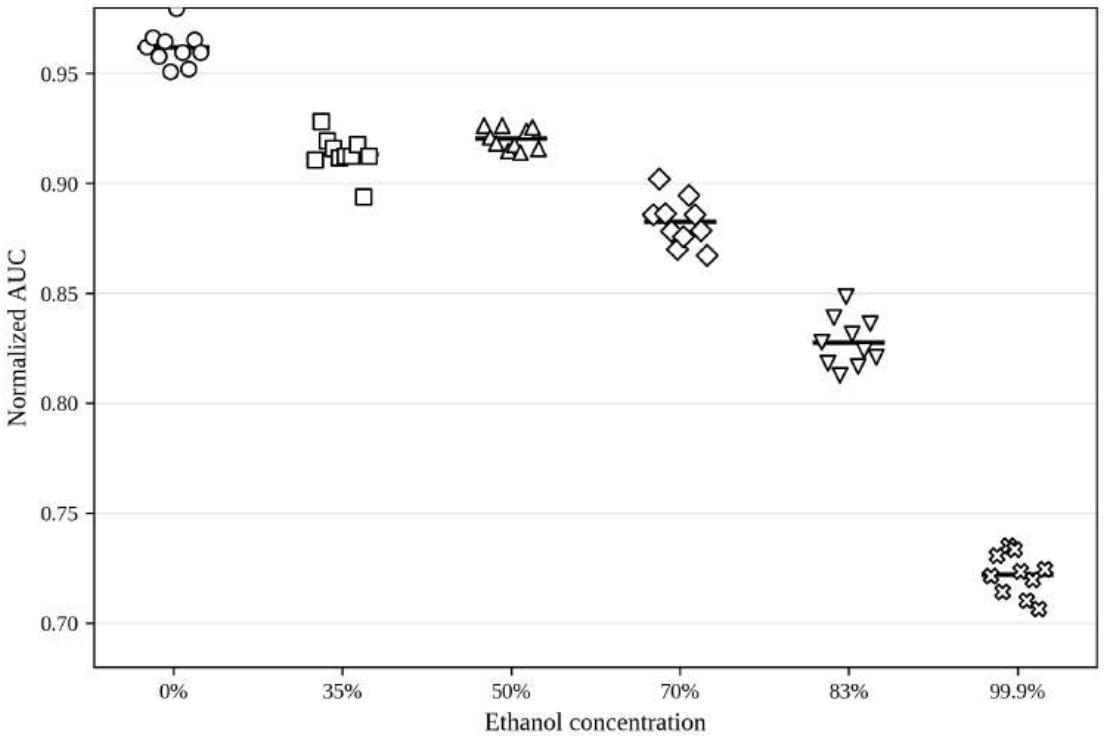

Fig 4: Normalized area under the remaining-mass curve (AUC) across ethanol concentrations. Symbols represent independent samples; horizontal bars indicate means.

3.3 Exploratory integrated and sensitivity analyses

The concentration-level protein and persistence estimates showed an opposing pattern (Figure 5). The integrated ranking changed across the four prespecified definitions (Table 3 and Figure 6). In the reference linear-plus-AUC model, 99.9% had the highest point estimate (0.850) and ranked first in 100% of bootstrap iterations. When scores 3 and 4 were treated as a plateau, the AUC-based model ranked 83% highest (0.910), but the top-rank probabilities were divided between 83% (56.3%) and 70% (43.7%). Both P60-based models ranked 70% highest, with top-rank probabilities of 86.1% for the linear transformation and 86.4% for the plateau transformation (Figure 7).

For all four definitions, the bootstrap confidence interval for the difference between 70% and 83% included zero. In the linear-plus-AUC model, 99.9% exceeded both 70% and 83%. In the plateau-plus-AUC model, both 70% and 83% exceeded 99.9%. In the plateau-plus-P60 model, the 70%-99.9% difference was 0.147 (95% confidence interval, 0.101–0.184), and the 83%–99.9% difference was 0.124 (0.103–0.142).

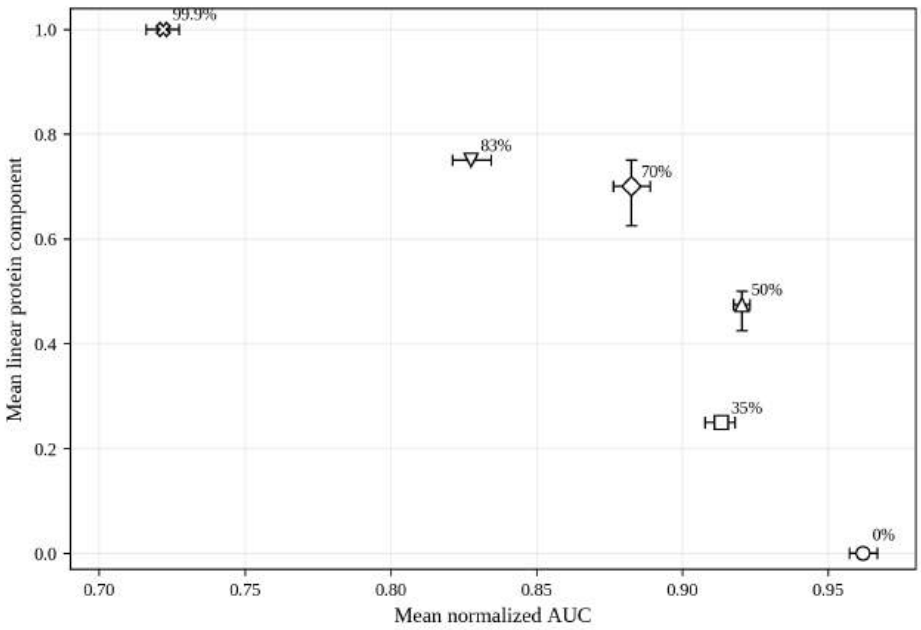

Fig 5: Trade-off between concentration-level protein response and solution persistence. Error bars show independent-bootstrap 95% confidence intervals.

Table 3. Exploratory balance indices for the three higher ethanol concentrations.

Definition	70%	83%	99.9%	Highest point estimate	Top-rank probability
Linear + AUC	0.786 (0.743–0.815)	0.788 (0.785–0.791)	0.850 (0.846–0.853)	99.9%	100.0%
Plateau + AUC	0.908 (0.858–0.941)	0.910 (0.906–0.913)	0.850 (0.846–0.853)	83%	56.3%
Linear + P60	0.732 (0.694–0.761)	0.712 (0.702–0.722)	0.698 (0.685–0.716)	70%	86.1%
Plateau + P60	0.845 (0.801–0.879)	0.822 (0.811–0.834)	0.698 (0.685–0.716)	70%	86.4%

Note: Values are point estimates (95% bootstrap confidence interval). AUC models used normalized AUC; P60 models used the 60-min remaining fraction. Plateau models treated protein scores 3 and 4 as equivalent. Top-rank probability is the percentage of 20,000 bootstrap iterations in which the listed concentration ranked first.

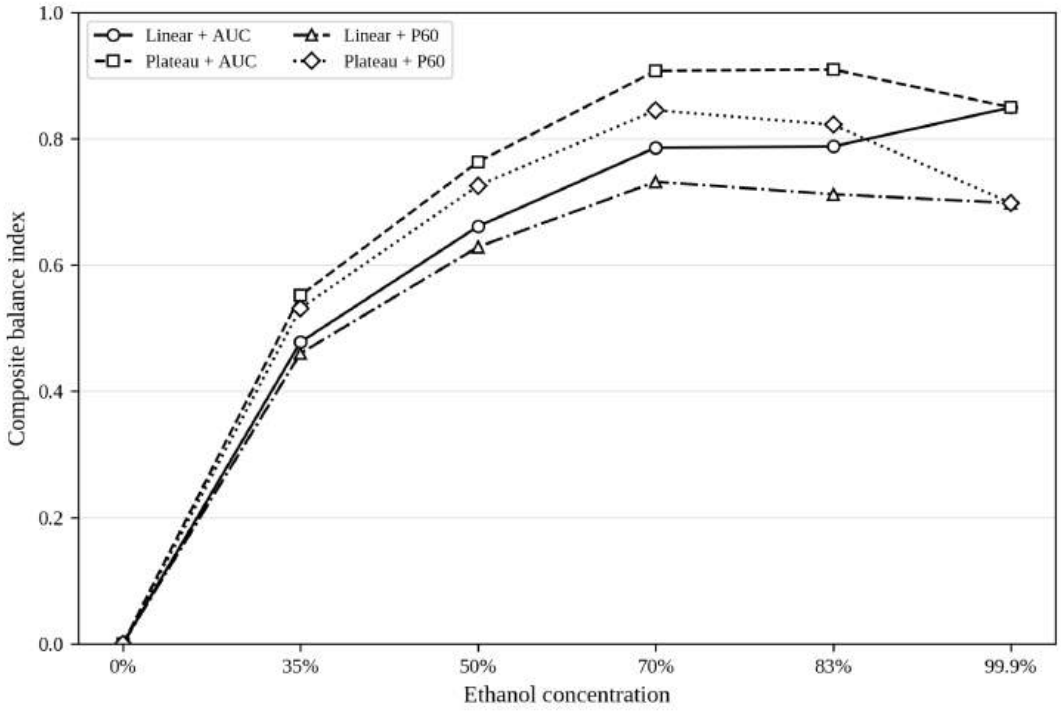

Fig 6: Sensitivity of exploratory balance-index values to the protein-score transformation and persistence endpoint.

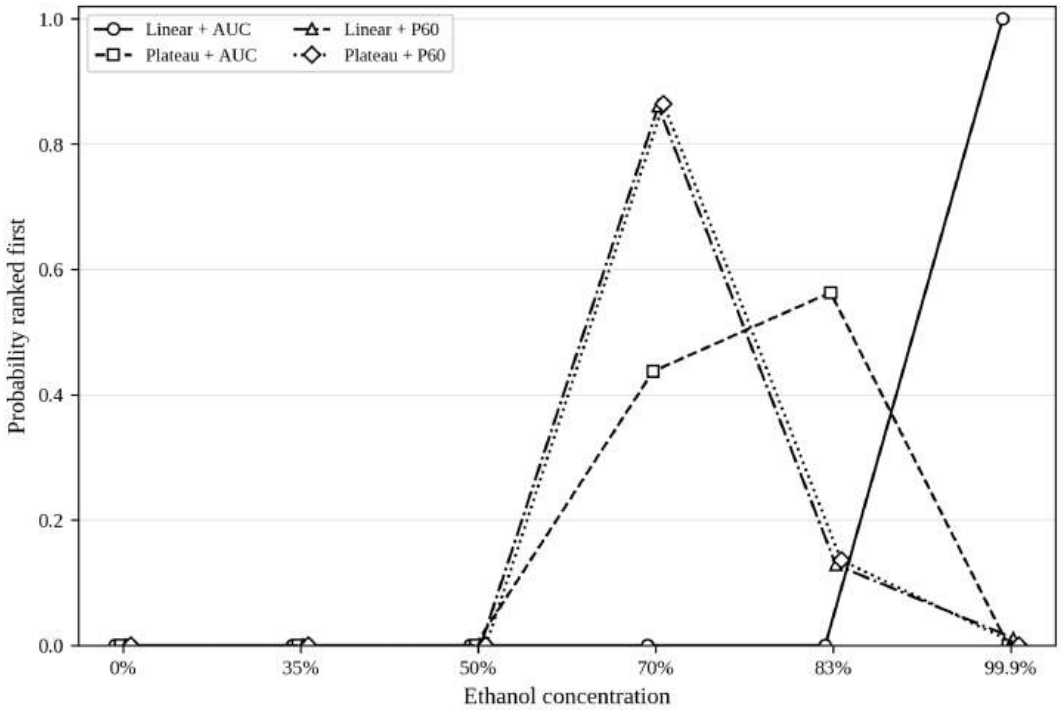

Fig 7: Bootstrap probability that each ethanol concentration ranked first under each exploratory balance-index definition.

4. Discussion

4.1 Principal findings

This independent-sample study identified two strong but opposing concentration-dependent patterns. Visible albumen response increased with ethanol concentration, while total solution-mass persistence decreased at higher concentrations. These primary results were supported by large Kruskal-Wallis effect-size estimates and by adjusted pairwise comparisons among the higher concentrations. However, the exploratory integration did not identify a single concentration that remained highest under all reasonable definitions. Linear scoring with AUC favored 99.9%, plateau scoring with AUC favored 83%, and both P60-based definitions favored 70%. The most defensible overall conclusion is therefore that concentration created a trade-off between the two measured proxy properties and that any composite optimum depended on analytical assumptions.

4.2 Interpreting the visible protein response

The increasing albumen score was directionally consistent with the known ability of alcohols to disrupt protein structure and promote aggregation (McDonnell and Russell; Maillard and Pascoe; Tanaka et al.). Egg albumen provided a low-risk, accessible mixture of proteins rather than a purified target, and visible whitening or coagulation likely reflected a combination of unfolding, association, phase separation, and precipitation. The assay therefore should not be interpreted as a direct measurement of molecular denaturation or microbial inactivation. The 99.9% samples received score 4 because they formed sharply localized opaque clumps with a clearer surrounding liquid, whereas scores of 3 at 70% and 83% represented extensive coagulation distributed throughout the tube. These are morphologically distinct outcomes, and the ordinal scale does not prove that score 4 represents a proportional biological improvement over score 3.

This distinction explains why the score transformation strongly affected the integrated ranking. The linear component assigned 99.9% a full value of 1.0 and assigned a mean score of 3 at 83% a value of 0.75. The plateau component instead treated all scores of 3 or 4 as adequate and therefore removed the extra credit assigned to localized score 4 coagulation. Once this assumption was changed, the greater persistence of 70% and 83% became more influential. Reporting both transformations made the dependence on the scoring model visible rather than hiding it within a single index.

4.3 Interpreting AUC and P60

The normalized AUC summarized the complete seven-point remaining-mass curve as the mean fraction of initial solution mass present during the 60-min period. It therefore incorporated both early and late mass loss and reduced reliance on one final measurement. P60 answered a different question: how much total solution mass remained at exactly 60 min. The two measures were highly related in this experiment, but they did not weight time identically. Selecting AUC

or P60 changed the balance-index rankings because P60 more directly penalized concentrations with low final persistence, while AUC retained information from the earlier portion of the curve.

The persistence findings were consistent with prior observations that alcohol concentration can influence dry time and with the broader physicochemical literature on ethanol-water evaporation (Macinga et al.; Sefiane et al.; O'Hare et al.; Pandey and Chandra). Nevertheless, binary-mixture evaporation is more complex than the loss of a single pure liquid. Ethanol and water do not necessarily leave the liquid at the same rate, and the composition of the remaining liquid changes during evaporation. The present balance measurements quantified only total mass; they could not determine how much ethanol remained at each time. Accordingly, normalized AUC and P60 should be described as solution persistence proxies, not ethanol-exposure curves.

4.4 Meaning of the sensitivity analysis

A composite index can appear objective because it produces one number, but it necessarily embeds choices about scaling, endpoints, and weights. The reference model assumed that the 0–4 score was approximately linear and that full-curve persistence was the relevant complementary property. Under those assumptions, 99.9% ranked highest. The plateau-plus-AUC model assumed that the visible response had already reached an adequate ceiling at score 3; under that definition, 70% and 83% became the leading concentrations, with no stable distinction between them. The P60 models emphasized final persistence and favored 70%, again without a bootstrap confidence interval that separated 70% from 83%.

These findings do not show that one model is correct and the others are incorrect. Rather, they demonstrate that the claim of a single best concentration cannot be supported by the two proxy experiments without an externally validated rule for converting morphology and persistence into antimicrobial performance. The four models are useful as a sensitivity analysis because their disagreement identifies the assumptions that control the conclusion. The direct primary measurements—score distributions, normalized AUC, and P60—remain more fundamental than any composite ranking.

4.5 Relationship to hand-hygiene literature

The observed trade-off is broadly compatible with the fact that effective alcohol products are usually aqueous formulations rather than undiluted ethanol. CDC guidance describes an optimal bactericidal range of 60–90% in water, and the WHO local-production formulation uses 80% volume/volume ethanol (Centers for Disease Control and Prevention; World Health Organization, *Guide to Local Production*). Standardized studies have also shown that concentration, applied volume, application duration, and formulation jointly influence dry time and measured efficacy (Suchomel et al.; Macinga et al.). However, agreement in general direction is not external validation of the present index. Public-health recommendations are based on microbiological testing, organism-specific evidence, user technique, safety, and

formulation performance. This study could not measure any of those endpoints directly due to high school-level setting limitations.

The broader literature also cautions against treating a single concentration as universally optimal. Ethanol susceptibility varies among viruses (Kampf), and biocide action depends on multiple targets and microbial structures (Maillard and Pascoe). The current results therefore should not be used to recommend a hand-rub formulation or to predict performance against a specific pathogen. Their value lies in experimentally separating two properties that can move in opposite directions and in demonstrating how an apparently simple combined conclusion changes when reasonable assumptions are altered.

4.6 Limitations

Several limitations restrict interpretation. First, egg albumen is a heterogeneous food protein mixture and is not a microorganism. The visual score was ordinal and observer-dependent, and no spectrophotometric measurement or inter-rater reliability assessment was performed. Scores 3 and 4 represented different morphologies, making a linear numerical interpretation uncertain. Second, Experiment B measured total solution mass on stainless steel for 60 min. It did not measure ethanol concentration, microbial exposure, evaporation on skin, rubbing, spreading, or the clinically relevant dry time of a formulated hand rub. Although they can influence evaporation, ambient temperature and humidity data were not obtained. Third, the nominal ethanol solutions were not analytically verified after dilution. The 50% preparation was approximately 49.95%, and stock-solution tolerances could have introduced additional uncertainty. Fourth, although n = 10 per concentration improved the estimate of experimental variability, all observations were produced within one experimental setting and may not generalize to different egg batches, containers, surface areas, environmental conditions, or formulations. Fifth, bootstrap confidence intervals quantified sampling uncertainty under the observed experimental design but could not correct model misspecification or measurement bias.

4.7 Future research

A stronger follow-up study should replace the albumen proxy, where feasible, with a direct antimicrobial endpoint using an appropriate safe organism and a validated quantitative assay. If the albumen model is retained as a preliminary or complementary measure, its response should be quantified using optical absorbance or image analysis, with randomized samples, blinded raters, and predefined criteria distinguishing diffuse coagulation from localized precipitation. For the persistence experiment, ethanol and water composition should be measured over time rather than inferred from total mass alone, and shorter intervals relevant to hand rubbing should be evaluated under controlled temperature and humidity using a consistent surface area and realistic gel or liquid formulations. Finally, any integrated index should be prospectively validated against microbial reduction before being interpreted as a measure of antimicrobial efficacy.

5. Conclusion

Across independent samples, increasing ethanol concentration increased the visible egg-albumen protein response score but reduced mass-based solution persistence. The primary outcomes differed strongly among concentration groups. Exploratory integration produced different leading concentrations depending on whether score 4 received additional weight over score 3 and whether persistence was summarized by the full 60-min AUC or by P60. Linear scoring with AUC favored 99.9%, plateau scoring with AUC favored 83%, and both P60-based definitions favored 70%; none of the four definitions reliably separated 70% from 83% by the bootstrap difference interval. These results do not establish direct disinfectant efficacy, support a pathogen-specific recommendation, or identify a universally optimal ethanol concentration. Within the limits of this experimental model, however, they support a concentration-dependent trade-off between protein response and solution persistence.

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Inhibition of *Escherichia coli* Adhesion Using Novel Quaternary Ammonium Silane-Functionalized Carbon Nanotubes

By Chelsea Lian & Mark Ellison PhD

Abstract

Nosocomial infections, or healthcare-associated infections, are acquired in hospital settings and affect more than 680,000 patients in the U.S. each year. Many are catheter- and intravenous line-associated infections caused by bacterial contamination on polydimethylsiloxane (PDMS), a flexible plastic widely used in medical devices. The purpose of my project is to study the inhibition of *Escherichia coli* growth on PDMS using single-walled carbon nanotubes (SWCNTs), with the potential application of reducing nosocomial infections. My hypothesis is if the novel quaternary ammonium silane (QAS)-functionalized SWCNTs are coated on PDMS, then they will inhibit *E. coli* growth more effectively compared to non-functionalized P2-SWCNTs because the introduced ammonium cation can damage bacterial membrane, leading to cell death.

The synthesis of functionalized SWCNTS was successfully completed through a condensation reaction with QAS and confirmed by FTIR spectra showing a new characteristic Si-O-C stretching peak at ~923 cm^{-1}. For the inhibition experiments, *E. coli* cultures were incubated on coated and uncoated PDMS for 3 hours at 37°C, serial-diluted, and plated on agar. Colonies were counted after 24 hours. Colony counts were converted into Colony Forming Units (CFU/mL), and the data was evaluated for significance using one-way ANOVA and post-hoc tests. The results strongly supported my hypothesis with statistical significance. QAS-SWCNTs were the most effective in inhibiting *E. coli*, with the lowest average CFU/mL of 1.1×10^8, compared to 2.2×10^9 for P2-SWCNTs and 6.3×10^9 for the plain PDMS control.

Introduction

Nosocomial infections, commonly known as healthcare-associated infections (HAIs), are acquired in hospital settings and affect more than 680,000 patients in the U.S. each year.[3] Many HAIs are caused by bacterial adhesion on polydimethylsiloxane (PDMS), a flexible silicone polymer used for implants, catheters, IV lines, and other medical instruments. However, the surface of PDMS provides an ideal environment for attachment and growth of bacteria colonies. The ability of *Escherichia coli* (E. coli), a Gram-negative microorganism linked to HAIs, to adhere to material surfaces such as PDMS emphasizes the importance of developing strategies that prevent initial bacterial attachment. Given that adhesion is largely dictated by surface chemistry, modifying the surface offers a viable approach for mitigating bacterial colonization. In this project, bacterial adhesion is tested, which does not include long-term biofilm formation, a possible future expansion of this project.

This experiment explores modifying PDMS surfaces with carbon nanotubes to hinder bacterial adhesion.[2] Carbon nanotubes have a rolled structure consisting of graphene sheets,

which is a sheet of hexagonally-arranged carbons, with carbon atoms in the sp2 hybridization. They have a wide range of properties, including being lightweight, durable, conductive, but most importantly, having antimicrobial properties. They consist of single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). SWCNT are one sheet of rolled graphene and typically have a diameter of 1-2 nm, hence the name nanometer. MWCNT are less stable because of possible structural defects and undefined diameter.[4] Specifically, single-walled carbon nanotubes (SWCNTs) are tested in this experiment, as opposed to larger multi-walled carbon nanotubes, who have a smaller surface-area-to-volume ratio. P2 SWCNTs have very few functional groups, and will be tested in this experiment. Some limitations found in research studies are the toxicity and hydrophobic nature of CNTs. However, these barriers can be reduced with their functionalization to allow for the use of these nanomaterials on medical surfaces. A novel functionalization of SWCNTs with a quaternary ammonium cationic group could potentially enhance antimicrobial properties.[1] Additionally, the presence of a long carbon chain in the functional group could increase hydrophobicity, potentially further improving effectiveness.

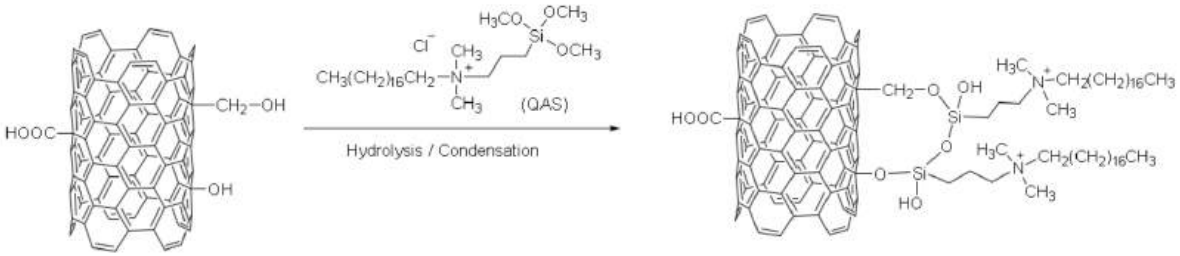

Figure A: Quaternary ammonium silane (QAS) functional group undergoes hydrolysis reaction, and forms a covalent bond with acid-oxidized single-walled carbon nanotubes through a condensation reaction.[5]

The positive charge on the ammonium cation will be attracted to the negatively-charged bacterial membrane. Therefore, the quaternary ammonium groups are hypothesized to disrupt bacterial membranes upon contact, leading to cell lysis and eventual death of bacterial cells.[1] This modification is hypothesized to reduce *E. coli* adhesion, advancing the development of safe medical materials.

Methods

Nanotubes are functionalized with dimethyloctadecy [3-(trimethoxysilylpropyl)] ammonium chloride. 10 mg COOH-SWCNT are combined with 10 mL methanol and sonicated for 30 minutes at 30ºC. This solution, 200µL QAS solution, 20 µL acetic acid, and 5 mL methanol are combined in a round-bottom flask. Then, it is covered with parafilm and spun at 40ºC for 24 hours. After the reaction is finished, the solution is filtered and washed with ethanol and deionized water through a vacuum. For a similar reaction, see: Wang et al. *Applied Surface Science* vol 707, 2025, 163658. An FTIR scan was run to confirm successful covalent bonding.

Figure B: The broad peak at 923 cm-1 demonstrates Si-O-C stretching, which is characteristic of functionalized nanotubes. Peak at 1570 cm-1 represents C-N stretching, and a peak at 2916 cm-1 shows stretching of methyl and methylene groups. The FTIR scan showed effective functionalization.

Additionally, a leach test was performed to ensure successful functionalization and that the coating would be safe for medical use. PDMS were emerged in sterile Phosphate Buffered Saline for 15 minutes. Bacteria culture is prepared 24 hours before testing by mixing one inoculation loopful of bacteria with 7 mL of LB Broth in a centrifuge tube. Bacteria are allowed to grow in a 37ºC incubator for 24 hours. Then a vortexed solution of 50 µL of the saline and 50 µL of bacteria culture were pipetted onto an agar petri dish and placed in the incubator for 24 hours. There was no significant difference between plain, P2 SWCNT, and QAS-functionalized SWCNT PDMS disks. Therefore, I could conclude that there was no leaching and the coatings would be safe in hospitals.

With safety and functionalization confirmed, the experimental procedures were started. PDMS disks are made using a 10:1 ratio of SYLGARD 184 Silicone Elastomer Base and SYLGARD 184 Silicone Elastomer Curing Agent. The resulting mixture is stirred, spun in a centrifuge, poured into a circular mold (1 cm in diameter), and cured in an oven at 77ºC for 2 hours.

PDMS disks were then spin-coated with P2 and QAS-functionalized SWCNTs until reaching five coats each. Then, PDMS were sterilized in a petri dish under UV/ozone light for 10 minutes. Previously prepared E. *coli* culture was standardized to reach an absorbance of 1.0 in a Genesys 20 spectrophotometer. 80µL of standardized bacteria culture was pipetted onto PDMS surfaces, and set in the incubator for three hours at 37ºC to allow bacteria to grow. Once incubated, 50µL of culture was extracted from the PDMS surface and added to a 2mL centrifuge tube, with 450µL LB broth. Then, the solution serial diluted until 10^{-8} dilution was reached. 50µL of culture was extracted and pipetted from 10^{-5} to 10^{-8} dilutions onto labelled and sterile agar plates. After 24 hours, plates were removed from the incubator, and colonies were counted. Any dilutions with more than 250 colonies were recorded as Too Numerous to Count (TNTC).

Results

Raw colony counts were recorded for n = 28. Then, they were transformed into Count Forming Units (CFU) per milliliter and averaged per each surface's dilutions. Standard error was calculated; CFU/mL and standard error were plotted on a bar chart below.

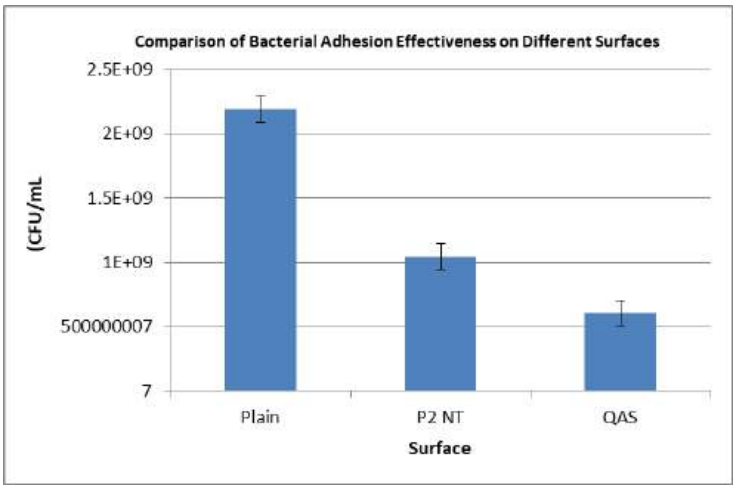

Figure C: Average CFU/mL for Plain, P2 SWCNT, and QAS-functionalized SWCNT surfaces demonstrated a significant decrease in bacterial adhesion. One-way ANOVA test resulted in a p-value of 2.19126E-9 <<< 0.05, demonstrating statistical significance. Additionally, further statistical tests with Tukey Post-Hoc HSD Test determined significance between Plain and P2 SWCNT, and between P2 SWCNT and QAS-functionalized SWCNT with p-values of 0.00002242 and 0.02853, respectively.

Discussion

1.1×10^8, compared to 2.2×10^9 for P2-SWCNTs and 6.3×10^9 for the plain PDMS control.

QAS-functionalized SWCNTs were the most effective at reducing bacteria adhesion with the lowest average CFU/mL count of 6.06E8 compared to plain PDMS with 2.19E9 CFU/mL and P2 SWCNTS with 1.09E9 CFU/mL; therefore, my hypothesis was supported and proven to be correct. The needle-like single-walled carbon nanotubes (P2 SWCNTs) punctured bacterial membranes and led to a significant decrease in *E. coli* colonies when compared to plain PDMS. Additionally, the quaternary ammonium group found in QAS-functionalized carbon nanotubes were even more effective than P2, supporting that the positive nitrogen's attraction to negatively-charged membranes led to cell death. Statistical analysis demonstrated that there was a significant difference between the three surfaces. Overall, continued testing may lead to novel developments in the medical field to reduce nosocomial infections in hospitals.

Future experiments can expand this experiment and the safety of materials in the medical field. This includes testing with Gram-positive bacteria such as *Staphylococcus epidermis*. Moreover, different or additional functionalizations could enhance bacterial inhibition.

Acknowledgements

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Sex Differences in Comorbid Conditions Among Children and Adults Diagnosed With ADHD By Sai Maheshwari Reddy Kola

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder characterized by persistent patterns of inattention, hyperactivity, and impulsivity that can interfere with an individual's functioning in daily life (National Institute of Mental Health). Symptoms typically begin in childhood and continue into adolescence and adulthood, although some individuals have symptoms that present differently across the life span and different sexes (National Institute of Mental Health). For instance, some people are more likely to display an inattentive presentation of ADHD, while others are more likely to present with hyperactivity, or a combination (National Institute of Mental Health). ADHD is associated with difficulties in academic achievement, occupational performance, interpersonal relationships, and daily functioning when left untreated (National Institute of Mental Health). Because ADHD symptoms can significantly affect the quality of life for many, early recognition in childhood and appropriate treatment is key for improving long-term outcomes.

ADHD rarely occurs in isolation and is frequently accompanied by one or more comorbid conditions (McGough et al.). These comorbid conditions are highly prevalent among individuals diagnosed with ADHD and often include depression, oppositional defiant disorder, anxiety, dysthymia, and conduct disorder (McGough et al.). The presence of these comorbidities can often make diagnosis of ADHD difficult as comorbidities can overlap or mask core symptoms of ADHD. In addition to diagnosis, having comorbid conditions also influences symptom severity, treatment, and long-term outcomes for individuals diagnosed with ADHD (McGough et al.). This evidence underscores why it is so important to gain a more in-depth understanding of comorbid conditions when diagnosing and treating individuals with ADHD.

As research on ADHD has been expanding, an increasing focus has been put on understanding whether comorbid conditions present differently in males and females, and more generally, how sex may influence the presentation and diagnosis of ADHD. Studies have shown that males with ADHD typically show comorbid externalizing disorders such as oppositional defiant disorder and conduct disorder, while females typically present internalizing disorders such as anxiety and depression (Bauermeister et al.). These differences are also reflected in academic functioning, where males with ADHD are more likely to exhibit disruptive behavior in school and face suspension (Bauermeister et al.; Biederman et al.). These differences in comorbidities may explain the finding that females are often underdiagnosed with ADHD compared to their male counterparts, as externalizing symptoms are easier to observe. By developing a better understanding of sex differences in comorbidities, research may help to improve earlier recognition and diagnosis of ADHD.

Despite growing research on ADHD comorbidities, the extent to which these conditions differ among males and females has not been fully synthesized. This review takes a developmental approach by examining these sex differences across childhood, young adulthood,

and adulthood to better understand how patterns in comorbid conditions may change or persist throughout the lifespan. Therefore, this review examines how sex differences in ADHD comorbidities develop across the lifespan, with females generally experiencing higher rates of internalizing conditions and males experiencing higher rates of externalizing conditions.

Methods

This literature review was conducted to examine the sex differences in comorbid conditions among children and adults diagnosed with ADHD by synthesizing findings from original, peer-reviewed research articles. Articles were found through Google Scholar and PubMed using search terms including ADHD, sex differences, comorbid conditions, comorbidity, ADHD in females, ADHD in males, anxiety, depression, oppositional defiant disorder (ODD), conduct disorder, autism spectrum disorder (ASD), children, and adults.

This study included only original peer-reviewed research articles that examined comorbid psychiatric conditions among people diagnosed with ADHD, and focused specifically on sex differences. One peer-reviewed systematic review was also included to provide additional evidence on the potential role of sex hormones in ADHD symptoms. Studies that did not include sex differences in comorbid psychiatric conditions among people diagnosed with ADHD were excluded.

Results

Sex Differences in Comorbidities Among School-Age Children

Research has consistently shown that sex differences exist in the comorbid conditions associated with ADHD, with females more likely to experience internalizing disorders and males more likely to exhibit externalizing disorders (Levy et al.; Bauermeister et al.; Biederman et al.). For instance, using data from children and adolescents in the Australian Twin ADHD Project, Levy et al. found that girls ages 5-19 were more likely than boys to be diagnosed with separation anxiety, while girls with the combined subtype of ADHD were also more likely to have higher rates of generalized anxiety disorder. Using a population-based sample of children in Puerto Rico, Bauermeister et al. also reported similar findings, showing that girls ages 4-17 with the inattentive subtype of ADHD were more likely to have been diagnosed with anxiety disorders than boys with the same subtype. Together, these studies suggest that girls with ADHD are more likely to be diagnosed with anxiety disorders than boys, although differences can occur based on ADHD subtype.

Findings regarding differences between school-age girls and boys in the presence of externalizing comorbidities have been less consistent across studies. Levy et al. found that among 5- to- 19-year-olds with ADHD, boys were more likely than girls to be diagnosed with oppositional defiant disorder and conduct disorder. Similarly, using a psychiatric clinic sample that included 140 boys and 140 girls with ADHD, Biederman et al. reported that boys ages 6-17 with ADHD exhibited higher rates of disruptive behavior disorders than girls. However,

Bauermeister et al. did not find significant overall sex differences in externalizing comorbidities among 4- to 17-year-olds with ADHD. Together, these findings suggest that although males with ADHD often exhibit higher rates of externalizing disorders, these differences may vary across studies and populations.

Researchers have increasingly focused on studying not only whether males and females differ in the prevalence of comorbid psychiatric conditions, but also in how ADHD affects different areas of functioning in childhood, including school performance. Bauermeister et al. discovered that 4- to 17-year-old boys with ADHD were more likely to be suspended from school than girls, while using a sample of 175 elementary school students with ADHD, DuPaul et al. found that both boys and girls with ADHD experienced difficulties with academics, behavior, and social functioning. These findings suggest that ADHD affects school functioning in both sexes, although some differences may exist in how these challenges are experienced.

Overall, studies have suggested that while boys and girls with ADHD share many similarities, there are also key differences to recognize that can improve recognition and treatment outcomes for both sexes. Studies have consistently found that girls are more likely to experience comorbid anxiety disorders than boys, while findings on externalizing disorders have been less consistent across studies. Researchers have also found that boys and girls experience difficulties with school functioning, although these challenges may present differently between the sexes. These findings provide a foundation to better understand how sex differences in comorbid conditions may continue to change throughout the lifespan.

Sex Differences in Comorbidities in Young Adults

As individuals transition from childhood to young adulthood, they often face new responsibilities, including college, employment, and increasing independence. These changes come with greater demands in school, work, and relationships. For people diagnosed with ADHD, this time also appears to be associated with worsening impairment in daily functioning (Howard et al.). Although many of the same sex differences in comorbidities seen in childhood continue into young adulthood, these comorbidities may become more apparent and impairing as individuals diagnosed with ADHD face greater social and academic demands.

Research shows that young adult females are more likely than males to experience internalizing comorbidities, particularly anxiety and depression (Anastopoulos et al.). Females with ADHD were also significantly more likely than males to have two or more comorbid disorders (43.9% vs. 18.9%; OR = 3.4) (Anastopoulos et al.). They also reported having higher rates of trauma- and stressor-related disorders (10.5% vs. 3.8%) and eating disorders (4.4% vs. 0%) (Anastopoulos et al.). Similarly, Fedele et al. found that young adult females with ADHD experience greater impairment across several areas of daily life, including academics, social relationships, and money management. Together, these findings suggest that internalizing comorbidities continue to have a greater impact on females with ADHD during young adulthood.

Although females were more likely to experience internalizing comorbidities, males with ADHD continued to report higher levels of conduct disorder symptoms (Anastopoulos et al.).

Together, these findings suggest that sex differences in ADHD continue into young adulthood, with females more likely to experience internalizing disorders and males exhibiting a greater tendency toward externalizing symptoms.

Overall, the findings suggest that many of the sex differences observed during childhood continued into young adulthood. Young adult females with ADHD experienced higher rates of several comorbid conditions as well as greater impairment across multiple areas of daily functioning. Males, however, continued to exhibit higher levels of externalizing symptoms, particularly conduct disorder symptoms. As individuals continue into adulthood, understanding these sex differences remains key, as they may influence diagnosis, treatment, and long-term outcomes for individuals with ADHD.

Sex Differences in Comorbidities in Adults

As individuals transition into adulthood, the effects of ADHD often extend beyond academic settings and begin to influence employment, relationships, independent living, and overall quality of life. Although some symptoms may change with age, ADHD frequently persists into adulthood and is often accompanied by one or multiple other co-occurring psychiatric conditions.

Research suggests that the higher prevalence of internalizing comorbidities among females with ADHD continues into adulthood. In a population-based longitudinal study of adults with persistent ADHD, females continued to be at greater risk for anxiety and depressive disorders than males, indicating that these sex differences often persist beyond childhood and young adulthood (Yoshimasu et al.). Similarly, Solberg et al. found that adult females with ADHD were more likely to be diagnosed with anxiety, depression, bipolar disorder, and personality disorders than men with ADHD. These differences in diagnoses may also extend to female-specific stages of adulthood. A recent study found that females with ADHD were at a significantly greater risk of developing postpartum depression and postpartum anxiety disorders than females without ADHD, suggesting that internalizing comorbidities continue to uniquely affect females throughout adulthood (Andersson et al.).

While females were more likely to experience internalizing comorbidities, adult males with ADHD continued to show higher rates of externalizing and other severe psychiatric conditions. Solberg et al. found that men with ADHD were more likely than females to be diagnosed with substance use disorders and schizophrenia spectrum disorders. The authors suggested that the higher prevalence of substance use disorders among men may partially contribute to the increased rates of schizophrenia spectrum disorders observed in males, although this relationship requires further investigation (Solberg et al.). These findings suggest that sex differences in ADHD continue into adulthood, with males more likely to experience externalizing and severe psychiatric comorbidities.

Overall, the findings suggest that sex differences in ADHD comorbidities continue into adulthood. Adult females remained more likely to experience internalizing psychiatric disorders, whereas males were more likely to exhibit externalizing and other severe psychiatric

comorbidities. Differences in ADHD presentation may also contribute to these outcomes, emphasizing the importance of considering both sex and ADHD subtype when evaluating adults with ADHD.

Discussion

Across childhood, young adulthood, and adulthood, a strong pattern emerged among individuals diagnosed with ADHD. Females were more likely than males to present with internalizing comorbidities such as anxiety and depression in childhood, along with eating disorders and other trauma- and stressor-related disorders in young adulthood (Levy et al.; Bauermeister et al.; Anastopoulos et al.; Solberg et al.). On the other hand, males typically presented with externalizing comorbidities such as oppositional defiant disorder and conduct disorder in childhood and adolescence, followed by higher rates of substance use disorders and schizophrenia spectrum disorders later in adulthood (Levy et al.; Solberg et al.). These findings suggest that sex differences in comorbidities among individuals diagnosed with ADHD are not limited to specific stages in development. At the same time, as individuals get older, they become more vulnerable to developing additional comorbidities that were not present during childhood, and existing comorbidities can become more impairing to daily functioning. These later-developing comorbidities still largely reflect the same patterns found during childhood and adolescence (i.e., externalizing for males and internalizing for females).

Sex differences can be attributed to various possible explanations. One possible explanation may involve differences in ADHD presentation and how these presentations are associated with comorbid conditions. For example, Levy et al. found that girls with the combined subtype of ADHD had higher rates of generalized anxiety disorder, while Bauermeister et al. found higher rates of anxiety disorders among girls with the inattentive subtype. In contrast, boys with ADHD were more likely to experience externalizing conditions such as oppositional defiant disorder and conduct disorder (Levy et al.; Biederman et al.). These findings suggest that ADHD presentation may be related to patterns of internalizing and externalizing comorbidities, although ADHD subtype alone does not fully explain these sex differences. Another possible explanation may involve biological factors such as sex hormones, as hormonal changes may be associated with changes in ADHD symptoms among females (Osianlis et al.).

Changes in the nature and severity of comorbidities across the lifespan are attributable to various possible causes. As individuals progress from childhood to adulthood, regardless of sex, increasing environmental demands (school, work, relationships, independent living) may make existing comorbidities more detrimental to daily functioning. These increasing environmental demands may also explain why additional comorbidities arise later in life, such as eating disorders and other trauma- and stressor-related disorders in females, and schizophrenia and substance use in men. Despite these changing life stages, the overall pattern of internalizing disorders in females and externalizing disorders in males remained relatively consistent.

These findings have important implications for the assessment and treatment of individuals with ADHD. Rather than using a one-size-fits-all approach, clinicians should

consider the different comorbidity patterns that may be more common among males and females when evaluating patients. For example, females with ADHD may benefit from additional screening for anxiety, depression, and other internalizing disorders, while males may benefit from screening for externalizing disorders and substance use. Recognizing these sex-specific patterns early may allow for more targeted interventions and improve academic, social, and long-term mental health outcomes across the lifespan. Targeted screenings based on sex may also improve earlier recognition of ADHD, especially in females. Females may experience delayed recognition of their ADHD, resulting in a later ADHD diagnosis. This delayed diagnosis can have significant consequences. Holden and Kobayashi-Wood found that women with delayed ADHD diagnoses reported negative effects on their mental health, quality of life, self-esteem, relationships, careers, and sense of self. Therefore, recognizing ADHD earlier may help females receive appropriate treatment and support before these difficulties continue into later stages of life.

While this literature review identified consistent sex differences in ADHD comorbidities, several limitations should be considered. Many of the studies included were conducted in Western populations, which may limit the generalizability of these findings. Additionally, fewer studies focused on young adults and adults than on children, highlighting the need for more research across later stages of development. Future studies should continue to examine the biological and developmental factors that contribute to these sex differences.

Conclusion

In conclusion, this literature review found consistent sex differences in the comorbid conditions associated with ADHD across childhood, young adulthood, and adulthood. Females were more likely to experience internalizing disorders while males more frequently exhibited externalizing disorders, suggesting that these patterns persist across development. Understanding these sex differences may improve the earlier recognition of ADHD and its co-occurring conditions, allowing for more targeted assessment and treatment. Ultimately, recognizing these differences may help improve outcomes and quality of life for both males and females with ADHD.

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Evaluating the Neurodevelopmental Effects of Early Childhood Exposure to General Anesthesia By Stephen Varghis

Abstract

Early Childhood is a period of rapid and sensitive brain development, which has led to concerns about whether general anesthesia might interfere with this process. Animal studies have shown that certain anesthetic drugs can trigger cell death. This raises questions about whether similar effects happen in humans. This paper reviews major human studies, including the Mayo Anesthesia Safety in Kids (MASK) Study, the General Anesthesia Vs. Spinal (GAS) Trial, the Pediatric Anesthesia NeuroDevelopment Assessment (PANDA) Study, and other systematic reviews. Across these sources, single short exposures to anesthesia do not seem to harm general intelligence. In contrast, multiple or prolonged exposures may be associated with small differences in processing speed, motor skills, or behavior. These findings suggest that exposure characteristics, such as duration, frequency, and developmental timing, are more important than anesthesia itself. The paper concludes with clinical implications, limitations in current research, and recommendations for future studies.

Introduction

General anesthesia is a crucial part of modern surgery, allowing patients of all ages to undergo procedures safely and without pain. In pediatrics, its importance is evident in the fact that infants and young children often require surgeries, imaging, and emergency interventions that cannot be performed without anesthesia (McCann et al., 2019). Additionally, early childhood is a period of critical brain development. Neurons rapidly form new synapses and strengthen pathways that support memory, language, attention, and motor skills (Frontiers Review, 2022). Because this developmental window is so sensitive, researchers have questioned whether anesthetic drugs might interfere with normal brain growth (Cureus Review, 2025).

Each year, millions of children undergo procedures that require anesthesia, making early childhood exposure far more common than many people realize (Frontiers Review, 2022). The most frequent surgeries in young children include ear tube placement, hernia repair, circumcision, tonsillectomy, adenoidectomy, and imaging procedures such as MRIs, which often require anesthesia to keep children still (Cureus Review, 2025). These procedures are routine parts of pediatric care, which means even small potential risks matter at a population level.

During early childhood, the brain goes through what researchers call the “brain growth spurt,” a period when synapses form at extremely high rates (Frontiers Review, 2022). GABA receptors transition from being excitatory in the fetal period to inhibitory after birth, while NMDA receptors guide synaptic plasticity, learning, and memory formation, as illustrated in *Figure 1*. When anesthetic drugs enhance GABA activity and block NMDA receptors, they temporarily silence large networks of neurons. In adults, this is safe and reversible. In infants, however, prolonged silencing may interfere with normal developmental signals. Because these molecular effects occur during a period of rapid brain growth, it becomes important to

understand how often young children actually receive anesthesia and why these exposures are so common.

To understand why anesthesia might influence development, it is helpful to examine how these drugs act at the molecular level. Most general anesthetics act on two major neurotransmitter systems: gamma-aminobutyric acid (GABA) receptors and N-methyl-D-aspartate (NMDA) receptors. GABA is the brain's main inhibitory neurotransmitter, and anesthetic drugs increase GABAergic inhibition, slowing neural activity (Frontiers Review, 2022). Many anesthetics also block NMDA receptors, which normally help neurons communicate and form new connections. In infants, these systems are still developing, and altering them may affect how neurons grow, migrate, and connect (Cureus Review, 2025).

These concerns originally came from animal studies. In rodents and primates, exposure to certain anesthetic drugs during peak brain development caused widespread cell death and disrupted synaptogenesis and dendritic spine formation (Frontiers Review, 2022). Although animals are not perfect models for humans, these findings raised important questions about whether similar effects could occur in human infants.

Neurodevelopment is also highly individualized, and children do not respond to anesthesia in exactly the same way. Factors such as age, genetics, underlying medical conditions, and the timing of exposure can influence how the developing brain reacts to anesthetic drugs (Frontiers Review, 2022). For most children a single brief exposure does not appear to cause lasting harm (McCann et al., 2019; Sun et al., 2016). However, children who experience multiple or prolonged exposures may be more vulnerable to subtle changes in processing speed, motor coordination, or behavior (Warner et al., 2018).

Several major studies have shaped current understanding of how anesthesia might affect neurodevelopment. Together, they provide a clearer picture of which exposures are safe and which may require caution.

The General Anesthesia vs. Spinal (GAS) Trial compared infants who received sevoflurane general anesthesia with infants who received regional spinal anesthesia. At age five, the two groups had similar IQ scores (McCann et al., 2019), supporting the idea that a single short exposure is safe.

The Pediatric Anesthesia NeuroDevelopment Assessment (PANDA) Study compared children who had a single anesthesia exposure before age three with their unexposed siblings. PANDA found no difference in IQ, memory, or attention (Sun et al., 2016). This sibling-matched design helps control genetics and environment.

The Mayo Anesthesia Safety in Kids (MASK) Study examined 997 children with no exposure, a single exposure, or multiple exposures before age three. Children were tested at ages eight to twelve or fifteen to twenty using standardized neuropsychological assessments. The study found that “IQ did not differ significantly according to exposure status” (Warner et al., 2018). However, it also reported that “Children with multiple exposures had decreased processing speed and fine motor abilities.” Parents of multiple exposed children described more issues with executive function, behavior, and reading (Warner et al., 2018).

A 2022 systematic review published in Frontiers in Molecular Neuroscience analyzed seventy-two studies from eighteen countries. The authors reported “Two thirds of the included studies reported negative neurocognitive effects after general anesthesia exposure" (Frontiers Review, 2022). However, they also noted that “The risk of adverse outcomes was more frequently observed in children with multiple or prolonged exposures. “Children younger than seven appeared more susceptible to potential effects.

A 2025 structured review from Cureus examined both retrospective studies and major prospective trials. It concluded that “current evidence does not support a significant neurodevelopmental risk from a single, brief exposure to general anesthesia” (Cureus Review, 2025)/ However, it also stated that “Repeated or prolonged anesthetic exposures have been associated with learning difficulties and behavioral problems.”

Despite the growing body of research, there is uncertainty about whether differences observed after multiple or prolonged exposures are directly related to anesthesia or to other factors. Differences in study design, exposure duration, age at exposure, and underlying medical conditions make it difficult to isolate the effects of anesthesia itself. Across all studies, a consistent pattern emerges: single short exposures appear safe, while repeated or prolonged exposure may carry small risks.This review examines the current evidence regarding the neurodevelopmental effects of general anesthesia exposure during early childhood. It first examines evidence regarding single and brief exposures, then evaluates findings associated with multiple or prolonged exposures. The review also considers how exposure duration and age may influence outcomes.

Figure 1 illustrates how anesthetic drugs such as sevoflurane interact with GABA-A and NMDA receptors at the molecular level, increasing inhibition and reducing excitatory signaling during early brain development

Methodology

This paper synthesizes evidence from prospective trials, retrospective cohort studies, and systematic reviews. Studies were selected based on sample size, design quality, and relevance to early childhood anesthesia exposure. Instead of generating new data, the goal is to compare existing findings and identify patterns that appear consistently across different types of research.

Results

Across all sources, several consistent findings emerged. The results of the studies are depicted in Table 1. A single short exposure to general anesthesia was not associated with significant differences in IQ or general intelligence (Warner et al., 2018; McCann et al., 2019; Sun et al., 2016). Multiple exposures may be associated with small differences in processing speed, motor skills, or behavior (Warner et al., 2018). Longer exposures, especially those lasting more than three hours, may carry more risk (Frontiers Review, 2022). Younger children may be more vulnerable to potential effects, particularly those under age seven (Frontiers Review, 2022).

Confounding factors such as underlying medical conditions, socioeconomic status, and surgery type make research challenging (Cureus Review, 2025).

Evidence from the GAS, PANDA, and MASK studies suggests that a single brief exposure to general anesthesia is not associated with significant differences in general intelligence. In the GAS Trial, infants who received sevolfurane general anesthesia had similar IQ scores at age five compared with infants who received awake-regional spinal anesthesia (McCann et al., 2019). Similarly, the PANDA Study found no significant differences in IQ, memory, or attention between children who had a single anesthesia exposure before age three and their unexposed siblings (Sun et al., 2016). The MASK Study also found that IQ did not differ significantly between children with no exposure, a single exposure, or multiple exposures before age three (Warner et al., 2018). Together, these findings across randomized, sibling-matched, and observational research provide relatively consistent evidence that a single brief exposure was not associated with measurable differences in general intelligence.

Findings were less consistent among children who experienced multiple exposures. In the MASK Study, children with multiple exposures had lower processing speed and fine motor abilities, while parents reported more difficulties with executive function, behavior, and reading compared with children who had no exposure or a single exposure (Warner et al., 2018). The 2022 Frontiers systematic review found that adverse outcomes were more frequently observed following multiple or prolonged exposures, while the 2025 Cureus review similarly reported associations between repeated or prolonged exposures and learning difficulties or behavioral problems (Frontiers Review, 2022; Cureus Review, 2025). However, these findings do not establish that anesthesia itself caused these differences because children requiring repeated procedures may also have underlying medical conditions or other characteristics that influence neurodevelopment.

Exposure duration and developmental age may also influence observed outcomes. The Frontiers systematic review found that potential adverse outcomes were more frequently reported among children who experienced prolonged exposures, particularly those lasting more than three hours. The review also identified younger children, particularly those under age seven, as potentially more susceptible to neurocognitive effects (Frontiers Review, 2022). These findings suggest that the frequency and duration of exposure may be more relevant than exposure alone. However, differences in study designs, neurocognitive tests, medical conditions, and exposure characteristics make it difficult to determine whether duration or age independently predicts developmental outcomes.

Table 1. Results from literature review.

Study	Design and Population	Main Outcomes	Limitations
MASK Study (Warner et al., 2018)	997 children; no exposure vs. single vs. multiple	No IQ differences across groups; multiple exposures linked to lower processing speed, fine motor skills, and more	Multiple exposures often tied to more complex medical histories; observational design

	exposures before age 3	parent-reported behavioral issues	
GAS Trial (McCann et al., 2019)	Randomized trial; infants receiving sevoflurane GA vs awake-regional spinal anesthesia	No difference in IQ at age 5; single short exposure appears safe	Only evaluates single, brief exposure; not powered to detect subtle specific effects
PANDA Study (Sun et al., 2016)	Sibling-matched group single exposure before age 3	No difference in IQ, memory, attention, or behavior	Only includes healthy children undergoing hernia repair; results may not generalize to medically complex populations
Frontiers Systematic Review (2022)	72 studies from 18 countries	Two-thirds reported negative neurocognitive effects, risks higher with multiple or prolonged exposures; younger children more vulnerable	Heterogeneous study designs; inconsistent neurocognitive tests; variation in exposure duration
Cureus Structured Review (2025)	Review of prospective + retrospective studies	Single brief exposures not linked to harm; repeated or prolonged exposures associated with learning and behavioral differences	Many retrospective studies rely on medical records; prospective studies are small and expensive

The figure shows the pattern that a single short exposure to anesthesia does not appear to harm general intelligence, while repeated or prolonged exposures may be associated with small differences in specific cognitive or behavioral aspects. At the same time, each study comes with limitations that make it difficult to isolate anesthesia from other medical or environmental factors, which is why continued research is necessary

Discussion

Taken together, these studies suggest that anesthesia itself is not harmful when used once for a short procedure. This is reassuring for parents and clinicians, since many surgeries in early childhood may be important. However, repeated or prolonged exposures may have small effects on certain cognitive or behavioral skills. These findings match what animal studies show about how prolonged neuronal silencing can disrupt synaptogenesis (Frontiers Review, 2022).

Human studies come with several limitations. Retrospective studies rely on medical records and school data, which can vary in accuracy. Prospective studies are stronger but often smaller and more expensive to run. It is also difficult to separate the effects of anesthesia from the effects of the underlying medical condition or the surgery itself. Factors like socioeconomic status and genetics add additional complexity.

Future research should focus on exposure duration, dose, and the specific developmental period when children may be most vulnerable. Larger prospective studies and more sibling-matched designs would help control genetics and the environment. Standardized neurocognitive testing across studies would also make results easier to compare.

Conclusion

Current evidence suggests that single short exposures to general anesthesia do not harm neurodevelopment (McCann et al., 2019; Sun et al., 2016). Multiple or prolonged exposures may be associated with small differences in specific cognitive or behavioral domains (Warner et al., 2018; Frontiers Review, 2022). Continued research will help clarify how exposure characteristics influence outcomes and guide safer clinical practices (Cureus Review, 2025).

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Does Dyslexia Have an Impact on Memory? By Vania Stiglich

ABSTRACT

Dyslexia is a neurodevelopmental learning disorder that is mainly associated with difficulties in phonological processing and reading. This systematic review examines the impact of dyslexia on memory, specifically short term, working, and long term memory. Out of the studies included, there is a particular focus on research using functional magnetic resonance imaging (fMRI) to investigate the neural mechanisms behind the memory impairments. Relevant studies for this review were identified using Google Scholar and PubMed, of which 5 were selected for inclusion. The findings show that individuals with dyslexia have impairments in verbal memory, as well as difficulties in accuracy and processing speed on demanding working-memory tasks. fMRI findings supported this by showing differences in activation within frontal, parietal, and cerebellar regions associated with working-memory processes. This review also showed that there is an impact on visuospatial long term memory in individuals with dyslexia, but that visuospatial short term memory is minimally affected. Overall, the findings suggest that the memory impairments in individuals with dyslexia extend past verbal memory and involve broader working memory difficulties, as well as disruptions in other neural processes. However, disparities in methodologies, small fMRI sample sizes, and the limited number of studies that met the criteria indicate that future research is needed.

INTRODUCTION

Dyslexia

Developmental dyslexia, commonly referred to as dyslexia, is an inherited learning disorder characterized by severe and persistent problems in reading skills and overall phonological processing (Fischbach et al., 2014). Specifically, dyslexia is the most common learning disability and accounts for around 80% of all neurodevelopmental disorders (Lerner, 1989). In fact, more men have dyslexia than women, on the order of 3 to 1. (Liu et al., 2016). Children with dyslexia also deal with impairments in visual cognition and sequential motor skills (Marchand-Krynski, 2018). Symptoms of dyslexia can be recognized through difficulties in literacy, particularly in correctly identifying letters (Lipowska et al., 2011) spelling, difficulty recognizing words and their pronunciation (Tunmer & Greaney, 2010). Other symptoms include problems with memory and executing basic mathematics skills (Youman & Mather, 2013). Cognitive behavioral research has revealed that literacy skill deficits are a measure of dyslexia risk and can be measured as early as preschool age (Sanfilippo et al., 2020), leading to an early dyslexia diagnosis. However, the age at which children are diagnosed with dyslexia varíes significantly. Although dyslexia intervention programmes can be effective, students that showed gains in phonetic skills and other behavioral aspects required continuous intervention to become a self regulated learner (Tam and Leung, 2019).

Memory

Working memory refers to a limited capacity memory system that plays a part in the temporary storage and processing of information (Beneventi et al., 2010). The working memory model consists of multiple components: the verbal component, called the phonological loop; the visual and spatial component, called the visuospatial sketchpad (Baddeley, 1997); the episodic buffer, and the central executive, an attentional control system (Baddeley, 2000). Individuals with dyslexia experience impairments in the phonological working memory leading to literacy struggles (Schuchardt et al., 2008). The function of the phonological loop is primarily associated with increased activity in several brain regions, including the left inferior parietal lobe, posterior inferior frontal gyrus, premotor cortex and the cerebellum (Awh et al., 1996). Furthermore, implicit memory regards nonconscious recall of motor skills and other tasks whilst explicit memory is the conscious recall of information about people, places and things (Bailey et al., 1996)

Functional Magnetic Resonance Imaging

Functional activation of the brain can be detected using a non-invasive neuroimaging technique called functional Magnetic Resonance Imaging (fMRI). The activation of brain regions can be detected via direct measurements which include tissue perfusion, blood volume changes or oxygen concentration changes in the blood (Logothetis, 2008) called blood oxygen level dependent (BOLD) response. Measurement of BOLD in fMRI provides a mixed signal that depends on blood flow, blood volume and blood oxygenation (Heeger and Ress, 2002). Lastly, the relationship between fMRI and neural responses depends on the fMRI acquisition technique, behavioral and stimulation protocols, and the data analysis methods of the neuronal activity (Heeger and Ress, 2002). Tasks administered in the MRI target neural function of specific brain regions underlying specific cognitive domains, like working memory.

Given prevalence rates of dyslexia and the importance of working memory, a literature review was conducted to understand how dyslexia impacts memory with a specific focus on studies utilizing fMRI methods to interrogate neural mechanisms of dyslexia.

METHODS

Study design

A systematic review was conducted to investigate whether dyslexia impacts short term and long-term memory. Searches were conducted in Google Scholar and PubMed. A total of 110 potentially relevant research papers were identified and title screened. Following full text review, 35 reports sought for retrieval, of which 5 met inclusion criteria.

Search strategy

Search terms were carefully selected and added and removed as the data was collected to facilitate the searching process. Examples of search terms included: “fMRI,” “dyslexia,” “ long

term memory," "short term memory," "working memory," "visuospatial memory," "implicit memory," "cerebellar deficit hypothesis," and "skill automatization". These terms were combined in strings of 2 to 4 at a time to create efficient searches. For example, " fMRI and dyslexia and working memory" or "dyslexia and long term memory".

Inclusion criteria

Studies were selected for inclusion based on specific inclusion and exclusion criteria. To be included, each study had to have a group of individuals with dyslexia and the group of participants with dyslexia had to have been assessed for IQ, processing speed, and screened to confirm their diagnosis with dyslexia. These were inclusion criteria because they ensured that the participants being evaluated were diagnosed with dyslexia and there were no other factors which may influence memory ability. Additionally, each study needed to examine the short term or long term memory performance and/or ability of participants with dyslexia without influence of additional factors (e.g., head injury) to be considered for inclusion. Finally, studies using behavioral approaches and fMRI were considered. Studies were excluded if they utilized a neuroimaging technique other than fMRI; did not report neural activity in any of the target areas; focused on typical motor skills, social skills or emotional processing rather than processing speed, phonological processing or skill automatization related to the set task. Additionally, peer reviewed studies published between 2001 and 2019 available in English were included. Although the exclusion of older and non-English work introduces bias, the criteria above was strictly followed to maintain consistency as much as possible.

Analytical approach

Studies were analyzed by identifying recurring patterns and themes within the data. Research papers were grouped depending on whether they focused on long term memory or short term memory and then further separated depending on whether the data was behavioral or whether it was gathered using fMRI.

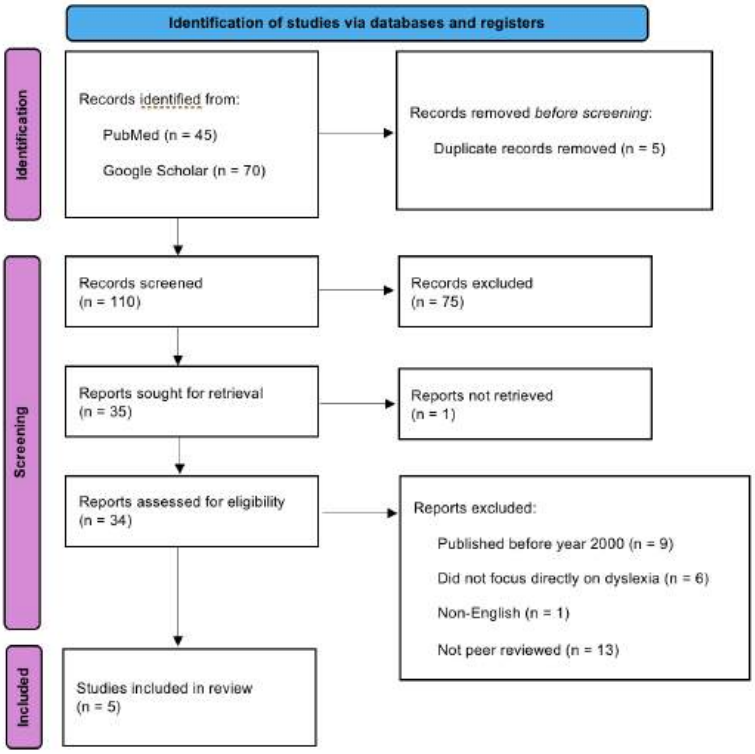

Fig. 1 PRISMA flowchart

RESULTS

Out of the 5 studies included in this review, they discussed at least one of the following themes 1) the relationship between individuals with dyslexia and processing speed in working memory 2) the relationship between brain activation and functioning in individuals with dyslexia 3) the differences in impairments across phonological tasks in the control group and the dyslexic groups. Additionally, The fMRI studies focused on the target areas in the brain relevant to both short term and long term memory. These include regions in the frontal, temporal or parietal lobes. INCLUDE COMMON AGES

Author & Year of publication	Title	Study design	Age group	Summary of findings
Alsulami, 2019	The role of memory in dyslexia	Literature review on 2 studies	8-15	Found a consistent association between dyslexia and impairments in working and short-term memory, especially those affecting phonological processing, reading, and recall, favoring the need for early intervention.
Beneventi et al., 2010	Working Memory Deficit in Dyslexia: Behavioral and fMRI Evidence	fMRI study	Mean age of 13.2 for individuals with dyslexia and 13.5 for controls	Children with dyslexia showed poorer working memory performance and reduced activation in prefrontal, parietal, and cerebellar brain regions during working memory tasks, supporting a neurobiological explanation for working memory deficits in dyslexia.
Beneventi et al., 2010	Executive working memory processes in dyslexia: Behavioral and fMRI evidence	fMRI study	Mean age of 13.2 for individuals with dyslexia and 13.5 for controls	Children with dyslexia showed poorer working memory performance, with lower accuracy and slower responses than controls. Dyslexic participants did not increase brain activation as cognitive demand increased, suggesting deficits in executive working memory processes.
Garcia et al., 2014	Visuospatial working memory for locations, colors, and binding in typically developing children and in children with dyslexia and non-verbal learning disability	Behavioral study	8-10	The spatial memory of children with dyslexia was relatively intact and did not show significant impairments compared to typically developing children. Children with dyslexia showed little decline in backward versus forward color recall, suggesting greater reliance on non-verbal strategies to compensate for phonological difficulties.
Menghini et al., 2010	Developmental Dyslexia and Explicit Long-Term Memory	Behavioral study	Age Range of 8.1-17.6 years old across all 4 groups	Children with dyslexia showed reduced long-term memory across verbal, visual-object, and visual-spatial tasks, indicating a generalized memory impairment rather than a purely phonological deficit. Non-verbal memory abilities also predicted word-reading difficulties.

Fig.2 Summary table of studies included

DISCUSSION

The impact of dyslexia on verbal memory

Previous research has primarily focused on the impact of dyslexia on verbal memory and one of the included studies examined this relationship in more depth by exploring the connection between phonological processing and working memory. This review assessed two studies, overlooking one hundred and fifteen students whose ages ranged from eight to 15 years. The first had two participant groups, a group of participants with dyslexia and a control group without dyslexia. Participants were tasked with repeating pseudowords (made up words) and were tested for their listening and reading comprehension. The results suggest that when children with dyslexia were asked to repeat pseudowords or number sequences, they performed significantly worse than the control group. Furthermore, the dyslexic group also performed worse on a reading comprehension task and when answering text related questions, but performed well on questions related to auditory narrative. Together, these findings contribute to the conclusion that there were

associations between dyslexia and working memory deficits (Alsulami, 2019). The second study in the review also examined a group of individuals with dyslexia compared to a control group. Participants engaged in a series of reading tasks. The results suggest that the group of participants with dyslexia performed significantly worse on all reading tasks compared to controls (Beneventi et al., 2010b). Taken together, these results align with the conclusion that those with dyslexia have deficits in short term memory related to phonological processing.

The impact of dyslexia on working memory

Other works have begun assessing the impact of dyslexia on working memory. One study, by Beneventi and colleagues, compared n-back task performance in the fMRI scanner between children with dyslexia and age matched controls. There were 26 participants in this study, with 14 individuals in the control group and 12 individuals in the dyslexia group. The n-back task is a measure of increasing working memory demands which allows researchers to examine working memory ability. The results suggest that the group of participants with dyslexia performed significantly worse on the n-back task as compared to controls at the more challenging levels of the task . The fMRI results showed that within the overall activation pattern during task performance, the control group showed significantly more activation than the group with dyslexia bilaterally in the posterior middle frontal gyrus (MFG), the superior parietal lobe (SPL), and the left cerebellum. The finding that the MFG and the SPL were more activated in the control group provides evidence of neural correlates that may be linked to a working memory deficit in individuals with dyslexia (Beneventi et al., 2010a), which aligns with previous research which found that the MFG and the SPL have been associated with working memory processes (Wager & Smith, 2003).

Additionally, in a similar study, researchers comparing 2-back task performance between a group of participants with dyslexia to those without found that when individuals with dyslexia performed the 2-back task, they have a different pattern of neural activation from the control group. They found this via their study conducted on 24 participants with 13 individuals in the control group and 11 individuals in the dyslexia group. Specifically, the participants in the dyslexia group displayed less activation in the superior/inferior parietal lobes, middle/superior frontal gyri, cingulate gyrus and cerebellum (Beneventi et al., 2010b). This finding suggests reduced recruitment of the typical working memory network and cerebellum in individuals with dyslexia. Therefore, this supports the conclusion that dyslexics have impairments in phonological processing but also in executive, fronto-parietal processes, suggesting altered neural pathways in working memory.

The impact of dyslexia on visuospatial memory

In addition to short term and working memory, prior works have assessed impacts of dyslexia on visuospatial memory as well. A study conducted involved 45 individuals with 15 typically developing (TD) children, 15 children with dyslexia, 15 children with non-verbal learning disability (NLD). In assessment of individuals with dyslexia compared to controls in a

visuospatial memory task, it was found that the dyslexia group performed similarly to the controls on the visuospatial memory task (Garcia et al., 2014). This finding suggests that the main difficulties of individuals with dyslexia are not in the visuospatial domain but rather in memory. In fact, this study argued that children with dyslexia may rely on visuospatial strategies to compensate for phonological-based weaknesses (Garcia et al. 2014). Therefore, the conclusion that dyslexia affects all types of short term memory does not include visuospatial memory.

Another study examined whether individuals with dyslexia have long term memory deficits that extend beyond verbal memory to visuospatial and visual object memory. The researchers compared three groups of children, with a total sample size of 125 individuals. It was found that children with dyslexia performed significantly worse on all three long term memory tasks by remembering fewer items and showing slower learning. (Menghini et al., 2010). These findings support that children with dyslexia have an explicit impairment in visuospatial and visual object long term memory and that dyslexia is not just a phonological disorder. In fact, these visuospatial impairments could contribute to the reading difficulties that individuals with dyslexia suffer from.

Limitations

The results of this review should be considered in the context of its limitations. One limitation is the heterogeneity of the methods used across current research studies. In particular, a recurring flaw is that a lot of the methods used only measure the impact of a single cognitive domain but dyslexia is a complex developmental condition. For example, there are methods that examine the impact of dyslexia on working memory yet the idea that the central executive and other structures in the brain may also be a contributor in the task presented was not considered. Another limitation is that not all the studies focused solely on dyslexia, but on other neurodevelopmental disorders as well. This was dealt with by focusing solely on the findings related to dyslexia. Furthermore, most fMRI studies have a much smaller sample size in relation to the behavioral studies. This disparity is explained due to fMRI studies being much more expensive to conduct and therefore not as many participants were recruited.

Future directions

It is important to note that there is substantially less reason for the impact of dyslexia on long term memory than on short term memory and this is an area future research should examine. Additionally, there are fewer studies utilizing fMRI than with behavioral tasks, and future research should expand fMRI work to better understand the neural impacts of dyslexia on the brain. Additionally, future works should focus on utilizing longitudinal paradigms to measure the impact on long term memory over time. There are also very few studies on the impact of dyslexia on episodic memory as working memory dominates the majority of the research. Therefore, future efforts should assess episodic memory ability in individuals with dyslexia. Furthermore, future research should utilize larger sample sizes, especially in the fMRI studies, a larger variety of ages, and a longer monitoring period for the individuals being tested. Lastly, the

majority of the dyslexia studies in relation to memory are done on children, so expanding the age group in future research could remove this bias.

Conclusion

In conclusion, the aim of this systematic review was to examine the impact of dyslexia on memory. The five studies included suggest that dyslexia strongly impacts memory. Individuals with dyslexia had deficits in working memory and were slower in processing speed and accuracy in comparison to typically developing (TD) children. The findings provide support that the impairments in individuals with dyslexia extend beyond verbal memory and the phonological loop and affect the central executive, and visual spatial memory. Therefore, the evidence in this review supports the importance of understanding the underlying neural mechanisms in memory that play a large role in the cognitive difficulties associated with dyslexia.

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