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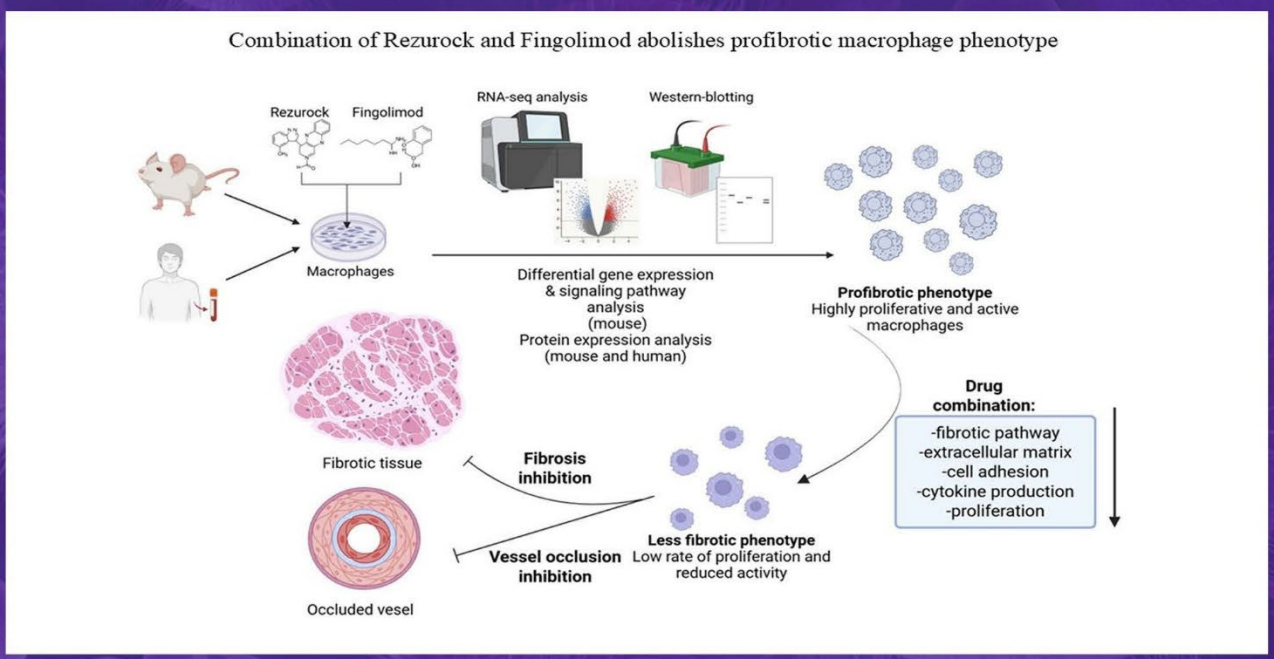

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EDITORIAL

Impact of climate change on clinical medicine

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1. Introduction

Climate change poses profound and multifaceted health challenges that are reshaping all aspects of our lives, including clinical practice. This unprecedented global crisis extends far beyond environmental degradation, fundamentally altering the landscape of human health, disease patterns, and healthcare delivery systems worldwide. This editorial seeks to outline these emerging health threats and evaluate our current level of preparedness to address them.

2. Direct heat-related illness

Extreme heatwaves are increasing in frequency, duration, and intensity. This can cause heat exhaustion, heat stroke, and exacerbate cardiovascular and respiratory conditions. Vulnerable populations include the elderly, outdoor workers, and individuals without access to air conditioning. The rising global temperatures are directly affecting human physiology and survival. Extreme heat events strain the body's thermoregulatory mechanisms, leading to dehydration, electrolyte imbalances, and multi-organ failure in severe cases. The 2024 Lancet Countdown on Health and Climate Change documented record-breaking temperatures, with 2023 reaching 1.45°C above the pre-industrial baseline, presenting unprecedented health threats from climate inaction.[1] Clinicians are confronted with more emergency presentations during heat events. Therefore, the healthcare systems face surges in emergency department visits during heatwaves, requiring new protocols for triage, treatment, and prevention. This phenomenon is already widely recognized, both within the clinical community and beyond it.

3. Shifting disease patterns

Climate change is dramatically altering the geographic distribution and seasonality of infectious diseases. Vector-borne diseases such as malaria, dengue fever, Zika virus, and Lyme disease are expanding into previously unaffected regions as warming temperatures allow disease-carrying mosquitoes, ticks, and other vectors to survive and reproduce in new areas.[2,3] This geographic shift means that clinicians must now consider tropical diseases in their differential diagnoses even in temperate regions, a shift in practice that requires enhanced surveillance systems, diagnostic capabilities, and treatment protocols. Waterborne diseases are also increasing due to flooding events and compromised water infrastructure, while changing precipitation patterns affect the prevalence of diseases such as cholera and leptospirosis.

4. Air quality and respiratory disease

Increased wildfires, longer pollen seasons, and higher ground-level ozone concentrations worsen asthma, chronic obstructive pulmonary disease (COPD), and allergies.

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Particulate matter from fires is linked to cardiovascular events and premature mortality. Air quality deterioration represents one of the most immediate health threats posed by climate change. Increased wildfire frequency and intensity, exacerbated by drought and rising temperatures, release massive quantities of particulate matter and toxic compounds into the atmosphere. Extended pollen seasons and higher pollen concentrations are intensifying allergic diseases.[4] The resulting air pollution contributes to asthma exacerbations, COPD progression, respiratory infections, and cardiovascular events, including heart attacks and strokes. Thus, clinicians are witnessing a constant increase in both acute presentations and chronic disease burden related to poor air quality.

5. Food and water security

Changing precipitation patterns, droughts, and floods affect food production and water safety. This impacts nutrition and increases waterborne disease risks, particularly gastroenteritis and cholera in vulnerable regions. Climate change also threatens the global food systems through altered growing conditions, extreme weather events, and degraded soil quality. Crop failures and reduced nutritional content in staple foods lead to malnutrition, micronutrient deficiencies, and food insecurity, particularly in vulnerable populations. In addition, rising food prices and supply chain disruptions disproportionately affect low-income communities.[5] Clinicians are increasingly encountering malnutrition-related conditions, calling for exploration of social determinants of health and implementation of community-level interventions.

Global major events, such as glacier melting and rising sea levels, are affecting both water quantity and quality. Droughts reduce access to clean water for drinking and sanitation, increasing the risk of dehydration and waterborne diseases in areas that have never experienced such disasters. Conversely, flooding contaminates water supplies and overwhelms sewage systems. Saltwater intrusion into coastal freshwater sources threatens drinking water security for millions. These changes require clinicians to consider water access and quality in patient assessments and public health planning.

6. Mental health impacts

"Climate anxiety" or eco-anxiety is emerging, particularly among young people suffering from depression, anxiety, and increased rates of substance abuse and suicide. In addition, natural disasters cause post-traumatic stress disorder (PTSD), depression, and community trauma.[6] According to the World Health Organization (WHO), climate change poses a rising threat to mental health and psychosocial well-being, from emotional distress to anxiety, depression, grief, and suicidal behavior. Communities experiencing climate disasters suffer from PTSD, depression, anxiety, and increased rates of substance abuse and suicide.[7] Loss of homes, livelihoods, and community ties has lasting psychological effects. Thus, the psychological toll of climate change is emerging as a significant public health concern. Climate anxiety and eco-distress affect millions who face an uncertain future. Healthcare providers must now integrate climate-related mental health screening and support into routine practice.

7. Climate migration health needs

Population displacement due to environmental degradation creates healthcare challenges, including infectious disease spread, interrupted chronic disease management, and increased trauma care needs.[8] Climate change disproportionately affects certain populations, exacerbating existing health inequities. Children face developmental risks from heat exposure, malnutrition, and infectious diseases. The elderly have reduced adaptive capacity to temperature extremes. Pregnant women experience increased risks of adverse outcomes. Low-income communities and marginalized groups often live in areas most vulnerable to climate impacts while having the least resources for adaptation. Due to climate change, indigenous populations also face growing threats to their traditional livelihoods and food sources. Clinicians must recognize and address these disparities through targeted interventions and advocacy.

8. Healthcare infrastructure strain

These challenges are fundamentally reshaping clinical practice. Healthcare providers must develop new competencies in climate-related health risks, incorporate environmental assessments into patient evaluations, and participate in preventive public health initiatives.[9] Medical education curricula are evolving to include climate health content. Clinical guidelines are being updated to address climate-related considerations. The concept of "planetary health" is emerging, recognizing the inseparable connection between human health and the health of natural systems.

Thus, climate-related disasters are directly impacting healthcare facilities and operations. Hurricanes, floods, wildfires, and extreme heat events can damage hospitals, disrupt power supplies, compromise pharmaceutical storage, and prevent patients from accessing care. Healthcare systems must invest in climate resilience, such as emergency preparedness, infrastructure strengthening, heat action plans, backup power systems, and telemedicine capabilities. The increasing frequency of mass casualty

events requires enhanced surge capacity and disaster response protocols, while physicians are required to counsel patients on climate health risks and advocate for policy changes. Together, these efforts underscore the urgent need for clinical healthcare system adaptation.

9. Conclusion

Addressing climate-related health challenges requires action at multiple levels. Individual clinicians can educate patients about climate health risks, prescribe climate-resilient care plans, and advocate for environmental health policies. Healthcare institutions must reduce their own carbon footprints while building resilience to climate impacts. The healthcare sector, which contributes significantly to greenhouse gas emissions, has both a responsibility and an opportunity to lead by example in climate change mitigation.

Ultimately, the health implications of climate change demand an integrated response that combines clinical adaptation with urgent climate action. Healthcare professionals are uniquely positioned to communicate the human health consequences of climate change and advocate for the systemic changes necessary to protect both current and future generations. The transformation of clinical practice in response to climate change is not optional—it is an essential evolution to meet the defining health challenge of our time.

Conflict of interest

Jacek Z. Kubiak is the Editor-in-Chief of this journal. The author declares that he has no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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REVIEW ARTICLE

The role of large language models in induced pluripotent stem cell-derived cardiomyocytes research and clinical translation

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(This article belongs to the *Special Issue: Exploring the Potential of Large Language Models (ChatGPT) in Cardiovascular Disease Management*)

Abstract

Background: Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are redefining cardiovascular regenerative medicine, yet challenges in differentiation fidelity, functional maturation, and scalable production restrain their full clinical potential. **Aim:** This review evaluates the pioneering integration of large language models (LLMs)—including GPT-4, BioGPT, and BioMedLM—into iPSC-CM research and translational therapeutics, with a focus on advancing precision, efficiency, and patient-specific care. **Methods:** Structured searches across biomedical and artificial intelligence-focused databases were conducted to map how LLMs augment literature mining, experimental design, multi-omics integration, and clinical translation, including personalized therapy prediction and drug safety assessment. **Results:** LLMs demonstrably surpass traditional tools in identifying gene-phenotype links, refining clustered regularly interspaced short palindromic repeats-based differentiation protocols, and merging patient-level datasets with iPSC-CM outputs. Limitations include model interpretability, reproducibility across genetically diverse populations, and ethical considerations regarding data privacy and bias. **Conclusion:** Despite these barriers, early translational applications demonstrate that LLMs can accelerate hypothesis generation, optimize laboratory-to-clinic pipelines, and enable high-fidelity, patient-specific cardiomyocyte modeling. **Relevance for patients:** The synergy of LLM intelligence and iPSC-CM biology has the potential to deliver safer, more effective, and deeply personalized regenerative cardiac therapies—moving the field closer to truly bespoke heart repair.

Keywords: Artificial intelligence; Large language models; Biomedical natural language programs; Induced pluripotent stem cells; Cardiac regenerative medicine

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1. Introduction

Cardiovascular diseases (CVDs) remain the leading cause of mortality worldwide,[1] necessitating continuous advancements in therapeutic strategies. According to the World Health Organization, CVDs account for 32% of global deaths,[2] with Indonesia

recording 651,481 CVD-related deaths (38.2%),[3,4] the United States 957,455 deaths (35.7%),[5] and Japan 372,483 deaths (28.0%).[6] In high-performing healthcare systems, CVD-related fatalities can be reduced below 20% through advanced preventive strategies, early intervention, and innovative therapeutic solutions.[7-10] One such breakthrough is the development of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs),[11] which offer potential applications in disease modeling, drug testing, and cardiac tissue engineering. However, challenges persist, including variability in differentiation protocols, limited functional maturation, and obstacles to large-scale clinical application.[12]

The rise of artificial intelligence (AI)[13] and large language models (LLMs)[14] has opened new avenues to accelerate iPSC-CM research and clinical translation. Historically, AI-driven innovations have reshaped cardiovascular medicine.[15] Machine learning has enhanced diagnostic imaging,[16] refined risk prediction models,[17] and optimized surgical planning in cardiothoracic procedures.[18] More recently, LLMs, such as ChatGPT (OpenAI), DeepSeek (Hangzhou DeepSeek AI Company), Bard (Google AI), and GROK (xAI),[19] have revolutionized biomedical research by enabling large-scale data analysis,[20,21] optimizing differentiation strategies,[22-25] and predicting patient-specific responses to regenerative therapies.[26,27] Since its release on November 30, 2022, ChatGPT reached one million users in just five days—far surpassing the growth of platforms, such as Facebook, which took nearly 10 months to reach the same milestone. Remarkably, ChatGPT has also demonstrated performance comparable to a 3rd-year medical student on the National Board of Medical Examiners assessments and passed the United States Medical Licensing Examination Step exams,[25] underscoring its potential to contribute meaningfully to high-accuracy domains, such as stem cell-based cardiovascular research.[28] Despite these advancements, current applications of LLMs in iPSC-CM research remain underexplored, with key gaps in long-term validation, reproducibility, and standardization.

This review critically examines the evolving role of LLMs in iPSC-CM research and translation. Through targeted analysis of current literature, it explores how LLM-based frameworks can enhance differentiation strategies, uncover functional biomarkers, and bridge lab-based insights with clinical application, laying a foundation for more scalable and precise cardiovascular regenerative solutions.

2. Methods

To synthesize a comprehensive view of LLM applications in iPSC-CM research and clinical translation, a narrative review methodology was adopted. Relevant studies were identified through targeted searches across PubMed, Google Scholar, arXiv, and Web of Science using combinations of the following terms: "LLM," "large language model," "iPSC-CM," "induced pluripotent stem cell," "cardiomyocyte differentiation," "regenerative cardiology," "cardiotoxicity," "CRISPR screen," "single-cell RNA-seq," and "deep learning." Additional queries incorporated more specific phrases, including "LLM in clinical genomics," "cardiac lineage specification," "iPSC-CM drug screening," "electronic health records," "BioBERT," "BioMedLM," and "protein structure prediction."

Inclusion criteria comprised: (i) peer-reviewed articles, preprints, or white papers describing the use of AI or LLMs in cardiovascular, stem cell, or regenerative research; (ii) studies involving iPSC-CMs in disease modeling, drug screening, or translational applications; and (iii) sources published in English from 2018 onward to reflect the advent of transformer-based architectures.

Exclusion criteria included: (i) studies not involving cardiovascular applications or not using iPSC-CMs; (ii) non-AI-based reviews or purely theoretical discussions without applied methodology; and (iii) articles lacking relevance to clinical translation or omics-driven discovery.

No strict limitations on publication types were imposed, allowing the inclusion of preclinical, computational, and translational studies. Approximately 150 sources were screened, with 45 core references included in the final synthesis based on thematic relevance, methodological quality, and impact on the evolving role of LLMs in cardiovascular regenerative medicine.

3. Results and discussion

Although structured as a narrative review, we integrate comparative insights and propose a scaffolding for future benchmarking protocols in iPSC-CM applications of LLMs.

3.1. Summary of key findings

LLMs, such as ChatGPT, are increasingly integrated into clinical and research workflows, supporting peer discussions, complex decision-making, and interdisciplinary planning. In cardiothoracic contexts, they assist with surgical preparation, data analysis, literature synthesis, and knowledge translation—supporting expertise sharing, collaborative planning, and innovation in iPSC-CM research.[24,26] The visual overviews of this pipeline are shown in Figures 1 and 2.

Figure 1 illustrates the progressive specialization of AI tools–from general-purpose AI to clinical personalization through LLM-enhanced multi-omics modeling customized for cardiac regenerative contexts. Figure 2 outlines the

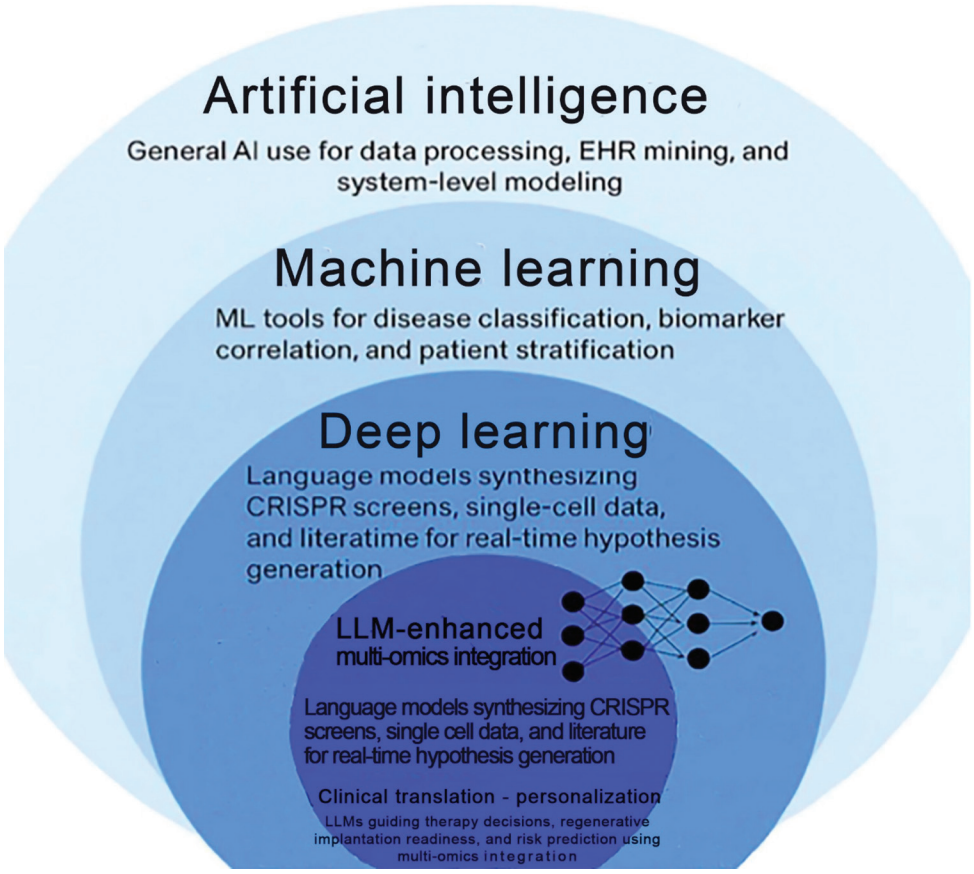

Figure 1. Layered AI-LLM integration in iPSC-CM research and clinical translation. Image created by the authors.
Abbreviations: AI: Artificial intelligence; CRISPR: Clustered regularly interspaced short palindromic repeats; EHR: Electronic health record; LLM: Large language model; ML: Machine learning.

integration of LLMs across the five-phase iPSC-CM research and clinical translation workflow: (i) literature mining and knowledge extraction: LLMs, such as BioGPT and ChatGPT, summarize protocols, annotate biomarkers, and extract disease-gene associations from biomedical corpora, (ii) target and pathway discovery: deep generative models, such as BioMedLM and AlphaMissense, prioritize variants, and signaling axes (e.g., *PGC1α* and *SIRT3*) relevant to mitochondrial maturation, (iii) *In silico* modeling of molecular interactions: Structure predictors (AlphaFold and RoseTTAFold) map mutation-driven conformational changes, while JAX and PyTorch simulate cardiomyocyte differentiation trajectories, (iv) functional testing in iPSC platforms: AI-guided experiment planners optimize clustered regularly interspaced short palindromic repeat (CRISPR) screens and electrophysiological readouts using tools like scGPT and DeepChem, and (v) clinical translation and risk prediction: multimodal fusion of omics + electronic health records (EHR) data supports transplant safety scoring, arrhythmia prediction, and therapy personalization through platforms, such as REALM and CardioGenAI. Arrows denote LLM-facilitated knowledge flow. Annotations highlight model-specific tasks. This framework emphasizes interpretability, reproducibility, and predictive fidelity across patient-specific and population-scale applications. This figure also illustrates the methodological diversity across international studies, enabling comparison between molecular-targeting and clinical-triage LLM use cases.

LLMs have become key tools in modeling iPSC-CM maturation. They also support clinical translation by handling complex, multi-layered datasets. At the molecular level, key maturation hallmarks, such as sarcomere alignment, T-tubule formation, and mitochondrial biogenesis, are increasingly understood through integration of single-cell transcriptomics,[29,30] epigenomic atlases,[31-35] and proteomic datasets. In particular, mitochondrial maturation has gained central focus, as iPSC-CMs transition from a glycolytic, fetal-like metabolic profile to one reliant on mitochondrial oxidative phosphorylation, characteristic of mature cardiomyocytes. Recent studies, including a study in Spain by Zamora-

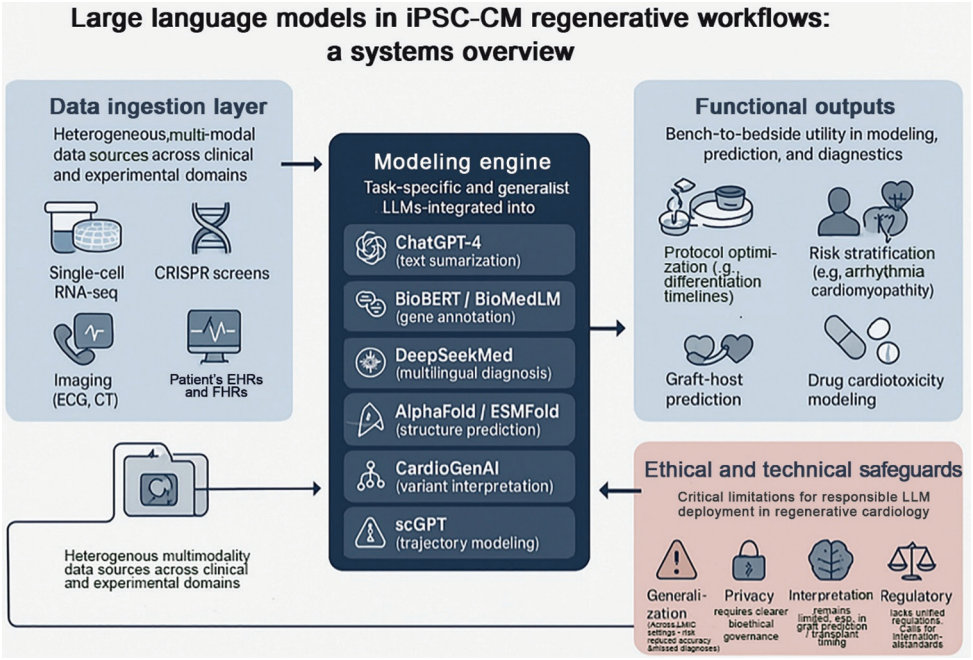

Figure 2. LLM-Augmented iPSC-CM Research and Translation Pipeline. Arrows denote the flow of data and knowledge; annotations highlight the model's functions and limitations. Image created by the authors.

Abbreviations: CRISPR: Clustered regularly interspaced short palindromic repeats; CT: Computed tomography; ECG: Electrocardiogram; EHRs: Electronic health records; FHRs: Functional heart readouts; LLM: Large language model; LMIC: Low- and middle-income countries; ML: Machine learning.

Dorta *et al.*,[36] utilized time-resolved metabolomics and CRISPR libraries to trace metabolic reprogramming (e.g., identifying *RTN4IP1* and *ECHS1*), providing a functional contrast to Liu *et al.*'s[37] broader transcriptomic atlas in iPSC-CM maturation. Together, these findings pave the way for using similar CRISPR-based tools to determine whether temporal activation and modulation of *PGC-1α*, *MFN2*, and *SIRT3* can enhance post-transplant integration and functional maturation.

Table 1 summarizes key studies that directly incorporate AI and LLMs into cardiovascular research, highlighting their methodologies, systems used, key findings, and limitations. While this paper follows a narrative review structure, the comparative table serves to clarify specific contributions and gaps across the current literature. Rather than providing a quantitative meta-analysis, it distills representative examples to scaffold the discussion that follows. These studies demonstrate a growing yet uneven integration of LLMs in clinical and experimental cardiology, often limited by a lack of benchmarking, small sample sizes, or conceptual framing without implementation. This underscores the need for more rigorous computational evaluation and real-world application trials.

On the computational front, advancements in LLM programming—including transformer-based architectures and integration with programming libraries, such as PyTorch,[40] JAX,[41,42] and HuggingFace transformers[43]—have enabled more efficient modeling of high-dimensional omics data. LLMs trained on scientific literature, laboratory records, and genomic annotations support the generation of hypotheses, design of protocols, and annotation of maturation-specific expression networks. For example, Google DeepMind's use of reinforcement learning on amino acid-specific datasets, AlphaMissense,[44] combined with LLM-assisted literature mining and streamlining CRISPR-based editing and functional assays, has reconstructed cardiac gene regulatory networks involving *NKX2-5*, *GATA6*, and *MYL2*, providing a systems-level view and enabling efficient mapping of variants affecting these regulators in cardiomyocyte differentiation. Cross-institutional efforts in Japan are now implementing LLM-assisted pipelines in pursuit of their first model suite, which will further impact the iPSC-CM studies and clinical translation while positioning themselves at the forefront of global AI-powered biomedical discovery.[45]

Table 2 compares the traditional workflows in iPSC-CM research and clinical translation using LLM-enhanced workflows. This table compares the evolution of iPSC-CM research with the support of AI. In the traditional workflow, each stage—such as reading papers, designing experiments, and analyzing data—relies heavily on manual labor and human memory. With AI integration, especially language models, tasks become faster, smarter,

Table 1. Comparative evaluation of AI and LLM studies in cardiovascular and biomedical research

Study	Methodological approach	Model system	Key finding	Notable limitation
1. Liu *et al.*[37]	CNN on echocardiograms	Human echo datasets	Outperformed cardiologists in detecting HCM	Reduced accuracy on underrepresented ethnicities
2. Panahiazar *et al.*[38]	Random forest and EHR	Retrospective EHR	Predicted heart failure six months in advance	No external validation across systems
3. Olawade *et al.*[15]	Narrative review of AI in cardiology in general	AI in general	Reviewed current AI trends in cardiology, highlighting LLMs' potential in diagnostics and clinical support	Lacked specific model validation or benchmarking
4. Tolu-Akinnawo *et al.*[16]	Systematic literature review	AI in non-invasive cardiac imaging	Improved cardiac image analysis accuracy, including LLM support in annotation and automation.	Applied heterogeneous validation metrics across studies.
5. Kasartzian and Tsiampalis[17]	Review	ML/AI for cardiac risk prediction	Outperformed traditional risk calculators in CVD risk assessment.	Limited real-world implementation
6. Leivaditis *et al.*[18]	Review	AI in cardiac surgery	LLMs supported surgical planning and patient stratification	Need for data standardization and a regulatory framework
7. Salihu *et al.*[24]	Pilot study	ChatGPT for heart team decision making	ChatGPT enhanced team communication in evaluating severe arctic stenosis cases	Limited by a small sample size and a qualitative nature
8. Ahmed *et al.*[25]	Opinion peace	ChatGPT in cardiothoracic surgery	Outlined the potential of ChatGPT to improve pre-/post-operation patient communication	No empirical data or case application
9. Clark[27]	Perspective	ChatGPT in cardiac surgery and transplantation	Proposed integration of LLMs in education and procedural support	Remained conceptual, no implementation data.
10. Chen *et al.*[21]	Model development	Multi-role ChatGPT framework	ChatGPT can assist in clinical summarization and medical data structuring	Dependent on prompt engineering
11. Iqbal *et al.*[20]	Umbrella review	LLM in healthcare (focus on ChatGPT)	Strong potential for clinical communication, patient interaction, and record summarization	Unresolved hallucination and bias mitigation
12. Pan *et al.*[39]	Computational-expert hybrid pipeline	LLM human integration in EHR	LLMs boost disease detection accuracy when augmented with clinical oversight	Required clinical validation to avoid misclassification

Abbreviations: AI: Artificial intelligence; CNN: Convolutional neural network; CVD: Cardiovascular disease; EHRs: Electronic health records; HCM: Hypertrophic cardiomyopathy; LLMs: Large language models; ML: Machine learning.

and more personalized, from literature scans to clinical predictions. The AI-enhanced approach offers deeper insights, minimizes human bias, and brings research closer to real-world applications with unmatched precision. Clinically, these integrated tools support the refinement of maturation protocols and enhance patient-specific therapeutic planning. By mapping mitochondrial density and electrophysiological maturity across iPSC-CM cohorts, LLMs can identify underdeveloped grafts that are unsuitable for transplantation, ensuring safety and efficacy. This integration of cellular bioenergetics with computational modeling supports strategies for biologically aligned and data-driven cardiac regeneration. This integration provides a framework for advancing translational insight and supporting clinical standardization of regenerative therapies.

Comparative studies have begun to show the superiority of transformer-based models over traditional logistic regression or rule-based natural language processing (NLP) in tasks, such as myocardial infarction identification from clinical notes or gene-phenotype linkage in iPSC-derived platforms. For instance, BioGPT outperformed MetaMap and cTAKES in semantic accuracy when classifying drug-induced arrhythmia mechanisms from biomedical abstracts. The comparative evaluation of LLMs in cardiovascular applications is discussed in Section 3.8.

3.2. Predictive modeling and diagnostics for iPSC-CMs disease modeling and early intervention

LLMs are transforming the landscape of predictive and diagnostic cardiology by integrating patient-level, biomolecular, and physiological datasets (Table 2). In the context of iPSC-CMs, LLMs can forecast disease phenotypes by analyzing genomic instability, ion channel transcriptomics, and electrophysiological aberrancies associated with arrhythmogenic and dilated

Table 2. Traditional versus LLM-enhanced workflows in iPSC-CM research and translation

Research stage	Traditional workflow	LLM-enhanced workflow
1. Literature review	Manual curation across databases and time-consuming filtering of relevant studies, limiting systematic analysis	AI-driven comprehensive reviews of extensive biomedical databases; automated retrieval, summarization, and contextual comparison of numerous papers through NLP
2. Experimental design	Institution-based hypothesis formation, heavily reliant on prior lab protocols and trial-and-error	LLM-assisted generation of precise and testable research questions, AI-assisted hypothesis generation, and protocol optimization based on similar published data
3. Data analysis	Statistical tools-based analysis (e.g., R and SPSS) of omics/electrophysiology, requiring multi-tool integration and specialist knowledge	Integration of multi-omics, phenotypic, and high-resolution imaging datasets. Multimodal integration of scRNA-Seq, CRISPR, proteomics, and imaging through unified AI models
4. Interpretation	Results interpretation by human researchers and subject experts, with potential risk of bias or oversight	Model-driven mechanistic insights with reduced bias; LLMs highlight underreported pathways, variant impact predictions, and potential modifiers
5. Clinical translation	Limited predictive capabilities on patient-specific responses, manual correlation between lab results and clinical outcomes	Personalized modeling of disease states and therapeutics; predictive modeling of therapy outcomes using EHRs, patient genomics, and LLM-generated risk profiles

Abbreviations: AI: Artificial intelligence; CRISPR: Clustered regularly interspaced short palindromic repeats; EHRs: Electronic health records; iPSC-CM: Induced pluripotent stem cell-derived cardiomyocytes; LLMs: Large language models; NLP: Natural language processing; scRNA: Single-cell RNA; SPSS: Statistical Package for the Social Sciences.

cardiomyopathies.[44-46] These models have demonstrated efficacy in predicting calcium-handling dysfunctions, sarcomeric gene disruptions, and metabolic shifts during cardiomyocyte maturation.[47] Clinically, LLM-driven platforms support early identification of myocardial ischemia and hypertrophy by integrating wearable telemetry, EHR-derived hemodynamics,[48] and laboratory markers, such as N-terminal pro-B-type natriuretic peptide,[49,50] troponins, and C-reactive protein.[51] Their ability to continuously learn from cross-institutional datasets allows them to fine-tune treatment decisions—suggesting beta-blocker versus angiotensin-converting enzyme inhibitor therapy in hypertensive heart disease,[52] or even proposing individualized antiarrhythmic strategies based on ion channel mutations (e.g., CardioGenAI).[53,54]

3.2.1. Major AI and LLM tools and platforms

Various AI and LLM platforms are currently integrated into iPSC-CM research pipelines and clinical translation workflows. These tools span from structure prediction and language modeling to real-time diagnostics and simulation:

(i) AlphaFold: A deep learning (DL) system developed by DeepMind that predicts the three-dimensional (3D) structure of proteins. It uses DL algorithms and protein structure databases to accurately determine the folding patterns and spatial arrangements of amino acids in protein sequences. This has revolutionized discovery workflows in structural and molecular biology. Alphafold's exceptional performance in the Critical Assessment of Structure Prediction competition has garnered widespread recognition.[55,56]

(ii) AlphaMissense: AlphaFold-based DL used to model pathogenicity of missense mutations; integrated into LLM pipelines for variant interpretation in cardiac genes, such as *MYL2* and *NKX2-5*[44]

(iii) BioBERT: Biomedical-focused LLM that supports annotation, relation extraction, and hypothesis generation in iPSC-CM molecular modeling and literature mining[57,58]

(iv) BioGPT: Biomedical generative transformer LLM for summarizing research findings, generating hypotheses, and automating insight extraction from omics data[59]

(v) BioMedLM: Biomedical-focused LLM trained on biomedical literature; useful in LLMs tasked with summarizing cardiac differentiation protocols or interpreting biomarker literature[60]

(vi) Cardiogen AI: Developed by BGI Genomics, it is an automated interpretation AI system that links genetic variants to clinical phenotypes in monogenic CVDs. It assists clinicians in diagnosing conditions, such as cardiomyopathies and hypertension, by providing a comprehensive genotype-phenotype database, enhancing precision medicine approaches in cardiology[53,54]

(vii) ChatGPT: General LLM used in literature synthesis, research planning, protocol brainstorming, and peer discussions; not domain-specific but widely integrated in clinical planning[21-27]

(viii) Chemputer: AI-driven chemistry automation aims to revolutionize the field of chemistry by automating and digitizing the chemical synthesis process. The Chemputer system combines robotics, AI, and

machine learning to enable the automated design and synthesis of complex molecules. It allows chemists to program and control the synthesis of specific compounds, improving efficiency. The ultimate goal of Chemputer is to accelerate the discovery and development of new compounds for applications in drug discovery, materials science, and beyond[61]

(ix) ClinVar: National Institute of Health (NIH)-owned variant, a public database that archives reports of the relationships between human genetic variants and their clinical significance. It collects submissions from research, labs, clinics, and researchers, helping to classify whether specific variants are benign, pathogenic, or of uncertain significance[62]

(x) DeepChem: DL framework for *in silico* drug modeling and structure-activity prediction; integrated into iPSC-CM cardiotoxicity screening pipelines using LLM-generated compound profiling[63,64]

(xi) DeepSeek-Med: Chinese biomedical LLM initiative; mentioned as a potential future collaborator in international AI consortia for regenerative platforms and clinical translation[19,58]

(xii) Ensembl Genome Browser: A genomic data hub platform that provides integrated, annotated reference genomes for a wide range of species, including humans. It enables researchers to explore genes, variants, regulatory regions, and comparative genomic data. In cardiac research and clinical translation, Ensembl plays a crucial role in identifying genetic mutations and regulatory elements linked to heart diseases[65,66]

(xiii) ESMFold: DL model developed by Meta AI that predicts protein 3D structures directly from amino acid sequences-similar to AlphaFold, but optimized for speed and scalability. It uses LLM principles trained on millions of protein sequences to understand protein folding patterns without relying on multiple sequence alignments. In cardiac research and clinical translation, ESMFold can help predict how mutations in cardiac-related proteins, such as ion channels or sarcomeric proteins, alter their structure and function. This is crucial for understanding diseases, such as cardiomyopathies or channelopathies[69]

(xiv) GEO: NIH-owned high-throughput gene expression and sequencing database repository, such as RNA-seq and microarray results. Researchers submit datasets from various tissues, cell types, and experimental conditions, including cardiac cells and disease models. GEO enables scientists to explore gene regulations in heart disease, development, or drug response. It is used for discovering biomarkers, understanding disease mechanisms, and validating experimental findings in iPSC-derived cardiomyocytes[69]

(xv) GROK: LLM developed by xAI; referenced in the context of AI landscape expansion but not yet applied in iPSC-CM pipelines[19]

(xvi) HuggingFace Transformers: LLM library hub used for implementing transformer-based models, including BioBERT and REALM; provides the backbone for LLM fine-tuning in multi-modal omics data pipelines[43]

(xvii) JAX: High-performance ML framework used in cardiac LLMs for omics modeling and optimization tasks, including protocol efficiency simulations in iPSC-CM studies[41,42]

(xviii) PyTorch: DL library used to build and train LLMs for cardiac modeling, including time-series prediction and transformer network construction[40,70,71]

(xix) REALM: Retrieval-augmented language model combining LLMs with document retrieval—used in EHR mining and real-time diagnostic applications, including arrhythmia detection[72]

(xx) RoseTTAfold (Baker Lab): A DL tool that predicts protein structures from amino acid sequences with high accuracy. It utilizes a three-track neural network to integrate sequence, distance, and coordinate data, enabling rapid modeling of protein structures. In cardiac research, RoseTTAfol aids in understanding the structural implications of genetic mutations associated with heart diseases. By predicting how specific mutations affect protein folding and function, researchers can identify potential targets for therapeutic intervention. This is particularly valuable when experimental structures are unavailable, allowing for exploration of disease mechanisms at the molecular level[56,73-75]

(xxi) scFoundation: LLM foundation model for single-cell data integration; supports high-resolution subtype prediction and cardiac developmental mapping in iPSC-CM pipelines[47]

(xxii) scGPT: Generative LLM tailored for single-cell omics; used in predicting cell fate trajectories, cardiac subtype classification, and transcriptomic modeling[46]

(xxiii) TensorFlow: DL library used for implementing deep learning models, including convolutional neural networks and recurrent neural networks, for cardiac imaging, time-series EHR data, or iPSC-CM signal traces.[70,71,76]

3.2.2. Comparative utility of biomedical LLMs

While Table 2 outlines technical specifications and training corpora across a diverse range of LLMs—from general-purpose models, such as ChatGPT and DeepSeek, to domain-specific engines, such as BioGPT and ClinicalCamel—it is important to highlight their comparative utility in real-world cardiovascular contexts. For instance, BioGPT and PubMedGPT have demonstrated superior term-precision in omics literature mining, especially in identifying gene-regulatory networks relevant to sarcomeric function and cardiac reprogramming. In contrast, DeepSeekMed and DoctorGLM, optimized for multilingual corpora, have outperformed baseline models in extracting phenotypic annotations from iPSC-CM differentiation protocols in both Chinese and English datasets. Experimental benchmarks from Japanese and U.S. institutions have also reported LLM-enhanced accuracy in predicting arrhythmogenic gene clusters and drug-drug cardiotoxicity interactions when integrated with CRISPR screen outputs. These comparative findings support the translational validity of such models, moving them beyond theoretical constructs into tools with tangible experimental and clinical consequences.

LLMs are redefining the diagnostic and predictive capabilities of iPSC-CM platforms by merging computational insight with molecular fidelity. Conventionally, disease modeling using iPSC-CMs has faced challenges in achieving sufficient phenotypic fidelity, temporal resolution, and predictive scalability across genetically diverse patients.[17,77-79] However, LLMs, particularly those equipped with multi-modal embedding and transformer-based architectures,[80-82] are overcoming these limitations by parsing vast datasets that include single-cell RNA-seq, electrophysiological traces, and ion channel dynamics to generate high-resolution disease maps. These models are particularly valuable in predicting arrhythmogenic cardiomyopathy, long QT syndrome, and hypertrophic pathways by recognizing transcriptomic anomalies or delayed afterdepolarizations early in the iPSC-CM lifecycle.[83,84]

Diagnostic assistance has extended into automated interpretation of echocardiograms and coronary computed tomography angiography imaging, offering real-time triage support for acute coronary syndrome.[85] Clinically, the convergence of LLMs with real-time telemetry, EHR-derived biometrics, and wearable data streams is advancing early detection of ischemia, subclinical myocarditis, or mechanical desynchrony. In real-world applications, Japan's Keio University and the United States-based Stanford BioHub have documented significant improvements in outcomes using LLM-augmented surgical planning in congenital heart anomalies and heart failure risk scoring models.[85,86] These data pipelines to detect diastolic dysfunction signatures with a 30% improved lead-time over standard echo interpretations. These platforms employ supervised learning through attention-weighted tokenization of patient metadata, including age, genotype, medication history, and cardiac rhythm strips, resulting in temporally contextualized diagnostics. Natural language extraction from imaging reports and procedural notes also supports risk stratification in patients awaiting valve replacement or regenerative therapy.

In surgical contexts, LLMs are becoming indispensable to pre-operative planning for congenital heart disease and heart failure reconstruction. Here, iPSC-CM-derived functional readouts, integrated with 3D imaging and spatial transcriptomics, enable AI-generated surgical roadmaps.[87,88] Using reinforcement learning algorithms, platforms trained on surgical registries and intraoperative sensor data can recommend optimized graft placements, conduction system preservation strategies, or pharmacological adjuncts tailored to the patient's cellular profile.[89,90]

In addition to transcriptomic and electrophysiological modeling, LLMs have increasingly complement protein structure prediction tools, such as AlphaFold (DeepMind),[55,56] RoseTTAFold (Baker Lab),[56,73,79,80] and ESMFold (Meta AI),[67,68] to enable multi-layered diagnostics in iPSC-CM disease modeling. These AI-driven predictors decode 3D folding of cardiomyocyte-specific proteins, including titin (TTN),[91] myosin heavy chain 7 (MYH7),[92] sodium voltage-gated channel alpha subunit 5 (SCN5A),[93] and ryanodine receptors,[94] allowing structural annotation of patient-derived mutations and elucidating their pathogenic impact. For example, AlphaFold-enhanced variant analysis has been used to map missense-induced conformational changes in sarcomeric proteins, aligning well with LLM-predicted phenotypes, such as reduced contractility or altered calcium kinetics. This fusion of sequence-based and structure-based inference supports early diagnostics of inherited cardiomyopathies, including dilated or arrhythmogenic subtypes. Moreover, for Japan's Institute of Physical and Chemical Research and Germany's Max Planck Bioinformatics Lab, hybrid models integrating AlphaFold predictions with iPSC-CM drug testing platforms have identified altered drug-binding dynamics in mutated β1-adrenergic receptors, offering insight into individual therapeutic responsiveness.[95-98]

Clinically, this multilayered modeling assists surgical planning by flagging high-risk molecular defects before regenerative implantation, such as graft-host desmosome incompatibility in arrhythmia-prone myocardium. Thus,

predictive diagnostics now extend beyond transcriptomes into the structural proteome, enabling cardiology to move from symptomatology to atomic-resolution risk stratification.[98,99]

Together, these advances represent a paradigm shift: from descriptive cardiomyocyte modeling to predictive, action-oriented diagnostics. LLMs not only enhance the resolution and interpretability of iPSC-CM-based disease simulation but also usher in an era where computational frameworks intersect with cardiomyocyte differentiation pathways, where neural networks model the heart across molecular and clinical scales to inform patient care. These implementations exemplify the synthesis of computational intelligence with biomolecular insight, elevating care delivery from reactive to proactive. Ultimately, this fusion of AI and cardiac physiology reflects a refined, forward-thinking pursuit—where innovation, integrity, and patient-centered design come together with clarity, elegance, and meaningful clinical impact.

Across the cited studies, LLM integration varies by both task and setting. For example, transcriptomic modeling by Li *et al.*[48] emphasizes mapping the regulatory pathway, while the study by Grafton *et al.*[100] focuses on detecting early cardiotoxicity. Furthermore, while BioGPT shows strength in knowledge synthesis, CardioGenAI demonstrates clinical-genetic alignment. These contrasts illustrate a spectrum from foundational modeling to translational precision, underscoring the importance of tailoring AI tools to specific regenerative goals.

3.3. Integration with EHRs and biomarkers

The fusion of LLMs with EHRs and biomarker datasets is accelerating the shift toward predictive, personalized cardiovascular care.[101,102] By analyzing structured and unstructured clinical data, including discharge summaries, imaging reports, laboratory trends, and physician notes, LLMs can extract subtle, temporally correlated patterns often missed by traditional models. For instance, leveraging expansive, open-access datasets such as Medical Information Mart for Intensive Care IV[103,104] allows LLMs to elegantly interweave structured and unstructured clinical information, unlocking nuanced, temporally aligned insights that illuminate early-stage cardiac dysfunction, including subtle diastolic anomalies in heart failure with preserved ejection fraction or asymptomatic ischemia in diabetic populations.[104,105]

For instance, in a recent meta-analysis by Zaka *et al.*,[116] machine-learning frameworks demonstrated superior risk stratification following percutaneous coronary intervention, outperforming conventional clinical models across multiple cohorts. Synthesizing data from global centers, including advanced cardiac units in Asia and North America, the study demonstrated that AI-driven, multimodal pipelines enhance predictive precision for major adverse cardiovascular events, setting a new benchmark for data-integrated, patient-tailored cardiology.[109] These findings align with parallel advancements reported by Tremamunno *et al.*[117] in the context of computed tomography-planned transcatheter aortic valve replacement, and by Chung *et al.*,[118] who highlighted the expanding role of LLMs in perioperative risk prediction and individualized prognostication.

By extracting nuanced clinical trajectories from EHRs, LLM-integrated platforms such as REALM and models trained on multimodal data are elevating precision in iPSC-CM research, enabling early phenotype-genotype matching, streamlining patient selection, and accelerating translational pathways from regenerative hypothesis to bedside impact.[110-127]

3.4. Therapeutic response prediction and drug screening

LLMs are increasingly applied to iPSC-CM drug screening, offering new tools for personalized cardiology and regenerative pharmacology. LLMs can be combined with phenotypic data from iPSC-CMs, such as calcium transients,[121,122] action potentials,[123,124] and contractility waveforms,[125,126] to simulate therapeutic responses across diverse, patient-derived cardiomyocytes. These models stratify compounds early, identifying effective therapies and flagging cardiotoxic risks before *in vivo* testing.[127]

Recent studies have highlighted the translational potential of AI-enhanced frameworks in cardiac safety pharmacology using human iPSC-CMs. For instance, Grafton *et al.*[100] used deep learning to detect cardiotoxicity with a higher sensitivity than immunofluorescence assays. Their models captured subtle shifts, such as QTc prolongation and mitochondrial changes, and linked them to known clinical cardiotoxic profiles.[100] These models identified subtle phenotypic changes, such as QTc prolongation and mitochondrial disruption, and linked them to known cardiotoxic profiles. Similarly, research in Frontiers in Pharmacology by Shim *et al.*[128] demonstrated that computational models integrating transcriptomic data and mechanistic simulations could predict individual-specific cardiotoxic responses to tyrosine kinase inhibitors. When validated against patient-derived iPSC-CMs, these predictions aligned with observed electrophysiological abnormalities, supporting the use of AI to anticipate drug-induced arrhythmias in genetically predisposed populations. Moreover, a study in Pharmaceutical Research[129] introduced a hybrid

in silico platform that combined physiologically based pharmacokinetic and quantitative systems pharmacology models. By incorporating iPSC-CM-derived functional data, this platform accurately predicted the risk of systolic dysfunction in virtual patient cohorts receiving cardiotoxic chemotherapeutics, validating its utility against clinical endpoints. Collectively, these findings demonstrate how integrating AI and iPSC-CM platforms—especially with expanding capabilities of large-scale models—bridges predictive toxicology with regenerative medicine. The convergence of these technologies is paving the way for individualized drug safety screening and the rational design of therapeutics with minimized adverse cardiac effects.

Furthermore, LLMs' ability to consolidate multi-omic datasets—mining epigenomic, proteomic, and transcriptomic responses—enhances their utility in predicting adverse cardiac events with unprecedented temporal resolution.[127-132] *In silico* cardiotoxicity models, trained on extensive compound structure-toxicity literature, are now capable of flagging risks that might otherwise remain undetected in the early stages of screening.[133,134]

Real-world applications continue to emerge. For instance, Japan's collaboration between regenerative medicine institutes and AI developers has produced deep learning-assisted screenings of iPSC-CMs under anthracycline exposure, successfully predicting cardiotoxic thresholds in chemotherapy patients.[99] In parallel, an FDA-supported pilot study in the United States integrated LLM-driven safety models with iPSC-CMs from patients with complex arrhythmia syndromes, directly informing clinical decision-making by identifying therapeutic agents with both robust efficacy and minimal toxicity.[135]

Collectively, these advances not only shorten the bench-to-bedside timeline but also enhance patient safety by reducing the inherent trial-and-error burden in drug development. As precision therapies become increasingly molecularly targeted, LLMs are poised to propel cardiac regenerative medicine into a new era characterized by safer, more effective, and patient-responsive interventions.

3.5. Mechanistic and diagnostic integration through omics, CRISPRs, and NLP applications

LLMs are increasingly deployed to bridge mechanistic discovery and diagnostic translation in iPSC-CM research by systematically integrating CRISPR datasets, multi-omics layers, and clinical telemetry. These systems enable insight across three key domains: identification of transcriptional regulators, molecular mechanism mapping, and omics-enhanced diagnostics.[136-138]

For example, RoFormer-based and graph attention networks now facilitate high-resolution enhancer-promoter mapping, which has been validated in the context of Wnt and Notch signaling bifurcations. Likewise, transformer-based models, such as BioBERT and scGPT, have been integrated with ECG telemetry and transcriptomics to identify arrhythmic risk with lineage-specific precision,[118-125] successfully prioritizing core regulator genes—*TBX5*, *NKX2-5*, and *MEF2C*[66-77]—that define early cardiac lineage commitment. By mining large-scale literature corpora and chromatin interaction data, these models have also identified co-factors, including *GATA4*, *HAND2*, and *SIRT1*, which contribute to subtype specification and maturation.[141-143] Deep generative architectures, including RoFormer and graph attention networks, now enable high-resolution predictions of enhancer-promoter interactions, making them valuable tools for mapping mesoderm-to-cardiomyocyte transitions *in vitro*.[139,140]

Beyond regulatory insight, LLMs contribute to diagnostic augmentation by integrating multimodal omics data with patient telemetry and imaging. These models analyze ECG signals, cardiac CTs, and biomarker profiles to generate patient-specific readouts and multimodal disease signatures. This supports real-time triage and phenotype-genotype linkage in inherited cardiomyopathies, arrhythmia risk, and drug response profiling.[104-108,118-125] In particular, transformer models, including BioMedLM, LLaMA, and scGPT, demonstrate utility in combining transcriptomic features with electrophysiological telemetry from patient-derived iPSC-CMs to anticipate disease progression or treatment response.[118-122]

Recent translational efforts have extended this modeling to chromatin-level regulation. Japanese research teams, for instance, have integrated low-abundance enhancer data from patient-derived iPSC-CMs to reveal transcriptional noise patterns associated with dilated cardiomyopathy and impaired maturation signatures.[144] Concurrently, U.S.-based platforms have reconstructed mesoderm-to-cardiomyocyte developmental trajectories, uncovering regulatory bottlenecks in Wnt/β-catenin and Notch signaling cascades that influence fate decisions.[145,146] In rodents, long non-coding RNAs, such as Braveheart and histone demethylase-like lysine-specific demethylase 6A, have emerged as pivotal reprogramming regulators, suggesting that enhancer-focused LLMs may refine reprogramming fidelity at the chromatin interface.[141-143] LLMs analyze billions of molecular data points, enabling them to clarify complex biology and assist in mechanistic discovery, not just analytics. To streamline and avoid repetition, a consolidated table (Table 3) summarizes these

Table 3. Large language model functions across multi-omics integration, CRISPR insight, and diagnostic support

LLM function	Input data type	Models	Output/application
Gene editing target prioritization	CRISPR perturbation and scRNA-seq	BioBERT, scGPT	AI identified *TBX5*, *MEF2C*, and *NKX2-5* as core cardiac regulators, impacting the fate of iPSC-CM
Enhancer-promoter interaction mapping	Sequence, and epigenomic	Roformer, GAT (Graph Attention)	Predicted bifurcation nodes in Wnt/Notch pathways
Transcriptional co-factor discovery	Biomedical abstracts and protocol	BioMedLM	Revealed the influence of *GATA4*, *HAND2*, and *SIRT1* on subtype transitions
Lineage trajectory reconstruction	Chromatin maps, scRNA-seq, and ECG	Deep generative models	Modeled mesoderm-to-cardiomyocyte stages and stratified arrhythmia risk
Triage and diagnosis (biomarker inference)	ECT, CT, and telemetry	BiomedLM, LLaMA, and scGPT	Generated arrhythmia and cardiomyopathy risk profiles, predicted early fibrosis signal in cardiomyopathy
Variant interpretation	Multi-omics and phenotype	CardioGenAI	Linked gene variants to severity in inherited cardiac diseases

Abbreviations: AI: Artificial intelligence; CRISPR: Clustered regularly interspaced short palindromic repeats; CT: Computed tomography; ECG: Electrocardiogram; ECT: Electroconvulsive therapy; iPSC-CM: Induced pluripotent stem cell-derived cardiomyocytes; LLMs: Large language models; scRNA: Single-cell RNA.

integrated applications across mechanisms, models, and outputs.

3.6. Translational gaps and ethical risks

While the integration of LLMs into cardiovascular regenerative frameworks shows great promise, several systemic and technical limitations remain underexamined. These include generalizability across underrepresented populations, reproducibility of predictions in noisy or unstandardized datasets, and the interpretability of high-stakes clinical outputs, such as transplant decisions or differentiation outcomes.

A key concern is the validity of the cross-population model. Most LLMs in current use have been trained on data derived from high-income countries (HICs)—particularly the United States—European EHRs, biomedical literature, and clinical guidelines. As a result, model outputs may fail to generalize across populations with different genomic architectures, environmental stressors, and healthcare access patterns. For instance, LLMs trained exclusively on Western cardiac data have shown diminished sensitivity in detecting ischemic heart disease in Southeast Asian and rural African populations.[147-149] This bias not only impairs diagnostic accuracy but can also perpetuate disparities in regenerative therapy candidacy and outcome prediction.

Beyond data imbalance, biological noise and institutional heterogeneity also challenge reproducibility. iPSC-CM modeling involves variation across laboratory protocols, epigenetic memory effects, and differentiation batch variability.[150,151] These inconsistencies introduce latent confounders that can mislead LLM outputs, especially when working with small or institution-specific datasets. Furthermore, longitudinal datasets from low-resource regions remain scarce, limiting model calibration for predicting long-term outcomes, such as graft-host integration, ventricular remodeling, or sudden cardiac death.[152] Without multi-center validation pipelines and regionally calibrated metrics, LLMs risk producing brittle or misleading outputs under real-world biological and clinical complexity.

Ethical challenges compound these technical issues. LLMs trained on patient data raise privacy risks and call for enhanced frameworks for informed consent—particularly in iPSC-CM contexts where patient-derived cells are used for training predictive models.[156-158] In regenerative therapy, where interventions may be life-altering or irreversible, opacity of model logic is especially concerning. Clinicians must be able to interpret the reasons that a model recommends or predicts a given outcome; otherwise, reliance on black-box predictions in high-stakes decisions (e.g., transplant eligibility and cell graft rejection likelihood) could undermine patient safety and trust.

Finally, algorithmic bias remains a pressing concern. Models trained on skewed data distributions can unintentionally reinforce disparities in access to regenerative interventions, gender bias in diagnosis (e.g., underdiagnosis of women with microvascular disease), or triaging influenced by insurance status. These risks are magnified in low- and middle-income countries (LMIC) settings, where infrastructural gaps may be masked by generalized LLM outputs that do not account for resource constraints.

Moving forward, responsible deployment of LLMs in cardiovascular regenerative medicine demands global data equity, transparent architecture, and regulatory harmonization. Cross-continental consortia should be established to develop standardized, open-access cardiac datasets that incorporate genomic, imaging, and clinical

data from underrepresented regions—including Japan, Indonesia, and countries in Latin America and Sub-Saharan Africa.[153-155] Only through such interdisciplinary, decentralized collaboration can AI-enabled regenerative medicine evolve in a way that is not only innovative, but also just, safe, and globally relevant.

3.7. Future directions and global equity

To unlock the full therapeutic scope of LLMs in cardiovascular regenerative medicine—particularly within iPSC-CM-based interventions—the next leap demands an infrastructure that is as globally inclusive as it is scientifically robust. LMICs, such as Indonesia, other ASEAN members, and regions across Sub-Saharan Africa, remain underrepresented in both clinical trial participation and regenerative medicine access. To correct this, scalable LLM-driven systems must be embedded into public health frameworks where analog records, inconsistent connectivity, and resource constraints are the norm. By integrating mobile diagnostics, point-of-care telemetry, and cloud-based EHR repositories, these systems can automate disease stratification, forecast trajectory shifts, and personalize post-transplant management even in decentralized care models.

The strategic development of federated learning ecosystems, in which anonymized cardiovascular datasets from diverse regions are collaboratively trained without breaching data sovereignty, ensures performance parity across ethnic, linguistic, and socioeconomic boundaries. Mobile LLM diagnostics, co-trained on electrophysiological data from iPSC-CM laboratories in Tokyo, Boston, and emerging hubs, have begun enhancing arrhythmia and ischemia detection in rural clinics. Crucially, these systems must be co-designed with local clinicians and patient communities to encode culturally relevant phenotypes and avoid epistemic asymmetries—thereby maximizing trust, usability, and precision.

Capacity-building remains essential. Regional training pipelines for clinicians and technologists alike must match the deployment of AI-regenerative tools in LMICs. Tele-education modules, academic exchange programs, and regional centers of excellence can catalyze local expertise and leadership. These efforts are beginning to materialize: academic-industry partnerships from Yogyakarta to Nairobi are already developing curriculum-integrated LLM training that supports both clinical interpretation and translational research design.

Yet amidst this momentum, a measured realism is necessary. While theoretical tools, such as real-time iPSC-CM protocol optimization, AI-assisted cryopreservation mapping, and graft-host compatibility prediction frameworks show enormous conceptual promise, many remain preclinical or unpublished. The peer-reviewed literature currently offers limited prototypes. However, early signals are emerging. Japan's Center for iPS Cell Research and Application, for instance, has piloted closed-loop AI platforms that fine-tune cardiomyocyte induction based on real-time metabolomic feedback. At Stanford, reinforcement-learning algorithms are being trained to simulate post-graft electrical integration using iPSC-CM-derived bio-signatures. Bioreactor-based cryopreservation mapping projects, aimed at predicting graft viability and post-thaw functionality, are also in conceptual testing. While these efforts remain in development, their presence marks the beginning of a tangible shift: from theoretical modeling to translational pipelines.

Hardware innovation must follow suit. Offline-compatible LLM interfaces and solar-powered diagnostic systems can mitigate bandwidth and electricity constraints in remote settings. Open-source software, policy-aligned governance, and shared trial infrastructures—backed by AI consortia, such as OpenAI and Hangzhou DeepseekAI—must underwrite this democratization.

In summary, the future of cardiovascular regenerative medicine rests not only in molecular innovation or computational elegance—but in the shared will to heal. When LLMs are built, deployed, and trusted across every corner of the healthcare spectrum, they cease being tools of privilege and become instruments of equity. This is the true legacy of an ethically coherent, biology-aligned, and human-centered cardiac future—where regenerative therapies reach every heart they are meant to save.

3.8. A comparative overview of LLMs in cardiovascular and regenerative contexts

Despite the rapid proliferation of LLMs in biomedical research, few studies have conducted systematic, domain-specific evaluations of their performance across regenerative and cardiovascular contexts. This absence of standardized benchmarking frameworks presents a notable gap in the translational landscape—particularly when considering the diverse architectural designs, training corpora, and deployment pipelines that shape each model's clinical relevance.

Emerging comparative studies have demonstrated that general-purpose LLMs, such as ChatGPT-4, excel in contextualizing clinical guidelines and summarizing literature with high fluency. However, they may underperform in multi-omics data integration due to a lack of domain-specific fine-tuning. In contrast, models such as BioGPT (Microsoft Research) and BioMedLM (Stanford

Table 4. Benchmarking key LLM and AI tools across cardiovascular and regenerative contexts

Name	Domain specificity	Primary input modality	Cardiovascular application	Key advantage
AlphaFold	Protein structure prediction	Amino acid sequences	Accurate modeling of cardiac proteins (e.g., sarcomere variants TTN, and MYH7)	High-resolution protein folding for CMs variant interpretation
AlphaMissense	Variant pathogenicity prediction	Gene variants	Interpret missense mutations, for example, in cardiomyopathy-related genes	Enables classifications of VUS in cardiac genomics using ClinVar-linked benchmarking
BioBERT	Biomedical NLP	Scholarly biomedical text	Named entity recognition and relation extraction in cardiology studies	Domain-tuned language understanding for gene-disease mining
BioGPT	Biomedical LLM (text generation and mining)	Biomedical text	Gene-disease annotation, literature summarization	High precision and recall in domain-specific NLP tasks
BioMedLM	Biomedical LLM	Text corpora of medical publications	Competitive QA performance on medical exams (~57–69%), QA systems in medical informatics, preliminary cardiovascular insights	Strong domain-specific NLP for QA
Cardiogen AI	Cardiac genomics ML	Genomic variant profiles	Predicting disease phenotype severity in monogenic CVDs	Superior variant-to-outcome interpretation in cardiology
ChatGPT-4	General-purpose LLM	Broad text corpora and multimodal inputs	Clinical guideline interpretation, literature synthesis, and preclinical planning	broad fluency and multi-step reasoning capability
Chemputer	Automated synthesis	Chemical synthesis pipelines	*In silico* synthesis for cardiac-regenerative compound generation	Automated drug-generation workflows tied to target biology
ClinVar	Variant database	raw clinical variant records	Reference database for variant pathogenicity annotation	Standard resource for variant interpretation benchmarking
DeepChem	Drug modeling library	*In silico* molecular prediction	Toxicity screening of compounds in cardiac assays	Efficient compound efficacy and toxicity modeling tools
DeepSeek-R1/ Med	General-purpose LLM (China-owned)	Bilingual reasoning tasks	Potential use in the Chinese cardiovascular research context	Scalable, open-source model with strong multilingual NLP, rivaling GPT4
Ensembl Genome Browser	Genomic data platform	Genomic and transcriptomic query	Identifying regulatory regions and variants relevant to the iPSC-CM pipeline	Centralized gene/variant annotation hub
ESMFold	Structure-prediction and evolutionary LLM	Protein sequence	Efficient structure prediction aiding cardiac variant annotation	Fast and scalable folding predictions alternative to Alphafold
GEO	Public gene expression repository	Transcriptomic microarray and RNA-seq	Data source for cardiac gene expression variation and iPSC-CM training	Large-scale expression datasets for CM modeling
GROK	open source LLM (xAI)	Text reasoning	Emerging general reasoning tasks, limited iPSC-CM application yet	Early-stage reasoning capabilities in open models
HuggingFace Transformers	Model library and fine-tuning hub	NLP/ML frameworks	Used to fine-tune BioBERT/ BioGPT/REALM for cardiac-specific tasks	Ecosystem support with model sharing and fine-tuning infrastructure
JAX	ML computation framework	Neural network training	Training LLMs or multimodal models for iPSC-CM omics interpretation	High-performance, accelerated neural architecture support
PyTorch	ML Framework	Deep-learning neural modeling	Foundation of variant annotation and regression models in cardiac biology	Large community and ecosystem for model development
REALM	Retrieval-augmented LLM	Document retrieval and text modeling	EHR mining and real-time guideline retrieval in cardiology workflows	Efficient integration of large-text archives with LLM query mechanisms

(Cont'd)

Table 4. ***(Continued)***

Name	Domain specificity	Primary input modality	Cardiovascular application	Key advantage
RoseTTAfold (Baker Lab)	Protein folding tool	Protein sequence and distance embedding	Structural prediction for variant evaluation in cell modeling platforms	Efficient three-track network for accurate fold prediction
scFoundation	Single-cell foundation model	Single-cell transcriptomics	Embeddings used for drug response prediction and lineage inference in iPSC-CMs	Outperforming baseline models in cell population mapping
scGPT	Single-cell generative transformer	Multi-omic cell atlas modeling	Predictive modeling of cell fate trajectories and disease phenotypes in cardiomyocyte differentiation	Scalable multi-scale modeling across millions of single cells
TensorFlow	Deep learning framework	Neural network model construction	Used in image, sequence, and time-series modeling within cardiac AI	Widely supported, with high interoperability across platforms

Abbreviations: AI: Artificial intelligence; CMs: Cardiomyocytes; CVDs: Cardiovascular diseases; EHRs: Electronic health records; iPSC-CM: Induced pluripotent stem cell-derived cardiomyocytes; LLM: Large language model; ML: Machine learning; MYH7: Myosin heavy chain 7; NLP: Natural language processing; QA: Quality assurance; TTN: Titin; VUS: Variance of uncertain significance.

CRFM) have been specifically optimized for biomedical corpora, demonstrating superior performance in gene-disease association tasks and biomolecular annotation, particularly in cardiomyopathy and arrhythmia literature mining.[59,63,159] A comparative summary of key models and their cardiovascular applications is presented in Table 4 to illustrate these performance distinctions across architectural and functional axes.

DeepSeekMed, a bilingual biomedical LLM trained on Chinese and English datasets, has outperformed ChatGPT and BioBERT in cross-lingual phenotype extraction and EHR-based cardiovascular risk scoring, making it especially promising for LMIC and multilingual health systems.[160-162] Similarly, CardioGenAI, a machine learning framework developed by BGI Genomics, has demonstrated high predictive accuracy in linking genetic variants with phenotype severity in monogenic CVDs, offering a focused advantage in genotype-phenotype interpretation relevant to iPSC-CM disease modeling.[163,164]

In structural biology and regenerative cardiology, AlphaFold2, RoseTTAFold, and ESMFold represent next-generation protein structure prediction tools that have outpaced prior algorithms in predicting conformational changes in sarcomeric proteins, such as MYH7, TTN, and SCN5A, the key targets in inherited cardiomyopathies. These models have been successfully integrated into iPSC-CM variant modeling platforms to anticipate functional disruptions before *in vitro* phenotyping.[165-167]

Several frameworks—such as BioGPT, BioMedLM, and scGPT—are particularly optimized for biomedical corpora and high-dimensional omics data, enabling improved performance in gene-disease association mining, transcriptomic trajectory modeling, and drug response prediction. Meanwhile, foundational platforms, such as JAX, TensorFlow, and PyTorch, remain essential for training custom cardiac models from raw datasets, offering flexibility in integrating imaging, text, and bio signal data streams.

Despite these advances, there remains no unified evaluation framework to assess LLM performance across core regenerative tasks, such as differentiation protocol optimization, cardiotoxicity modeling, or graft-host interaction simulation. Each model tends to be benchmarked independently, often using proprietary metrics, narrow data types, or single-institution validation sets, limiting clinical transferability. The lack of harmonized performance indicators, common cardiac data benchmarks, and population-representative validation pipelines—especially in iPSC-CM contexts—continues to hinder reproducibility and deployment scalability in clinical-grade environments.

Therefore, this review serves to highlight not only the current strengths and domain-specific niches of various LLMs in regenerative cardiology but also the urgent need for cross-institutional benchmarking consortia. Such efforts should incorporate:

(i) Standardized cardiac datasets (e.g., iPSC-CM electrophysiology, omics, and CRISPR perturbations)
(ii) Multimodal integration tasks (e.g., combining text, imaging, and gene expression), and
(iii) Regionally calibrated validation protocols (especially across LMIC and diverse genetic populations).

The current review functions not only as a descriptive summary but also as a framework proposal—a scaffold on which future empirical benchmarking protocols and clinical-grade LLM applications in cardiovascular regenerative medicine can be systematically developed.

Table 5. Proposed LLM-iPSC-CM evaluation framework

LLM	Output types	Benchmark task	Suggested metric	Application in iPSC-CM
AlphaFold	Protein 3D structure prediction	Structural mutation mapping	RMSD/TM-score	Sarcomeric protein modeling for inherited cardiac diseases
AlphaMissense	Pathogenicity classification	Missense variant pathogenicity scoring	ClinVar concordance/PPV	Predicting clinical impact of sarcomeric mutations
BioBERT	Biomedical NER/relation extraction	Disease-gene-drug linkage mining	Precision/recall/F1 score	Mapping arrhythmia genes and drug interactions
BioGPT	Biomedical relation extraction	Gene-disease association mining	Precision/recall/F1 score	Prioritizing cardiomyopathy targets
BioMedLM	Literature summarization	Biomedical passage summarization	ROUGE-L/BERTScore	Rapid review of regenerative medicine papers
Cardiogen AI	Variant-phenotype linking	SNP-to-clinical outcome prediction	AUC/Matthews correlation coefficient	Personalized risk stratification using iPSC-CM
ChatGPT-4	Text generation/Q&A	Clinical Guideline interpretation	BLEU/ROUGE/expert rating	Patient education, therapeutic summarization
Chemputer	Chemical reaction planning	iPSC-CM-compatible media prediction	Reaction yield prediction accuracy	Media optimization for differentiation/stability
ClinVar	Clinical variant database	Variant validation for disease relevance	Overlap with patient variant sets	Validation of iPSC-CM patient-derived mutations
DeepChem	Molecular graph prediction	Drug-toxicity prediction	ROC/AUC/ sensitivity-specificity	Cardiotoxicity modeling via iPSC-CM
DeepSeek-R1/Med	Bilingual phenotype extraction	EHR-to-concept mapping in a multilingual setting	Exact match/recall	LMIC-compatible phenotype extraction
Ensembl Genome Browser	Gene annotation/visualization platform	iPSC-CM-related gene discovery	Annotation depth/retrieval accuracy	Regulatory target mining for cardiac differentiation
ESMFold	End-to-end structure generation	Cell lineage reconstruction	Trajectory concordance/ PAGA metrics	Maturation-state-specific folding (e.g., fetal vs. adult CM)
GEO	Omics data repository	Benchmarking gene expression in iPSC-CMs	Expression match score/ TPM fold-change	Model training dataset for transcriptomic-based prediction
GROK	Explainable AI output	Interpretability of iPSC-CM risk models	SHAP/LIME agreement with expert annotations	Enhancing transparency in regenerative risk models
HuggingFace Transformers	Model zoo and training framework	Deployment of biomedical transformer-based LLMs	Adaptability/API integration score	Hosting custom cardiac LLMs like fine-tuned BioGPT
JAX/PyTorch/ TensorFlow	Backend frameworks (for training custom models)	Custom LLM implementation/ fine-tuning	Neural FLOPs/time to convergence/accuracy	Infrastructure layer for cardiac LLM pipelines
REALM	Document retrieval	Omics data-linked literature navigation	Recall@10/NDCG	Evidence mining for protocol optimization
RoseTTAfold (Baker Lab)	Protein-protein interaction prediction	Binding site inference	Interface RMSD/DockQ	Drug-target screening via iPSC-CM
scFoundation	Single-cell foundation model	Generalization across cardiac single-cell datasets	Silhouette score/batch effect reduction	Cross-cohort prediction in cardiac developmental states
scGPT	Single-cell trajectory generation	Cell lineage reconstruction	Trajectory concordance/ PAGA metrics	iPSC-to-cardiomyocyte fate modeling

Abbreviations: 3D: Three-dimensional; API: Application programming interface; AUC: Area under the curve; BERT: Bidirectional encoder representations from transformers; BLEU: Bilingual evaluation understudy; CM: Cardiomyocytes; EHR: Electronic health record; FLOPs: Floating point operations per second; iPSC-CM: Induced pluripotent stem cell-derived cardiomyocytes; LIME: Local interpretable model-agnostic explanations; LLM: Large language model; LMIC: Low- and middle-income countries; NDCG: Normalized discounted cumulative gain; NER: Named entity recognition; PAGA: Partition-based graph abstraction; PPV: Positive predictive value; Q&A: Question and answer; RMSD: Root mean square deviation; ROC: Receiver operating characteristic; ROUGE: Recall-oriented understudy for Gisting evaluation; SHAP: Shapley additive explanation; SNP: Single-nucleotide polymorphism; TM: Template modeling; TPM: Transcripts per million.

4. Conclusion

4.1. Synthesizing the path forward

LLMs have emerged not as passive computational tools but as cognitive collaborators in the evolution of cardiovascular regenerative medicine. Their symbiosis with iPSC-CM technologies has redefined the possibility of bridging molecular depth with clinical foresight, transforming static data into dynamic, patient-specific insight. From decoding transcriptomic vulnerabilities to simulating drug responses and unmasking hidden cardiac signaling cascades, LLMs elevate regenerative cardiology from a discipline of promise to a praxis of precision.

Through the lens of iPSC-derived cardiomyocytes, LLMs do not merely predict outcomes—they actively co-shape them. Whether parsing the hidden linguistics of cardiac electrical signals or annotating the silent language of mutated sarcomeric genes, these models act as translators between biology's complexity and medicine's intent. Institutions in Japan, the United States, and others are already weaving LLMs into clinical pipelines, illustrating a future where human intuition and machine intelligence are harmoniously aligned in rhythm and resolution.

4.2. Limitations of this review

While this review presents a comprehensive outlook on the applications of LLMs in iPSC-CM research, it is not without limitations. First, the field is rapidly evolving, and novel models—particularly multimodal foundation models integrating text, images, and omics—emerge at a pace that risks outdating current interpretations. The current analysis also leans heavily on literature and infrastructural models from HICs, which may not fully account for the logistical and technological constraints present in LMICs. Challenges, such as limited access to high-throughput iPSC-CM platforms, fragmented EHRs, and low local computational capacity, may hinder the real-world use of LLM-based tools in these regions. While ethically aligned LLM deployment is emphasized, this review cannot substitute for the legal, clinical, and sociotechnical audits necessary before practical implementation in research or care. It does not offer a validated benchmarking pipeline, nor does it provide quantitative evaluations of model accuracy. Major technical limitations include the lack of validation across genetically diverse populations, insufficient quantification of uncertainties in LLM-driven predictions, and nascent regulatory frameworks for AI-based regenerative therapies. These gaps impede reproducibility and hinder clinical translation, particularly in the context of LMICs. Furthermore, the interpretability of transformer-based predictions remains a black-box challenge, demanding post-hoc explainability layers before regulatory approval. Nonetheless, by framing both upstream and downstream application nodes, this review offers a conceptual scaffold to examine ethical, biological, and translational fault lines in LLM-guided regenerative medicine.

Other important limitations remain, such as ethical risks (e.g., erroneous decisions made based on automated predictions), issues of bias in biological data, the need for rigorous regulation and clinical validation, and institutional resistance to AI integration.

This review should be seen as both a map and a mirror—a reflection of current achievements and a roadmap for future empirical validation. Although fundamentally theoretical, it is grounded in real-world implementations of LLMs and structured to highlight performance differentials, practical gaps, and future benchmarks across AI platforms in cardiovascular regenerative medicine.

4.3. Future directions

The next frontier lies in the intentional integration of LLMs with wet-lab protocols and clinical trials. This includes dynamic LLM-based systems that adjust differentiation protocols in real-time based on omics feedback from iPSC-CM cultures, AI-assisted cryopreservation mapping to ensure graft integrity, or predictive frameworks for long-term graft-host interactions post-transplantation. In addition, LLMs must transition from being interpreters of known science to generators of new hypotheses, supporting regenerative surgeons and electrophysiologists in exploring novel frontiers of cardiac identity, within the constraints of current interpretability and validation frameworks.

Globally, the emphasis must shift toward algorithmic equity, particularly through the development of federated and multilingual models so that iPSC-CM-based therapeutics do not remain a privilege of academic elites but a birthright of every human heart—regardless of geography, gross domestic product, or genetic background. A cross-continental commons for cardiac data, rooted in transparency and cultural humility, could democratize access and imagination.

To catalyze translational acceleration, we propose a modular evaluation framework that cross-references LLMs by output type (e.g., protocol optimization, variant annotation, and predictive modeling), validation method (e.g., wet-lab cross-check and patient data alignment), and regulatory stage (research-only, preclinical, or investigational use) (Table 5).

To summarize, LLMs are currently supporting cardiovascular regenerative research by interpreting omics data, optimizing iPSC-CM differentiation protocols, and simulating clinical outcomes. Their most immediate promise lies in transforming static cardiac datasets into

real-time, patient-specific insights—especially when integrated with iPSC-CM platforms and longitudinal health data. Critical gaps remain in model reproducibility, equity in data representation, and clinical translation—particularly in low-resource settings and non-Western genomic contexts. To move from theoretical potential to clinical practice, the field must prioritize federated learning, interdisciplinary education, ethical model design, and globally inclusive infrastructures that decentralize innovation and democratize access.

4.4. Final reflection on AI in the service of empathy

The integration of LLMs into regenerative cardiology offers a powerful means to enhance clinical precision and therapeutic foresight. Rather than replacing human judgment, these systems are designed to augment clinicians' capabilities—supporting nuanced interpretation and broader reach. As medicine enters an era shaped by both molecular insight and computational intelligence, a balanced and ethically guided approach is essential.

The success of LLMs in cardiovascular applications will depend not only on their technical performance but also on their alignment with human values. Empathy, transparency, and accountability must remain central, ensuring that these models are developed and deployed in ways that respect patient agency and clinical nuance.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Alterations in vaginal and urinary microbiota in menopause and associated pathologies: A narrative review

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Abstract

Background: In the premenopausal stage, the vaginal microbiota is characterized by a high abundance of *Lactobacillus*, a key genus for preserving a healthy vaginal environment. However, the estrogen decline associated with menopause modifies this microbial community, leading to a reduction in *Lactobacillus* and promoting the proliferation of anaerobic bacteria, thereby increasing the risk of dysbiosis, as observed in bacterial vaginosis. Likewise, the urinary microbiota undergoes alterations that heighten the susceptibility of postmenopausal women to urinary tract infections. Hormonal changes also cause symptoms such as vaginal dryness, irritation, and dyspareunia, resulting from urogenital atrophy, which affects not only physical health but also emotional well-being and quality of life. **Aim:** The aim of the study was to describe the changes of the vaginal and urinary microbiota's associated with estrogen deficiency in menopause, as well as their relationship with relevant clinical conditions, including pelvic floor diseases, genital infections, periodontal disease, and gynecological cancers. **Relevance for patients:** Understanding these microbial changes is crucial for optimizing clinical management and improving the overall health of women in this stage of life, as these alterations represent an emerging field of research with important diagnostic and therapeutic implications.

Keywords: Vaginal microbiota; Urinary microbiota; Menopause; Associated pathologies

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1. Introduction

Menopause marks the end of the female reproductive stage. It is accompanied by a decrease in estrogen levels, which not only affects women's overall health but also alters the composition and diversity of the lower genital tract microbiota.[1] The development of molecular techniques has enabled a deeper understanding of the diversity and complexity of this microbial community.[1,2] In premenopausal women, the vaginal microbiota (VM) is predominantly composed of beneficial bacteria, particularly from the *Lactobacillus* genus, which help prevent infections through the production of hydrogen peroxide and lactic acid, creating a protective acidic environment.[1,3] After menopause, the decline in estrogen levels alters the vaginal microbial community, leading to a decrease in *Lactobacillus* abundance[4,5] and promoting microbial imbalance with an overgrowth of anaerobic bacteria (dysbiosis). This shift increases the risk of infections such as bacterial

vaginosis (BV).[1] Estrogen plays a key role in regulating vaginal pH and stimulates epithelial cells to produce glycogen, an essential nutrient for *Lactobacillus* survival.

The urinary microbiota is also affected by menopause, although its study has been less frequent. Urine in healthy women is generally sterile, but with the decrease in estrogen, the microbiota in the urethra and bladder change, and the pH is altered. Alterations in the urinary microbiota may facilitate colonization by uropathogenic bacteria, increasing the frequency of recurrent urinary tract infections (UTIs/rUTIs) in postmenopausal women.[6,7] In addition, hormonal changes also affect vaginal hydration and mucus production. These symptoms, including vaginal dryness, irritation, and pain during intercourse, result from vaginal atrophy caused by estrogen deficiency.[1,4] Recurrent genitourinary infections and irritation symptoms can negatively impact the emotional well-being of women, which is not always addressed in medical studies. This suggests that the VM contributes not only to protection but also to maintaining vaginal health and the overall quality of sexual function.[1] In this context, menopause has been associated with changes in the composition, diversity, and activity of the microbiota in different body regions—most notably the vaginal, intestinal, and urinary tracts—which may influence the general health of postmenopausal women.[8] The decrease in estrogen levels, along with the alteration of the vaginal and urinary microbiota, may influence a range of health conditions, including urinary incontinence, genital infections, genital discomfort, gynecological cancer, and periodontal disease.[5,8] This set of menopause-related diseases constitutes an emerging field of research that has begun to gain attention in recent years.[8]

This narrative review seeks to examine the alterations in the vaginal and urinary microbiota that occur during menopause and to identify the related conditions that may impact women's systemic health. Gaining insight into the underlying causes of these changes could support more effective strategies for managing these disorders.

2. Materials and methods

This review included the following types of publications: systematic reviews, narrative reviews, meta-analyses, and relevant original studies (cohort studies and case–control studies) that demonstrated a low or very low risk of bias and a high or moderate likelihood of establishing a causal relationship between the vaginal and urinary microbiota and disorders associated with menopause. Articles published from 1990 onwards were included, provided they were available in the following databases: PubMed, Elsevier, Science Direct, Wiley, Scopus, Ovid, and SciELO, and were directly and explicitly relevant to the subject of interest. All publications that did not meet the established criteria, as well as studies on the microbiome and conditions unrelated to menopause, were excluded.

3. Vaginal and urinary microbiota in fertile women

In healthy, fertile women, estrogen levels and glycogen availability directly influence the VM by promoting the dominance of *Lactobacillus*, thickening of the stratified squamous vaginal epithelium, and increasing cervical mucus secretion.[9,10] *Lactobacillus* species are essential in maintaining the vaginal ecosystem, as they produce lactic acid (which lowers vaginal pH), hydrogen peroxide (H_2O_2), and bacteriocins—compounds with antimicrobial, antiviral, and immunomodulatory properties.[9] They also compete for adhesion sites with other bacteria, helping to prevent sexually transmitted infections (STIs) and the overgrowth of endogenous opportunistic microorganisms.[11-14]

Among healthy women of European descent, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* are the predominant $H_2O_2^-$ and bacteriocin-producing species, while in African American women, *Lactobacillus iners* is most frequently detected.[15] Understanding the prevalence of species that colonize the vaginal ecosystem is useful for the development of products for *Lactobacillus* replacement therapy.[15]

This direct effect of estrogen on the quality and increase of *Lactobacillus* has been demonstrated in reproductive-aged women using contraceptives containing estrogen. According to a review conducted in Australia, contraceptive methods that include estrogen may support a favorable VM in certain groups of women. However, the effects of progestin-only contraceptives on the vaginal environment remain uncertain, and further research is required to clarify their potential role in negative reproductive and sexual health outcomes.[16]

Advances in bacterial gene sequencing, particularly targeting the *16S ribosomal RNA* (*rRNA*) gene, have enhanced our understanding of the diversity of microbial communities in the female genital tract.[2,4,9,15] A study conducted on asymptomatic women of reproductive age in North America, including participants of Asian, White, Black, and Hispanic backgrounds, identified five distinct types of microbial community structures. Four of these were primarily dominated by different *Lactobacillus* species: community state (CST) type I (*L. crispatus*), CST II (*L. gasseri*), CST III (*L. iners*), and CST V (*L. jensenii*). The fifth type, CST IV, consisted of a more diverse group of bacteria, mostly anaerobic species.[15] It was observed that the predominance of lactobacilli in the VM is higher,

and vaginal pH is lower, in White women. In contrast, the predominance of lactobacilli decreases and vaginal pH gradually increases in women of Asian, Hispanic, and Black descent, respectively. In addition, CST III (*L. iners*) is associated with microbial states of high diversity.[15] A bidirectional interaction exists between the female reproductive system and the VM. Physiological changes occurring from birth and extending beyond menopause can impact the VM; while conversely, the VM itself can affect reproductive functions.[9]

4. Vaginal and urinary microbiota in menopause

The decline in estrogen levels during menopause leads to a reduced presence of *Lactobacillus* species, decreased glycogen in vaginal epithelial cells, and lower lactic acid production. Consequently, vaginal pH rises, making the environment more prone to infections. This shift in VM increases the risk of conditions such as BV, aerobic vaginitis (AV), and vaginal candidiasis in postmenopausal women. In addition, estrogen deficiency and low glycogen contribute to vaginal atrophy, characterized by thinning of the squamous epithelium (mainly basal and parabasal layers), decreased vaginal secretions, dryness, and painful intercourse.[1,4] Several studies confirm that both the diversity and abundance of lactobacilli diminish after menopause. A study from Sweden comparing fertile and postmenopausal women found a higher frequency of *L. crispatus* colonization in fertile women (p=0.0036).[17] Similarly, Zhang *et al.*[18] reported a reduced diversity of *Lactobacillus* spp. in postmenopausal compared to premenopausal women (p<0.05). Another investigation demonstrated that premenopausal women had significantly greater free glycogen levels, which correlated with higher *Lactobacillus* counts, while postmenopausal women showed lower glycogen and *Lactobacillus* levels (p=0.03).[19] Using *16S rRNA* gene sequencing, it was shown that during menopause, CST IV becomes predominant, marked by diverse bacterial populations and a lack of *Lactobacillus*. CST IV-A contains a few lactobacilli and various anaerobes such as *Anaerococcus*, *Peptoniphilus*, and *Prevotella*. Conversely, CST IV-B is characterized by a large proportion of *Atopobium* along with *Prevotella*, *Parvimonas*, *Sneathia*, *Gardnerella*, *Mobiluncus*, and *Peptoniphilus*.[20] The emergence of CST IV is linked to BV, a microbiota imbalance that causes symptoms like unpleasant odor, discharge, and discomfort.[1,4] BV is more prevalent after menopause and is associated with increased risks of UTIs, STIs, and gynecological problems such as pelvic inflammatory disease (PID). Women who exhibit CST III vaginal profiles during perimenopause tend to shift toward CST IV-A after menopause, which is more closely related to atrophic vaginitis.[21] Although molecular methods to study the microbiome have advanced, most clinical settings worldwide still rely on traditional approaches such as Gram staining and Nugent scoring to evaluate VM.[1] Exogenous sex steroids used in hormone replacement therapy (HRT) for menopause are commonly employed to manage menopausal symptoms. There is growing evidence that estrogen-containing compounds may promote a healthier VM. In the previously mentioned Australian review, it was found that among postmenopausal women using HRT, topically applied exogenous estrogen was associated with an increased prevalence of *Lactobacillus*.[16]

Compared to the VM, information on the urinary microbiota remains limited. Some findings suggest that hormonal imbalances after menopause may lead to dysbiosis, potentially contributing to both anatomical and functional changes that impact women's general health. These alterations can compromise vaginal integrity and contribute to the onset of genitourinary syndrome of menopause (GSM). In addition, an imbalanced urinary microbiota has been linked to symptoms like urinary urgency and incontinence, as well as conditions such as interstitial cystitis, bladder pain syndrome, and neurogenic bladder. As these issues frequently occur in postmenopausal women, the influence of hormonal shifts on microbial composition may be significant. Menopause is associated with increased alpha diversity in the urinary microbiome and a reduced abundance of *Lactobacillus* in urine—variations that may precede rUTIs like cystitis. Further investigation is essential to clarify how menopause-related changes in urinary microbiota affect the development of urinary tract disorders.[22]

5. Gut microbiome

The importance of the gut microbiota in overall health and disease is now widely recognized. A recent editorial[23] discusses how the balance or imbalance of the intestinal microbiota affects immunity and general health. Factors such as genetics, diet, age, stress, medications, and mode of delivery determine the microbial composition of the gut and, consequently, its influence on immune responses. A microbiota in eubiosis, or in a balanced state, promotes the production of metabolites with immunoregulatory and protective effects, maintaining the organism's homeostasis and health. In contrast, dysbiosis or microbial imbalance can trigger inflammation and epithelial dysfunction.

The editorial brings together research linking the gut microbiota to a range of conditions, from viral infections and respiratory diseases to cancer and neuropsychiatric disorders. It also highlights the therapeutic potential of dietary bioactive compounds and beneficial

microorganisms in immune modulation. Taken together, the editorial underscores the importance of maintaining a balanced microbiota as a comprehensive strategy to preserve health, including the health of the urogenital ecosystem.

Although the primary focus is the gut, the authors suggest broader implications. The immunological effects of the microbiota may extend to other mucosal environments, such as the vagina, by influencing epithelial integrity, pH regulation, and susceptibility to infections. Thus, the state of the gut microbiome may indirectly affect the composition and stability of the VM.

5.1. Gut microbiome metabolites and the diversity of the vaginal and urinary microbiota

The gut microbiome produces a variety of metabolites with systemic effects, including on the vaginal and urinary ecosystems. These compounds include short-chain fatty acids (SCFAs), β-glucuronidases, urolithins, and other bioactive metabolites, which can modify the composition and stability of microbiota in other body sites, thereby influencing urogenital health.[23]

β-glucuronidases, enzymes produced by the intestinal estrobolome, enable the reactivation of estrogens in the gut, promoting their recirculation and exerting beneficial local effects on the vaginal epithelium, pH, and microbial composition.[24]

SCFAs such as butyrate, propionate, and acetate—produced through the fermentation of dietary fibers—act as immunomodulators and enhance epithelial integrity, exerting anti-inflammatory effects. These fatty acids play a protective role by helping to preserve microbial balance in the vaginal and urinary tracts.[22] This improvement in mucosal function may reduce the migration of intestinal microorganisms toward the urogenital tract.[7,25]

Urolithins are metabolites produced by the gut microbiota from ellagitannins, compounds found in foods such as pomegranates and walnuts. Among them, urolithin A is excreted in the urine and may act directly on the bladder, possibly promoting a urinary ecosystem dominated by *Lactobacillus*.[22]

A deficiency of these metabolites—related to inadequate diet, gut dysbiosis, or menopause—is associated with a more diverse microbiota, loss of *Lactobacillus*, and an increased risk of infections.[7,25]

5.2. Gut microbiome and vaginal and urinary microbiota in menopause

The human microbiome plays a fundamental role in women's health, particularly in the regulation and defense of the urogenital tract. The interaction between the gut, vaginal, and urinary microbiomes has gained relevance in understanding conditions such as UTIs, overactive bladder (OAB) syndrome, and disorders related to the climacteric period.[22]

There is a functional connection between these three microbial ecosystems, and their disruption may predispose individuals to recurrent or chronic urinary diseases. After menopause, VM shows reduced *Lactobacillus* dominance and an increased presence of anaerobes and *Gardnerella vaginalis*, facilitating colonization of the lower urinary tract. In turn, the gut microbiome serves as a reservoir for uropathogenic bacteria, such as *Escherichia coli*, which can translocate and disturb the balance of the urobiome.[7]

This gut-vagina-bladder axis is regulated through shared immunological and metabolic networks. Factors such as diet, antibiotic use, age, and hormone levels exert a joint influence. A gut microbiota rich in *Lactobacillus* and Bifidobacterium species is associated with a lower risk of vaginal dysbiosis and recurrent cystitis, whereas its alteration may promote low-grade systemic inflammation, a condition commonly observed among postmenopausal women.[8]

In functional disorders such as OAB, the urinary microbiome is characterized by reduced *Lactobacillus* abundance and greater microbial diversity[25]—patterns that are often mirrored in the vaginal and gut microbiomes. These parallels support the existence of a shared microbial axis that modulates urogenital function.

The gut also plays a role in hormonal regulation through the estrobolome, a collection of bacterial genes capable of metabolizing estrogens.[24]

This microbiome-hormone axis is also implicated in the development of gynecological cancers. Alterations in the gut and VM can affect local immunity, promote chronic inflammation, and modify estrogen availability—factors that are all key to carcinogenesis.[26,27]

Overall, the gut, vaginal, and urinary microbiomes are closely interconnected. An integrated analysis of these ecosystems provides a more comprehensive understanding of urogenital diseases and opens new avenues for developing innovative, personalized therapeutic strategies in women's health.

6. Vaginal and urinary microbiome and interaction with the immune system in menopause

During menopause, hormonal changes induce profound remodeling of the urogenital tract, including alterations in

the vaginal and urinary microbiomes, as well as in local immunity. These synergistic changes have a significant impact on women's urogenital health during this stage, increasing the incidence of genitourinary symptoms, recurrent infections, and persistent inflammatory states.

Both the vaginal and urinary microbiomes exert immunomodulatory functions through interactions with the mucosal epithelium, resident immune cells, and soluble mediators such as cytokines, antimicrobial peptides, and immunoglobulins.

In states of eubiosis, *Lactobacillus* species predominate in the VM. The acidic environment they produce promotes the expression of antimicrobial peptides such as beta-defensin-2, which regulates the activation of pattern recognition receptors, including Toll-like receptor (TLR) 2 and TLR4, enabling the detection and control of pathogens.[3,11]

During postmenopause, the VM becomes less dominated by *Lactobacillus* and richer in anaerobic bacteria characteristic of CST IV, which favors genitourinary infections.[8] This microbial transition is accompanied by a less effective immune response, characterized by increased levels of inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and decreased production of defensins and other innate antimicrobial molecules.[7,26]

The urinary microbiome also undergoes dysbiosis. A decline in *Lactobacillus* in both the vaginal and gut microbiota contributes to urinary symptoms such as dysuria, urgency, or recurrent infections.[25] Moreover, this dysbiosis may negatively modulate the activity of resident immune cells, such as macrophages and dendritic cells, compromising tissue homeostasis and promoting low-grade chronic inflammation.[27]

In summary, menopause leads to the loss of vaginal and urinary eubiosis, accompanied by an immune imbalance that favors chronic inflammatory states and recurrent pathologies.

7. Vaginal and urinary microbiota in genitourinary infections in menopause

7.1. Urinary infections

The study by Naji *et al.*[7] emphasizes the important contribution of the intestinal, vaginal, and urinary microbiomes in the origin and persistence of UTIs, particularly rUTIs in women. Disruptions in these microbial communities are thought to facilitate both the onset and recurrence of such infections. In women of reproductive age, the urinary microbiota is mainly composed of bacteria from the phylum *Firmicutes.*[28] Its composition and richness are influenced by variables such as age, hormonal status, ethnicity, and sexual behavior, all of which undergo significant changes during menopause.[29] At this stage, the reduction in *Lactobacillus* and changes in urinary microbial balance are linked to an increased risk of bladder dysbiosis, incontinence, and rUTIs.[28,29] Moreover, antibiotic treatments can disrupt the urinary microbiota, encouraging the growth of resistant uropathogens and the formation of biofilms—factors that contribute to persistent or recurrent infections.[30] The VM is also closely involved in rUTIs, as it shares several bacterial species with the urinary tract. The presence of *Lactobacillus* in the vagina is crucial for preventing the colonization of pathogens such as *E. coli*, a major cause of UTIs.[31] In women with vaginal dysbiosis or low *Lactobacillus* levels, especially during menopause, the risk of UTIs increases.[32] In addition, factors such as sexual activity and the use of vaginal douches can facilitate bacterial transfer between the vagina and urinary tract.

Estrogen replacement in postmenopausal women has been shown to reduce the incidence of UTIs by restoring *Lactobacillus* levels and promoting a healthy VM, thus lowering the risk of rUTIs.[7]

Probiotic therapies and the use of *L. crispatus* suppositories have shown promising potential in preventing rUTIs, although further research is needed to confirm their effectiveness.

The gut microbiome is also a key contributor to the development of UTIs, as bacteria residing in the intestine can translocate to the urinary tract and cause infections. Disruptions in gut microbial composition may influence both the vaginal and urinary microbiota, increasing susceptibility to UTIs. Natural defense mechanisms of the intestinal microbiota, such as the production of bacteriocins and SCFAs, help limit the growth of uropathogenic bacteria and reduce the likelihood of rUTIs.[33]

In this context, biotherapeutic strategies, including probiotics and fecal microbiota transplantation, have demonstrated encouraging outcomes in preventing rUTIs by reducing bacterial adhesion, impairing biofilm development, and enhancing host defenses.[34]

Recurrent UTIs are particularly prevalent in postmenopausal women, affecting over half of this population and significantly impacting quality of life, as well as increasing the risk of serious complications such as urosepsis. Recent studies using quantitative urine culture and *16S rRNA* gene sequencing in women over 55 years old with rUTIs (some receiving daily antibiotic prophylaxis and all on vaginal estrogen therapy [ET]) found no major differences in the total number of microbial species, including *Lactobacillus*.

However, genomic analysis revealed differences in specific bacterial populations, such as *Bacteroidales*, *Prevotella*ceae, and *Actinobacteria*. These findings underscore the need for further research to clarify the role of the urinary microbiome in rUTIs among postmenopausal women.[6] The rise in antimicrobial resistance has intensified efforts to develop strategies aimed at modifying the urogenital microbiota as a therapeutic approach for rUTIs. The interconnection between the vaginal and urinary microbiota is key, as both contain *Lactobacillus*, which offers protection against pathogens. Hormonal therapy with estrogens, both systemic and vaginal, has been associated with an increase in *Lactobacillus* abundance and a reduced incidence of rUTIs. However, in women with a history of rUTIs, a higher presence of pathogens and antimicrobial resistance genes has been observed, suggesting that microbiota alterations may contribute to infection persistence. In this regard, it has been shown that ET can modify the urogenital microbiota, promoting a healthier microbiota environment and protecting against rUTIs in postmenopausal women.[35] In summary, the intestinal, urinary, and vaginal microbiomes play an interconnected role in the pathogenesis of rUTIs in postmenopausal women. Therapies aimed at restoring microbiota balance, such as ET and probiotics, show promising potential for preventing and managing these infections.

7.2. VM and recurrent vaginal candidiasis

Vulvovaginal candidiasis (VVC) is one of the most common vaginal infections; however, there is limited data on its impact in postmenopausal women. The decline in estrogen levels during menopause alters the vaginal environment, increasing susceptibility to VVC. Nevertheless, the likelihood of developing VVC decreases by approximately 7% for each year after age 57, likely due to lower glycogen levels in these women. Factors such as medications (e.g., tamoxifen, antibiotics, HRT) and comorbidities like diabetes or immunosuppression can increase the prevalence of infection. Despite these associations, little research exists on the prevalence, risk factors, treatment, and recurrence of VVC in postmenopausal women. Given the changes in both the vaginal environment and the characteristics of *Candida* species, the disease is not accurately diagnosed, emphasizing the need for further studies and patient education to support appropriate treatment in this population.[36]

7.3. VM and BV - pathogenic mechanisms of *G. vaginalis*

G. *vaginalis* is included in CST IV of the VM, even among healthy women, complicating the interpretation of its role in the pathogenesis of BV. However, evidence suggests that G. *vaginalis* comprises virulent subtypes with distinct genetic and phenotypic characteristics. Recent research has identified 10 different strains, some of which produce β-galactosidase. Notably, strains that express the *sialidase a* gene are associated with BV and exhibit the ability to form biofilms. This enzyme cleaves sialic acid residues from glycoproteins in the vaginal mucus, exposing binding sites that facilitate G. *vaginalis* adhesion, support its nutrition acquisition, and protect it from host immune defenses. As a result, the bacteria can proliferate and compromise the protective mucosal barrier.[9]

A review by Daniel *et al.*[37] explored the association between intrauterine device (IUD) use and BV. Out of 1140 identified articles, 15 studies were included, comprising cross-sectional, case-control, cohort, quasi-experimental, and randomized trials. These studies examined BV prevalence in women using copper IUDs (Cu-IUDs) and levonorgestrel-releasing IUDs (LNG-IUDs), organizing the data into three categories: (i) point prevalence of BV among IUD users, (ii) incidence and prevalence of BV in Cu-IUD users, and (iii) incidence and prevalence in LNG-IUD users. The findings suggest that Cu-IUDs may increase the incidence of BV. However, there was insufficient evidence to establish a definitive relationship between LNG-IUD use and BV onset, largely due to variability in study designs and diagnostic criteria.

Vaginal dysbiosis, particularly BV, is associated with increased risk of acquiring urogenital infections, including STIs such as HIV. Studies have shown that women with a normal VM are less likely to contract HIV-1 than those with BV.[38]

7.4. Microbiota of the reproductive tract and PID

PID is an infection of the upper genital tract caused by pathogens ascending from the vagina and cervix, affecting the uterus, fallopian tubes, and ovaries. These pathogens may be endogenous, such as *Staphylococcus aureus*, *E. coli,* coagulase-negative *Staphylococcus*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Proteus mirabilis*, which are common in AV, or exogenous, mainly *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. BV is also associated with an increased risk of PID. Furthermore, dysbiosis in the VM, especially the decline in *Lactobacillus* species, facilitates the growth of pathogens and increases the likelihood of inflammation in the upper reproductive tract. Women with vaginal dysbiosis are at higher risk of bacterial colonization, which can lead to pelvic infections. It has been proposed that *Lactobacillus* protects the host by reducing the ability of *C. trachomatis* to infect epithelial cells.[39,40]

A prospective study investigating the microbiota of the upper and lower genital tracts in patients with acute

PID found an association between BV, AV, cervical inflammation, and PID. The study included both PID patients and a control group of women undergoing tubal sterilization. PID was diagnosed through laparoscopy, culdocentesis, ultrasonography, and endometrial biopsy, and microbiological cultures of abdominal and cervical samples were conducted to identify the causative microorganisms. In the PID patients, the most frequently isolated abdominal microorganisms included *Bacteroides*, *Peptostreptococcus*, *E. coli*, *Ureaplasma urealyticum*, and *Mycoplasma hominis*. Sexually transmitted pathogens such as *N. gonorrhoeae* and *C. trachomatis* were detected in 17% and 28% of patients, respectively. In the control group, no abdominal microorganisms were isolated. PID was attributed to endogenous bacteria in 48% of cases, and to sexually transmitted bacteria in 54%.[39]

A retrospective cohort study reported a higher incidence of PID among women diagnosed with BV. Among 2956 participants, the presence of BV, as determined by Nugent's score (adjusted hazard ratio [aHR] 1.53) and Amsel's criteria (aHR 2.15), and the use of vaginal douches (aHR 1.47) were independently associated with an increased risk of PID, regardless of sexual activity patterns or coexisting STIs.[41] Another study revealed an association between prolonged use of Cu-IUDs and the development of tubo-ovarian abscess (TOA) in postmenopausal women. Patients who had used an IUD for more than 10 years, without removal during menopause, showed a higher frequency of TOA and pelvic actinomycosis.[42]

Collectively, PID involves infection of the upper genital tract due to the ascending spread of pathogens linked to STIs, BV, and AV. Its development is influenced by factors such as genital tract inflammation, hormonal changes during menopause, and prolonged use of Cu-IUDs.

8. Vaginal and urinary microbiota in menopause and genitourinary syndrome

8.1. Clinical presentation

The GSM refers to a collection of symptoms associated with reduced estrogen levels that impact the genital, urinary, and sexual health of women. While it can arise at various stages of reproductive life, it is most frequently observed during menopause. Before 2014, terms like vulvovaginal atrophy, atrophic vaginitis, and urogenital atrophy were commonly used. That year, the North American Menopause Society and the International Society for the Study of Women's Sexual Health introduced the term GSM to provide a more accurate definition. VM, particularly *Lactobacillus* spp., is crucial for genital health, but its levels decrease with menopause due to reduced estrogen. GSM is a progressive condition affecting between 67% and 98% of postmenopausal women, with 50% presenting symptoms; however, only 32% seek medical help. These symptoms are often not recognized as being related to menopause.[43]

GSM can lead to complications such as labial atrophy, vaginal prolapse, introital stenosis, and urethral issues. It also negatively affects quality of life, emotional well-being, sexual function, and self-esteem. The VM plays a fundamental role in defending against infections and preserving gynecological health. A decline in *Lactobacillus* is linked to symptoms such as vulvovaginal atrophy and vaginal dryness. Estrogen contributes to symptom relief and helps restore *Lactobacillus* dominance in the vaginal environment, supporting genital tract protection and overall vaginal well-being.[43]

8.2. Microbiota in GSM

Several investigations have explored the relationship between the VM and GSM. A 2-year follow-up study involving 750 women aged 35–60 revealed that postmenopausal women had a higher prevalence (49.7%) of vaginal microbial communities with low *Lactobacillus* levels, in contrast to 21.2% in premenopausal and 22.9% in perimenopausal women. Vaginal environments dominated by species like *L. crispatus*, *L. gasseri*/*jensenii*, and *L. iners* were linked to a lower likelihood of developing vaginal atrophy. In addition, *L. gasseri*/*jensenii* in postmenopausal women was associated with fewer symptoms of vaginal dryness and reduced libido, indicating the potential role of VM in managing and preventing GSM, especially after menopause.[44] In a separate cross-sectional analysis of 96 peri- and postmenopausal women, researchers examined the role of vaginal dysbiosis in GUSM. Among participants, 83.58% reported symptoms associated with the condition, and a greater microbial variety was observed in postmenopausal individuals. A decline in *Lactobacillus* levels correlated with both the onset and intensity of GUSM symptoms. Other microorganisms, including *E. coli*, *Shigella*, *Anaerococcus*, *Finegoldia*, *Enterococcus*, *Peptoniphilus* harei, and *Streptococcus*, were linked to genital and sexual complaints. Supplementation with *Lactobacillus* was found to ease genital discomfort and enhance sexual function, suggesting it could offer a non-hormonal therapeutic option for addressing GSM symptoms.[45]

8.3. GSM treatment

Managing GSM poses challenges due to the broad spectrum of available therapies and the necessity to tailor treatment to each patient's specific clinical profile. As noted by Cuccu *et al.*,[46] initial strategies typically involve the use of vaginal lubricants and moisturizers, particularly in cases of mild to moderate discomfort. In situations

where symptoms are more intense, hormonal treatment options are generally indicated. Topical hormonal options, such as vaginal creams, are preferred due to their lower risk of side effects compared to systemic treatments. Notable options include ospemifene and dehydroepiandrosterone (DHEA), which improve vaginal and urinary symptoms without significantly altering systemic estrogen levels.

Although hormone therapy is beneficial, it carries potential risks, particularly for women with a personal history of cancer or who are considered at elevated risk. The safety of these treatments remains a controversial topic, requiring further research, particularly in women with a history of breast cancer. It is crucial for physicians to consider the benefit-risk profile of each patient before prescribing hormonal treatments and to promote informed decisions based on available evidence.[46]

A multicenter study carried out across 28 sites in Spain involving 108 postmenopausal women found that using 0.005% vaginal estriol gel was effective in lowering the recurrence of UTIs. Participants treated with estriol experienced a notable decline in infection rates and an improvement in vaginal pH, supporting its potential as a safe and beneficial therapy for postmenopausal women affected by GSM.[47] Over the years, multiple clinical guidelines have addressed the treatment of this condition. The United States Food and Drug Administration (FDA) has authorized various therapies for managing vulvovaginal atrophy and vasomotor symptoms linked to menopause. Nonetheless, the FDA has also issued warnings about the potential risks of hormone therapy, including increased risks of cardiovascular events, breast cancer, and thromboembolism. These concerns led many women to discontinue systemic hormone treatment following the findings from the Women's Health Initiative.[43] For pharmacological treatments, systemic ET is the main option for vasomotor symptoms, while local ET is used for vaginal symptoms. Vaginal ET is considered the first-line treatment for GSM, as it involves lower doses and has fewer side effects. In women who have undergone hysterectomy, only estrogen is used, whereas in those with an intact uterus, it is combined with progestogens.[43]

DHEA, also known as prasterone, is effective for treating dyspareunia (pain during sexual intercourse) and other GSM symptoms. DHEA improves vaginal lubrication and epithelial function without affecting systemic estrogen levels. Ospemifene, a selective estrogen receptor modulator, is also effective in treating dyspareunia and vaginal dryness, offering beneficial effects on bones and anti-estrogenic effects on breast tissue.[43]

Estriol, a naturally occurring estrogen with mild potency, is commonly employed in countries outside the United States for managing GSM. Despite lacking FDA approval, its application as a vaginal gel has demonstrated positive effects on the vaginal maturation index and pH levels in postmenopausal women. The combination of estriol with *Lactobacillus acidophilus* has been reported to improve GSM symptoms and support the recovery of VM. In women with a personal history of breast cancer, hormonal therapies should generally be avoided. In these cases, non-hormonal alternatives such as lubricants, hyaluronic acid, pelvic floor therapy, and laser-based treatments offer reasonable and safe options.[43] Emerging research indicates that probiotics, either alone or used alongside ET, can help relieve symptoms of vulvovaginal atrophy. In postmenopausal women, oral probiotics like *Lactobacillus rhamnosus* Gr-1 and *Lactobacillus reuteri* RC-14 were associated with significant reductions in Nugent scores and notable improvements in GSM symptoms (p=0.0001). Another study found that the use of estrogen together with probiotics was especially beneficial for symptoms like vaginal dryness and painful intercourse. In ovariectomized rat models, supplementation with *Lactobacillus* decreased menopausal symptoms and enhanced intestinal barrier function.[48] Altogether, VM is crucial for genital health and is related to GSM. Estrogen deficiency in menopause reduces *Lactobacillus*, leading to symptoms such as vaginal dryness and urinary issues. Vaginal dysbiosis, characterized by fewer *Lactobacillus* and more pathogenic bacteria, exacerbates symptoms. Management strategies encompass vaginal estrogen formulations, ospemifene, DHEA, and estriol, along with non-hormonal therapies suitable for women diagnosed with breast cancer. Among the non-hormonal alternatives, laser-based treatments and *Lactobacillus* supplementation have demonstrated encouraging outcomes.

9. Vaginal and urinary microbiota in menopause and pelvic floor disorders

A systematic review[49] explored the influence of the microbiome on female reproductive and urological health, addressing conditions such as urinary incontinence, OAB, pelvic pain, fecal incontinence, and hypoactive sexual desire disorder. It underscores the relevance of microbial species like *Lactobacillus*, which are key to preserving microbial homeostasis. A significant association was observed between symptom severity in OAB and increased microbial richness and diversity. Genera including *Lactobacillus*, *Streptococcus*, *Gardnerella*, *Prevotella*, *Methylobacterium*, *Acinetobacter*, and *Sphingomonas* were linked to the intensity of OAB-related symptoms, suggesting a connection between bladder microbiota composition and clinical manifestations.[50] The work highlights how variations in microbial composition

between healthy individuals and those with certain conditions may offer diagnostic value. Specific bacterial profiles could act as indicators for disorders such as endometriosis and urinary incontinence. Adjusting the microbiota using probiotics or comparable methods is proposed as a possible therapeutic approach. Moreover, the relationship between microbial diversity and symptom severity is highlighted, as greater bacterial diversity might increase the severity of conditions.[49] The included studies utilized sophisticated methods, including *16S rRNA* gene analysis and focused metabolic profiling, which have contributed to a deeper understanding of the microbiome. It underscores the importance of a personalized treatment approach based on individual microbial differences. However, an important gap is identified—the lack of causal evidence between changes in the microbiome and pelvic floor dysfunctions. Longitudinal studies manipulating the microbiome to assess its direct effects on these dysfunctions are suggested.[49]

One study explored how the urinary and VM are linked to the intensity of mixed urinary incontinence (MUI) symptoms in women.[51] The study included 210 participants, with 126 diagnosed with MUI and 84 serving as controls. Researchers identified six distinct urinary microbiome profiles; one group, characterized by low *Lactobacillus* levels and increased microbial diversity, was correlated with more frequent and intense episodes of total and urgency incontinence. The reference group, dominated by *Lactobacillus*, showed less severe symptoms. Although vaginal community types were not related to the severity of incontinence, alpha diversity in urine showed that greater bacterial richness was associated with more incontinence episodes. The results suggest that lower *Lactobacillus* dominance and higher bacterial diversity may be linked to greater severity of urinary incontinence, but more research is needed to determine whether other bacterial genera also play a role.[51]

Before 2012, it was commonly assumed that the urinary tract of healthy individuals was sterile. However, metagenomic analysis has uncovered the presence of a distinct urinary microbiota, reshaping the understanding of lower urinary tract disorders (LUTD). Alterations in this microbial community, termed urinary dysbiosis, have emerged as a possible contributing factor to functional LUTD. A review encompassing 36 studies found that viable bacteria present in urine, but undetectable through traditional cultures, may play a central role in dysbiosis.[52] An important observation is that women experiencing OAB present distinct urinary microbial profiles when compared to asymptomatic women. Notably, there is a higher presence of genera like *Gardnerella* and a reduced abundance of *Lactobacillus*, indicating that such microbial imbalances may play a significant role in the development of OAB manifestations, particularly urgency in the absence of infection.[53] This pattern is also seen in urgency urinary incontinence, where urinary dysbiosis may influence bladder storage symptoms. Research has shown 80% bacterial growth in women with OAB, undetected by standard cultures, highlighting the need to improve diagnostic methods.[53]

Emerging evidence suggests that urinary microbiota may be pivotal in tailoring individualized therapies for women affected by urgency urinary incontinence, allowing more precise classification of subtypes and optimizing treatment strategies. This underscores the growing recognition of the microbiome's role in the development and management of urinary tract conditions.[54] In contrast, the systematic review conducted by Sze *et al.*[25] examined the relationship between dysbiosis in the gut, vagina, and urinary tract in women diagnosed with OAB. While a consistent bacterial profile was not observed among healthy participants, OAB patients displayed reduced microbial diversity. Although the overall bacterial composition between cases and controls did not differ significantly, the urinary microbiome of those with OAB appeared more susceptible to changes influenced by the intestinal or vaginal environment. These findings point to a possible interconnection between the three microbial ecosystems, but further studies are needed to clarify this association.[25]

In addition, Yu *et al.*[55] carried out a meta-analysis including 7298 Chinese women across eight studies, exploring the relationship between pelvic floor disorders and vaginal microbial alterations. The results indicated that a reduced presence of *Lactobacillus*, increased vaginal discharge, and a history of vaginitis were linked to higher pelvic floor disorder risk. The review highlights that imbalances in the VM may lead to inflammation and damage to pelvic support tissues, potentially contributing to the onset of pelvic organ prolapse. This condition results from weakening of the pelvic structures—ligaments, muscles, and connective tissue—often influenced by changes in extracellular matrix components such as collagen, elastin, and proteoglycans, all synthesized by fibroblasts and crucial for pelvic stability.

In summary, the urinary and VM play a significant role in women's urogenital and reproductive well-being during menopause. Variations in microbial diversity within these ecosystems have been associated with conditions such as urinary incontinence and OAB. Although notable associations have been reported, further studies are required to confirm causality. Modulating the microbiome, including through probiotic interventions, holds promise

as a preventive and therapeutic option for urinary and pelvic floor disorders.

10. Vaginal and urinary microbiota in menopause and gynecological cancer

The female reproductive tract contains a specialized microbiome crucial for maintaining health, especially in the lower tract, where *Lactobacillus* species dominate during the reproductive years. These bacteria interact beneficially with the host, preserving vaginal balance. When this harmony is disrupted, known as dysbiosis, it can impair immune and metabolic pathways, leading to processes associated with malignancy, such as persistent inflammation, genomic instability, and altered metabolism.[56-58] These disruptions may contribute to the onset and progression of gynecologic cancers, including cervical, ovarian, and endometrial cancers, potentially through both indirect and direct mechanisms.[56] While the involvement of specific bacterial pathogens in these cancers remains uncertain, broad shifts in microbial composition have been linked to tumorigenesis.[26] Multiple risk factors are implicated in these cancers, including STIs (human papillomavirus [HPV], *C. trachomatis*, HIV), use of postmenopausal hormones, obesity, tobacco use, and inherited genetic predispositions. More recently, research has started exploring how human-associated microbial communities might influence cancer development in the reproductive tract. Although it is still unclear whether microbial alterations are a driving factor or a byproduct of these malignancies, increasing data support the notion that the microbiota may foster tumorigenesis through mechanisms such as reduced apoptosis, enhanced cell proliferation, and genomic instability.[26,57]

The urogenital microbiota, influenced by factors such as sex and age, also plays an important role in gynecological carcinogenesis. In women with low estrogen levels, such as those before puberty or postmenopausal, a mixture of anaerobic bacteria predominates, potentially creating a less protective environment against infections and cellular alterations. In contrast, during pregnancy or in young women with high estrogen levels, the VM remains more stable and is dominated by *Lactobacillus*, which protects the reproductive tract against pathogens and reduces the risk of gynecological cancers.[56] Harmful bacteria significantly contribute to the weakening of the epithelial barrier by producing hydrolytic enzymes and promoting the release of proinflammatory cytokines like IL-6 and TNF.[58] These actions drive chronic inflammation and disturb local metabolic processes, creating conditions that may support carcinogenesis. They also induce genetic instability by either damaging DNA directly or interfering with its repair, thereby increasing mutation risk. This may lead to the activation of molecules such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which suppress programmed cell death and stimulate blood vessel formation, both of which are critical to tumor progression. Moreover, microbial metabolites from the gut, including deoxycholic acid and lipoteichoic acid, can circulate through the body and contribute to the emergence of cancers in distant organs, such as the liver.[26,59,60]

Various pathogenic bacteria are linked to carcinogenesis. For example, *Helicobacter pylori* is associated with gastric cancer,[61] while *Fusobacterium nucleatum* has been linked to colon cancer.[62] Research in animal models has shown that reducing the microbiota through antibiotics decreases tumor formation in organs such as the colon and liver, suggesting that a dysbiotic microbiota may promote tumor development.[26]

Alterations in microbial balance have also been associated with the initiation and development of tumors in other parts of the body, including the skin, mouth, respiratory system, and reproductive tract. Dysbiotic microorganisms can cause failures in the epithelial barrier, immune dysregulation, and genotoxicity, creating a microenvironment conducive to cancer. Chronic inflammation is one of the best-documented mechanisms modulating cancer characteristics. For instance, *F. nucleatum* in colorectal cancer activates the NF-κB pathway, promoting the production of inflammatory cytokines such as IL-6 and TNF, which in turn promote cell proliferation and angiogenesis, both of which are essential characteristics of cancer.[56]

C. trachomatis has been identified as one of the bacteria involved in the development of gynecologic cancers. It facilitates tumor initiation by triggering epithelial-mesenchymal transition, which reduces cell adhesion and disrupts mechanisms that repair DNA damage.[63] In addition, the interplay between the gut and VM can modulate estrogen concentrations, influencing hormone-dependent disorders like endometriosis and specific cancers. The estrobolome, comprising microbial genes responsible for estrogen metabolism, controls circulating estrogen levels via β-glucuronidase activity. Dysbiosis can interfere with this regulation, leading to hormonal imbalances that may contribute to gynecologic conditions.[64] Beyond its impact on cancer development, the microbiota also plays a role in treatment outcomes among women with gynecologic malignancies. Anticancer strategies, including chemotherapy and radiotherapy, can disrupt microbial communities, potentially affecting treatment effectiveness and side effects. Interventions such as probiotics or fecal microbiota transplantation

offer the potential to enhance therapeutic efficacy and improve patients' quality of life by restoring microbial equilibrium.[56] Research in this field is crucial as it could open new opportunities for the prevention and treatment of these cancers, improving clinical outcomes and the quality of life for affected patients.[56]

10.1. VM and cervical cancer: Role of HPV

Cervical cancer remains one of the leading malignant tumors affecting women and ranks fourth in global incidence, with around 342,000 deaths recorded in 2020.[26] More than 95% of cases are attributed to persistent infection with HPV.[65] Although cervical cancer affects women worldwide, disparities exist between racial and ethnic groups. For instance, Hispanic women in the United States face a 60% higher likelihood of being diagnosed and a 30% greater mortality rate than non-Hispanic white women.[66] Oncogenic strains such as HPV-16 and HPV-18 are the primary contributors to cervical cancer development.[26,56] The cervical transformation zone, where squamous and columnar cells meet, is particularly vulnerable to HPV and is the origin site for most cervical malignancies.[67] While many infections are cleared by the immune system, approximately 10–15% persist and may evolve into cervical intraepithelial neoplasia or invasive cervical cancer.[26] Several cofactors, including multiple births, tobacco use, hormonal contraceptive use, and coinfections with other sexually transmitted pathogens, increase the risk of disease progression.[56]

Recent investigations have emphasized the significant role of the VM in influencing the persistence of HPV infections and the development of cervical cancer. This association appears especially relevant among Hispanic women, who often present with reduced *Lactobacillus* dominance and increased vaginal pH—factors that may partly explain the higher incidence and mortality from cervical cancer observed in this population.[67] A meta-analysis based on longitudinal data supports the hypothesis that a vaginal microbial environment with high diversity and lacking *Lactobacillus* predominance favors the acquisition and persistence of HPV, as well as the development of precancerous cervical lesions.[56] Specifically, women whose microbiota is primarily composed of *L. iners* show a greater tendency toward persistent infection by high-risk HPV types and progression to malignancy compared to those dominated by *L. crispatus*. This may be due to the diminished protective function of *L. iners*, including its weaker ability to suppress harmful microbes and lower lactic acid production, which contributes to a microenvironment conducive to HPV survival and cervical neoplastic changes.[56] Disruption of the vaginal microbial balance, known as vaginal dysbiosis, significantly contributes to HPV persistence. This microbial imbalance fosters an inflammatory milieu that supports viral transformation, including the upregulation of oncogenic proteins like E6 and E7,[68] promotion of genomic instability, and activation of telomerase—processes central to cervical carcinogenesis. In addition, women whose VM is predominantly composed of *L. iners* have demonstrated increased concentrations of inflammatory cytokines such as TNF-α, IL-1α, interferon gamma, and IL-8, further illustrating the link between dysbiosis and heightened susceptibility to cervical abnormalities.[69]

C. trachomatis infection has also been implicated in enhancing HPV persistence and its progression to precancerous changes by disrupting epithelial integrity, increasing basal cell exposure to HPV, and triggering anti-apoptotic pathways that support ongoing infection.[70] Supporting this evidence, recent studies underscore the role of VM in cervical pathology. BV, characterized by a depletion of *Lactobacillus* species and an overgrowth of anaerobic bacteria, has been linked to increased susceptibility to HPV infection and reduced viral clearance. A greater diversity of non-*Lactobacillus* bacteria has been associated with persistent HPV infection and progression toward high-grade cervical intraepithelial neoplasia.[71] Cross-sectional analyses indicate that HPV-positive women without dysplasia tend to exhibit a more heterogeneous VM, with higher prevalence of BV-related bacteria such as *Gardnerella*, *Sneathia*, *Megasphaera*, *Dialister*, and *Atopobium* compared to HPV-negative women.[72,73] Furthermore, longitudinal data suggest that *L. gasseri* may facilitate viral clearance, whereas *Atopobium* species are more frequently associated with sustained HPV infection.[74] A decrease in *Lactobacillus* abundance and increased microbial diversity have also been associated with elevated vaginal pH, a condition linked to more severe cervical lesions.[73]

Taken together, these findings highlight the key role of the VM in HPV persistence and cervical oncogenesis. An imbalanced microbiota, particularly when dominated by *L. iners* and BV-associated anaerobes, promotes a proinflammatory state that supports viral persistence and transformation. Understanding these microbial dynamics is essential for advancing both prevention and therapeutic strategies targeting cervical cancer.

10.2. VM and endometrial cancer

Endometrial cancer predominantly affects women after menopause, especially those in their 60s and 70s. It ranks as the leading gynecological malignancy in developed nations and is particularly common among women in the United States. Although genetic and hereditary mutations account for only 10–20% of cases, sociodemographic

elements such as race, ethnicity, and economic status may elevate the risk.[56] In the United States, both Black and non-Hispanic White women exhibit high incidence rates of this disease; however, African American women face nearly double the mortality rate compared to other racial groups.[56,75] Key contributing factors include obesity, chronic inflammation, disruptions in estrogen pathways, and the use of ET after menopause. These elements are associated with alterations in both the gut[76] and VM,[77] indicating that microbial changes may play a role in the pathogenesis of endometrial cancer.[56] Obesity contributes to endometrial cancer development through multiple biological pathways, including elevated insulin levels and increased bioavailability of insulin-like growth factor 1, both of which stimulate cellular growth and reduce programmed cell death in the endometrium.[26,78] This condition is a major contributor to the rising incidence of endometrial cancer, partly due to enhanced estrogen synthesis by adipose tissue, which drives endometrial cell division and tumor progression.[78] Estrogens also play a critical role, reinforcing how external influences such as high-fat diets are associated with a heightened risk of the disease. While ET may help relieve menopausal symptoms, it has also been linked to a greater likelihood of developing endometrial cancer.[26,78] Recent studies have suggested that both the intestinal and VM could be indirect risk factors, highlighting their importance in the etiology of the disease.[79] In summary, endometrial cancer is a multifactorial disease in which genetic, hormonal, environmental, and microbiological components interact, underscoring the need for preventive and therapeutic approaches that consider this complex interaction of factors.

The long-held belief that the uterine cavity was sterile has been questioned by several studies using 16S rRNA sequencing, which confirmed the existence of a resident microbiota. Microorganisms may reach the uterus through hematogenous spread, ascend from the lower genital tract during different menstrual phases, or be introduced during gynecological interventions such as assisted reproductive technologies.[80] Compared to the vagina; the microbiota of the upper genital tract is less abundant but more diverse, whereas the vaginal flora is primarily dominated by *Lactobacillus* species.[81]

Chen *et al.*[82] reported that the composition of the microbiota differs along the female reproductive tract and undergoes fluctuations depending on the menstrual cycle phase. During the secretory phase, there is a notable increase in microbial presence, especially *Propionibacterium acnes*, along with heightened metabolic activity involving purines, pyrimidines, amino acids, and peptidoglycan synthesis. Furthermore, certain vaginal bacterial species such as *Prevotella*, *Porphyromonas*, *Firmicutes*, *Spirochaetes*, *Atopobium*, and *Bacteroides*, in combination with elevated vaginal pH, have been linked to the development of endometrial cancer.[56,77,83,84] These microorganisms trigger the production of proinflammatory cytokines like IL-1α, IL-1β, IL-17α, and TNFα, which are commonly overexpressed in various malignancies, including those of the endometrium. IL-17α in particular has been shown to stimulate endometrial cell growth and facilitate the progression of endometriosis through its role in promoting inflammation and angiogenesis.[26,85] Recent studies indicate that the gut-brain axis plays a role in controlling circulating estrogen levels, involving the "estrobolome," a collection of bacteria capable of modifying estrogen enterohepatic circulation.[86] These bacteria produce β-glucuronidase, an enzyme that reactivates estrogens, enabling their interaction with receptors and influencing estrogen-dependent biological functions.[24,26] Disruptions in the estrobolome, or dysbiosis, can cause imbalances that contribute to diseases, including cancer. Research has identified a distinct microbial profile in endometrial cancer tissues compared to adjacent non-cancerous tissue, with higher amounts of genera like *Prevotella*, *Atopobium*, and *Porphyromonas* in tumor tissues, while *Lactobacillus* predominates in surrounding tissues.[87] In addition, elevated *Prevotella* levels and increased D-dimer in cancer tissue have been linked to more advanced disease and poorer outcomes.[26] Various explanations for bacterial overgrowth in endometrial cancer include changes in the tissue environment that promote bacterial proliferation, weakened immune defenses, or altered bacterial adherence and colonization. These observations suggest that the microbiota may influence the development, etiology, and progression of endometrial cancer, a field that requires further investigation.

10.3. Microbiota and ovarian cancer

Ovarian cancer is the second most common cancer in women, with more than 313,000 new cases and 152,000 deaths in 2020.[26] The incidence varies across different demographic groups, being highest among non-Hispanic White women (11.6/100,000), followed by Native American, Hispanic, non-Hispanic Black, and Asian and Pacific Islander women.[87] The lack of specific symptoms often delays diagnosis, leading to 70% of cases being detected at advanced stages. Incidence increases in postmenopausal women, with various factors contributing to the risk.[26]

10.3.1. Risk factors

Recent investigations suggest that microbial communities may play a role in the onset and development of ovarian

cancer, though establishing direct causality remains challenging due to the complex interplay of multiple contributing factors and the high variability of individual microbiomes.[88]

Hormonal influences such as not having given birth, early onset of menstruation, and late menopause are associated with an elevated risk. In contrast, pregnancy and oral contraceptive use offer protective effects by limiting ovulatory cycles.[26] Oral contraceptives also influence the expression of transforming growth factor β isoforms, triggering apoptosis in ovarian epithelial cells. Environmental exposures, including diets high in animal fats and Westernized lifestyles, may increase susceptibility, while diets rich in vegetables are linked to reduced risk. Genetically, although most ovarian cancer cases are sporadic, about 10% are inherited. Mutations in the *BRCA1* and *BRCA2* genes are major contributors, conferring a 20–40% lifetime risk for *BRCA1* mutation carriers and a 10–20% for *BRCA2* carriers.[26] Having multiple pregnancies, undergoing tubal ligation, or using oral contraceptives has been shown to lower the risk of ovarian cancer.[56]

10.3.2. Oncobiome or the interaction between the human microbiome and carcinogenesis

The concept of oncobiosis refers to the interplay between the human microbiome and the development of cancer, and it has been observed in several anatomical sites, including the vaginal and cervicovaginal regions, the upper genital tract, ovaries, tumor tissue, peritoneal cavity, bloodstream, and fecal matter. This microbial imbalance is linked to a reduction in microbial diversity, particularly in the peritoneal and intratumoral microbiomes found in ovarian cancer cases.[88,89] Analyses of tumor specimens revealed a predominance of bacterial phyla such as *Proteobacteria* and *Firmicutes* compared to non-cancerous tissues.[88-91] These microbes can secrete genotoxins like colibactin and cytotoxic distending toxins, which cause DNA damage and trigger repair mechanisms.[26] Moreover, a decrease in microbial diversity has been observed in cancer cases, suggesting that changes in the microbiota could influence disease development.[26,88,91] On the other hand, a study revealed that malignant epithelial ovarian tumors harbored a more diverse and abundant microbiota, including members of the order Actinomycetales as well as genera such as *Acinetobacter*, *Streptococcus*, *Ochrobacterium*, and *Pseudomonas.*[92] They also identified that *P. acnes* might accelerate cancer development.

In the vagina, a low abundance of *Lactobacillus* is associated with ovarian cancer and *BRCA* mutations,[93] especially in younger patients.[94] Genital infections, such as *C. trachomatis* and *N. gonorrhoeae*, increase the risk of ovarian cancer,[93] as well as viral infections such as HPV, cytomegalovirus, Epstein-Barr virus, and HIV.[26,56,84] In addition, antibodies against *C. trachomatis* may be associated with a higher risk of ovarian cancer by promoting the survival of cells with damaged DNA.[95] Some microorganisms invade the tumor, generating an "intratumoral microbiota" that contributes to cancer progression through mutations in DNA, activation of carcinogenic pathways, and metastasis. Certain bacterial components, including *E. coli* lipopolysaccharide found in the VM, can trigger pro-inflammatory cytokine production, which supports tumor progression and resistance to chemotherapy.[26] Inflammation within the genital tract has been linked to carcinogenesis, as seen in PID, a recognized risk factor for ovarian cancer.[56] An imbalance in the VM—marked by a decline in *Lactobacillus* species and an increase in genera such as *Acinetobacter*, *Burkholderia*, G. *vaginalis*, and *Prevotella*—may promote a local environment prone to inflammation and malignancy. This alteration appears to be associated with metabolic shifts involving glycerophospholipids and tryptophan, which, in murine models, contribute to ovarian tumor development.[56,96] Local inflammation, induced by bacterial colonization, can promote carcinogenesis by activating pattern recognition receptors such as TLR2, 4, and 5, which respond to Gram-negative bacteria. The activation of these receptors triggers inflammation through signaling pathways like NF-κB, which favors oncogenesis. Tumor-associated macrophages are essential for the development of ovarian cancer,[93] and peritoneal colonization can drive metastasis formation, including interaction with the intestinal microbiome, promoting spread to the gastrointestinal tract.[56,88] Moreover, the microbiome affects the response to treatments such as chemotherapy. Modifying the microbiome with antibiotics, probiotics, and nutrients could be a promising therapeutic and diagnostic strategy.[89]

10.3.3. Summary

Research on the microbiota and ovarian cancer has identified a significant relationship between microbial alteration and disease progression. Dysbiosis affects areas such as the vagina, genital tract, tumor tissue, and intestines, contributing to the initiation and progression of cancer. Bacteria such as *Proteobacteria* and *Firmicutes* predominate in these tissues, and bacterial infections such as *C. trachomatis* and *N. gonorrhoeae* increase cancer risk. The reduction of *Lactobacillus* in women with *BRCA* mutations may be a key factor. Bacterial metabolites induce chronic inflammation, suggesting the use of probiotics and antibiotics as potential treatments.

11. Vaginal and urinary microbiota in menopause and periodontal disease

Periodontal disease, encompassing both gingivitis and periodontitis, is a chronic infection resulting from the build-up of bacterial plaque on the tooth surface.[97] This condition involves a persistent inflammatory response that affects the tissues supporting the teeth, including the gums, periodontal ligament, and alveolar bone.[97] It has a complex origin, but key pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are recognized as major contributors.[98-100] The interaction between these microorganisms and the host immune defense can trigger tissue breakdown and ultimately result in tooth loss. In addition, the disease can introduce anaerobic Gram-negative bacteria, toxins, lipopolysaccharides, and proinflammatory substances into the circulation, potentially influencing the onset or progression of systemic conditions. Menopause, associated with a decrease in hormone levels, particularly estrogen, has been identified as a risk factor for periodontal deterioration. Studies have shown that postmenopausal women have a higher prevalence of periodontal disease, which could be linked to increased systemic inflammation and hormonal changes that affect both vaginal and oral microbiota.[97,101]

A study from India found a link between menopause, periodontal tissue damage, and osteoporosis. When comparing premenopausal and postmenopausal women, measurements such as dental plaque, gingival inflammation, probing depth, and clinical attachment loss were significantly higher in postmenopausal women (p=0.01). These findings suggest that women after menopause have an increased risk of periodontitis, highlighting the need for preventive care and timely treatment of oral conditions.[97] In addition, another study concluded that steroid sex hormones, particularly estrogen, play a crucial role in modulating periodontal tissue responses to bacterial plaque. The decline in estrogen during menopause could alter these responses and contribute to the development of periodontal disease.[101] Although it is known that estrogen deficiency is associated with bone loss in the periodontium, the exact mechanism by which this deficiency leads to bone loss remains an area of research.[102]

The relationship between the VM and periodontal health has been increasingly recognized. Although they belong to different biological systems, it has been suggested that both microbiota could influence each other due to the interconnectedness of inflammatory processes.[103] Several studies indicate that dysbiosis in the VM, especially during menopause, can induce a systemic inflammatory response that affects not only the genital tract but also other tissues such as the gums, promoting the progression of periodontitis.[97,101]

In addition, certain bacteria frequently found in both the oral cavity and VM, including *P. gingivalis* and *F. nucleatum*, are linked to periodontal disease and may also colonize the vaginal environment.[98-100,103] This suggests that infections originating in the mouth might affect the VM, potentially impacting women's reproductive and gynecological health. BV and periodontal disease both involve an imbalance in microbial communities, known as dysbiosis. These conditions have been linked to a higher risk of pregnancy complications, although a clear causal link remains unproven. Research involving South African adolescent girls found that bacteria commonly linked to periodontal disease, including *Prevotella intermedia* and *Porphyromonas endodontalis*, were present in greater amounts in the oral microbiota of those with disrupted VM. This points to a potential connection between oral and vaginal microbial imbalances, highlighting the need for further studies to clarify any causal relationship.[104]

In a study conducted at the Hospital Clínico San Borja Arriarán, which included pregnant women with preterm labor before 34 weeks of gestation, a prevalence of periodontal disease of 93.2% was found. Furthermore, 27.1% of patients showed microbial invasion of the amniotic fluid, with 18.6% associated with periodontal pathogenic bacteria. Cervicovaginal infection was observed in 83.1% of patients, with BV present in 23.7%. Among the women with cervicovaginal infection, 72.9% also had periodontal disease. Preterm birth (<37 weeks) occurred in 64.4% of the patients and was significantly associated with generalized periodontal disease and the concurrent presence of ascending bacterial infection and periodontal disease. In addition, patients with preterm birth and generalized periodontal disease showed a higher frequency of chorioamnionitis and funisitis, suggesting that infection contributed to preterm labor.[99] This study highlights the interaction and importance of the periodontal and VM in pregnant women. This relationship is likely to be no different in postmenopausal women.

11.1. Biological mechanisms linking periodontal disease with genital microbiota

Several biological mechanisms have been proposed to explain the link between genital microbiota dysbiosis and periodontal disease, particularly in postmenopausal women:

(i) Systemic inflammation: Dysbiosis in the VM during menopause can induce a systemic inflammatory response that affects other tissues, including the gums, promoting the progression of periodontitis.[103]

Table 1. Pathologies associated with menopause and microbiota alteration

Pathology (references)	Microbiota alteration
Recurrent urinary tract infection[28-30,32,33]	Decrease in *Lactobacillus* spp. in the vagina and reduction in SCFAs and intestinal bacteriocins, which normally inhibit uropathogen growth in the vagina and bladder
Bacterial vaginosis[37,38]	Decrease in *Lactobacillus* spp. and increas e in anaerobes such as *Gardnerella vaginalis*, which expresses sialidase A—enhancing bacterial adhesion, biofilm formation, and compromising vaginal defenses
Pelvic inflammatory disease[39-41]	Decrease in *Lactobacillus* spp.; increase in endogenous pathogens (*Escherichia coli*, *Staphylococcus*) and exogenous ones (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*). BV promotes bacterial colonization and persistent inflammation, enabling ascending infections
Genitourinary syndrome of menopause[43-45]	Significant decrease in *Lactobacillus gasseri/jensenii* and *Lactobacillus crispatus*, contributing to vaginal atrophy, dryness, and sexual dysfunction. Increased microbial diversity with the presence of *E. coli*, *Shigella*, and *Streptococcus,* causing genital symptoms
Pelvic floor disorders—OAB, UI, POP[49-55]	Decrease in *Lactobacillus* spp. and increase in vaginal microbial diversity are associated with OAB and UI. Inflammation weakens pelvic connective tissue, favoring POP. Associations exist, but more research is needed to establish causal links
Gynecological cáncer[56-58,64]	Decrease in *Lactobacillus* spp. and increase in anaerobic vaginal bacteria promote dysbiosis, chronic inflammation, genetic instability, metabolic dysfunction, and cell proliferation. Disruption of the estrobolome reduces estrogen levels
Cervical cancer[56,68-74]	Loss of *Lactobacillus* promotes HPV persistence and cancer progression. Dominance of *Lactobacillus iners* facilitates inflammation, oncoprotein expression, and poor HPV clearance. BV with *Gardnerella* and *Atopobium* is linked to dysplasia
Endometrial cancer[56,76,77,79,83,84,86,87]	BV and the genera *Prevotella*, *Porphyromonas*, and *Atopobium* drive chronic inflammation and cancer. Induction of IL-1β and TNF-α promotes proliferation and angiogenesis. Estrobolome is disrupted
Ovarian cancer[88-96]	BV and the genera *Acinetobacter*, *Gardnerella*, and *Prevotella* are associated with inflammation and cancer. Dysbiosis in peritoneum and tumor tissue, with reduced microbial diversity, activates NF-κB. Intratumoral *Proteobacteria* and *Firmicutes* produce DNA-damaging toxins. *C. trachomatis* infection, *BRCA* mutations, and microbiota contribute to progression/metastasis
Periodontal disease[98-100,103,104]	*Porphyromonas gingivalis* and *Fusobacterium nucleatum* are found in both periodontal and vaginal microbiota. Vaginal dysbiosis may trigger systemic inflammation that exacerbates gum disease and periodontitis progression

Abbreviations: BV: Bacterial vaginosis; HPV: Human papillomavirus; IL-1β: Interleukin 1 beta; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; OAB: Overactive bladder; POP: Pelvic organ prolapse; SCFAs: Short-chain fatty acids; TNF-α: Tumor necrosis factor alpha; UI: Urinary incontinence.

(ii) Shared bacterial composition: Although oral and genital microbes originate from different environments, they share certain common pathogens. Bacteria such as *P. gingivalis* and *F. nucleatum*, associated with periodontal disease, can also be found in the microbiota of the female genital tract, suggesting an interaction between both microbiota.[99]
(iii) Impact of estrogens: Estrogens not only regulate the genital microbiota, but their decline also affects bone formation capacity.[102]

11.2. Clinical implications and management

The comprehensive management of postmenopausal women's health should consider both periodontal and vaginal health. Key recommendations include:
(i) Hormonal treatment: HRT may help restore estrogen levels, potentially improving both vaginal health and reducing the risk of periodontal disease.
(ii) Proper oral hygiene: It is essential to maintain rigorous oral hygiene, including regular brushing, flossing, and periodic visits to the dentist.
(iii) Probiotic supplements: Probiotics, especially those containing *Lactobacillus*, may be beneficial for balancing both vaginal and oral microbiota, reducing menopause-associated symptoms, and preventing periodontal disease.
(iv) Regular monitoring: Postmenopausal women should undergo regular gynecological and periodontal check-ups to detect and treat any signs of vaginal infection or periodontal disease early.

Menopause induces physiological changes that affect both the genital microbiota and periodontal health. The decrease in estrogens and the resulting systemic inflammation contribute to conditions like BV and periodontal disease, opening new possibilities for their preventive and therapeutic management. Table 1 provides an overview of the principal pathologies associated with menopause and microbiota alteration. These conditions include rUTIs, BV, PID, GSM, pelvic floor disorders, gynecological cancers (cervical, endometrial, and ovarian), and periodontal disease. All of these have been linked to shifts in microbial composition and function, which may contribute to their onset and progression.

12. Conclusion

The intestinal, urinary, and vaginal microbiomes form an interconnected ecosystem whose alteration during menopause, primarily caused by estrogen deficiency, is associated with various gynecological and urological disorders. Vaginal dysbiosis clinically manifested as dryness, atrophy, and urinary symptoms, significantly affects women's quality of life. Its management with local estrogens and probiotics has shown consistent benefits. Microbiota imbalance also influences urological health, and its modulation could serve as a promising tool against UTIs and pelvic floor dysfunctions, although further evidence is needed to confirm its effectiveness. Moreover, the VM appears to play a role in the progression of gynecological cancers such as cervical, endometrial, and potentially ovarian cancer, through mechanisms involving persistent HPV infection and the presence of bacteria associated with BV, opening the possibility for preventive strategies based on microbiome modulation.

This study's strengths include its narrative review design, which incorporates a rigorous selection of relevant, low-bias studies supported by current evidence from recognized databases, thereby reinforcing the clinical conclusions on postmenopausal microbiota. However, the narrative approach does not apply systematic quality criteria or a reproducible search strategy. In addition, the heterogeneity of the included studies and the lack of randomized controlled trials limit the ability to establish definitive causal relationships in all cases.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Metabolomics of healthy hematopoietic stem cells and leukemia stem cells

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Abstract

Background: Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) and sustain life-long hematopoiesis by balancing quiescence, self-renewal, and differentiation. A key feature distinguishing quiescent HSCs from their activated counterparts is a shift in their metabolic profile, including changes in glycolytic flux and mitochondrial oxidative metabolism. Disruption of HSC homeostasis can lead to hematologic diseases such as BM failure, clonal hematopoiesis, or oncogenic transformation into leukemia stem cells (LSCs). Similar to HSCs, LSCs retain stem-like characteristics but acquire malignant features, including drug resistance and a reprogrammed metabolism, which results in distinct metabolic profiles that contribute to their pathogenesis. **Aim:** This review summarizes the key metabolic characteristics distinguishing healthy quiescent and active HSCs from oncogenic LSCs. In addition, we highlight modern tools for investigating the metabolome, which enable the identification of novel metabolites, metabolic interactions, pathways, and potential targets for diagnosis or therapeutic intervention in hematologic diseases. **Conclusion:** Metabolic regulation is essential for maintaining HSC quiescence, self-renewal, and lineage commitment, whereas its disruption often underlies oncogenic transformation into LSCs. Advances in metabolic profiling reveal key differences between healthy HSCs and LSCs and identify LSC vulnerabilities that sustain survival and therapeutic resistance. Targeting these hijacked metabolic pathways may facilitate the development of LSC-specific treatment while preserving normal hematopoiesis. Further investigation of stem cell metabolism will be critical for translating these insights into effective treatments for hematologic malignancies. **Relevance for patients:** Understanding the metabolic profiles of healthy HSCs and LSCs can facilitate the development of innovative techniques, technologies, and therapeutics. These advances can be applied to the identification, treatment, and prevention of hematologic disease. By elucidating the metabolome of LSCs, therapies can be designed to selectively target their unique metabolic pathways, dependencies, and resistance mechanisms.

Keywords: Hematopoietic stem cells; Leukemia stem cells; Metabolism; Metabolomics

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1. Introduction

Hematopoiesis is a tightly regulated process that produces differentiated blood cells to meet the physiological demands of the human body.[1,2] Located within the human bone marrow (BM) niche, hematopoietic stem cells (HSCs) play pivotal roles in sustaining these demands by intricately balancing self-renewal and regenerative proliferation with differentiation and lineage commitment at an average rate of 1×10^6 cells/s.[1-4] At homeostasis, HSCs in the BM are maintained in a highly quiescent state, the G_0 phase of the cell cycle, and only enter proliferation or differentiation upon stimulation.[1,4,5] When stimulated by extrinsic and intrinsic factors (e.g., colony-stimulating factors, growth factors, and cytokines),[6,7] HSCs either transition into a state of self-renewal or differentiate heterogeneously through a hierarchy of multipotent progenitors into fully differentiated myeloid and lymphoid cells to adapt to changing physiological needs.[1,4,5] The exit of HSCs from quiescence is an energy-intensive process that coincides with a rapid and critical metabolic shift from glycolysis to mitochondrial metabolism, including increased mitochondrial biogenesis to support oxidative phosphorylation (OXPHOS).[1,5,8-10] This metabolic shift is a cornerstone of the unique self-renewal and differentiation capabilities of HSCs.[5,8-10]

However, dysfunction in the metabolic processes that guide HSC exit from quiescence often leads to metabolic hijacking and ultimately the transformation of HSCs into leukemia stem cells (LSCs).[1,3,11-14] LSCs are found in the BM niche and are characterized by stemness features, including drug resistance, self-renewal, and lack of differentiation, as well as highly heterogeneous phenotypes, genetic mutations, and metabolic alterations.[3,12,13] While HSCs and LSCs share fundamental self-renewal and drug resistance capabilities (i.e., high expression of ATP-binding cassette [ABC] transporters for genotoxin efflux),[1] their metabolic programs differ significantly to support the altered biosynthetic and energetic demands associated with malignant transformation.[3,5,12-14] Understanding the metabolic divergence between HSCs and LSCs can provide insight into stem cell homeostasis, leukemogenesis, drug resistance, and therapeutic targeting.[11,13,14]

Advances in metabolic profiling have enabled researchers to dissect these metabolic states and provide insight into what drives the pathological shift from HSCs to LSCs.[15,16] Through metabolic profiling, recent studies have confirmed that profound alterations in cellular metabolism are a key determinant of ultimate stem cell behavior. Meanwhile, several studies have developed emerging metabolic tools to shed light on the biochemical pathways and dynamic metabolic characteristics that guide the transition between quiescent and active HSCs and initiate oncogenic transformation into LSCs.[15-17] In this review, we summarize the key metabolic features that guide HSC activation and distinguish healthy HSCs from LSCs. Furthermore, we highlight how advances in metabolomic technologies can decode the metabolic framework and uncover novel biochemical pathways and therapeutic strategies for regulating and targeting HSC and LSC metabolism, respectively.

2. Metabolic landscape of healthy HSCs

2.1. Metabolic profile of HSCs within the BM microenvironment

Under normal homeostatic conditions, HSCs are preserved in extended periods of quiescence through a tightly regulated balance between glycolysis and OXPHOS dependence[1,4,5,9,10,14] (Figure 1). Quiescent HSCs primarily rely on glycolysis for the minimal energetic demands of stem cell maintenance, as previously stated, and allow for the minimization of reactive oxygen species (ROS) production commonly associated with fatty acid oxidation (FAO), such that quiescent HSCs can preserve their genomic integrity and protect themselves against metabolic stress and functional decline.[1,4,5,9,10,14] This level of sustained quiescence is largely due to the hypoxic microenvironment of the BM niche and provides protection against genomic instability, metabolic stress, and functional decline induced by ROS production.[1,8,14]

The BM niche provides HSCs with a stabilized and specialized microenvironment comprised of support cells and extracellular components. These components include a heterogenous array of osteoblasts, endothelial cells, mesenchymal stem cells, adipocytes, fibroblasts, macrophages, and extracellular matrix proteins that support the hypoxic microenvironment and provide favorable conditions to maintain HSC quiescence.[1,8,18] Furthermore, the BM niche provides HSCs with several critical niche factors, modulators of cell cycle progression, and developmental signaling pathways that work independently and often redundantly to maintain quiescence and stemness (Table 1).[1,19,20]

Niche factors, including transforming growth factor beta 1 (TGF-β1),[29-32] angiopoietin-1 (Ang-1),[33] stromal cell-derived factor-1α (CXCL12),[34,35] stem cell factor (SCF),[36-38] thrombopoietin (TPO),[39] and osteopontin (OPN),[40,41] work to enforce HSC quiescence by influencing the regulators of the cell cycle or acting as negative regulators of proliferation and differentiation. The TGF-β1 niche factor promotes HSC dormancy through the activation of the Smad signaling pathway, which ultimately inhibits HSC proliferation and differentiation.[29-32] Ang-1 is secreted

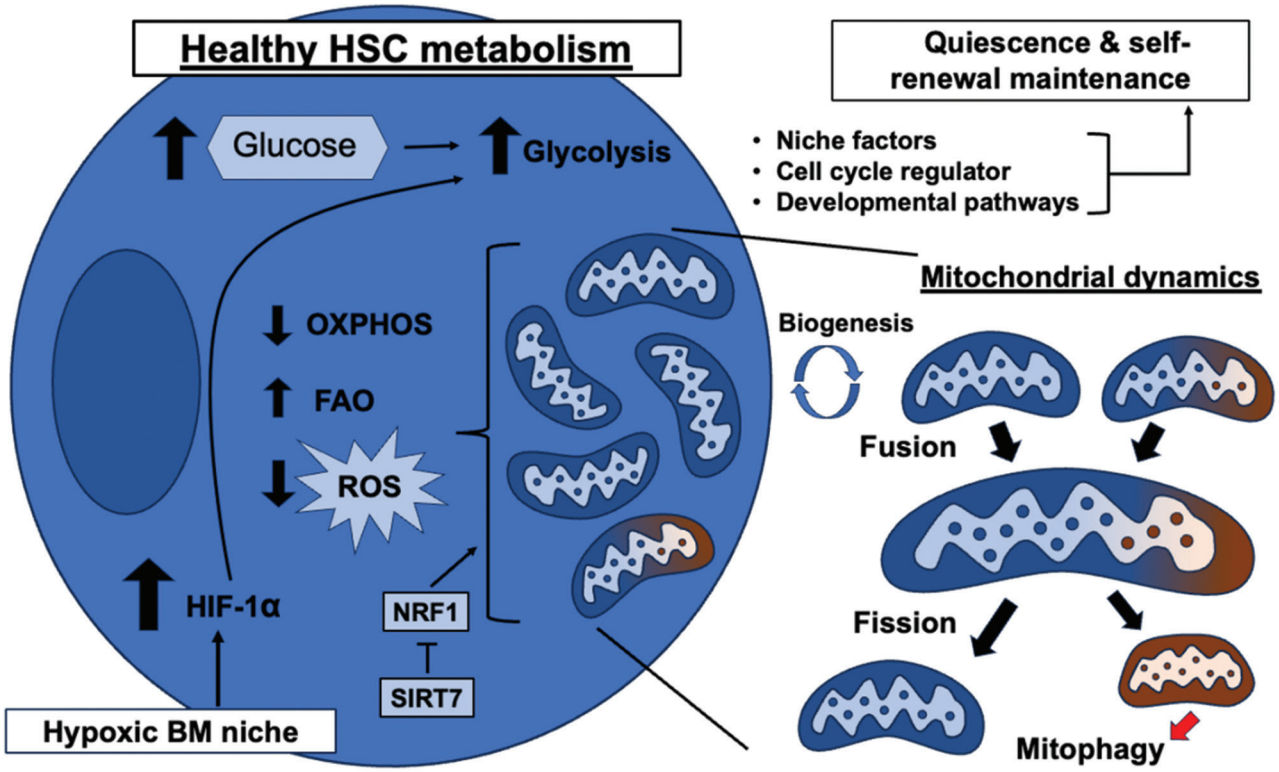

Figure 1. Healthy HSC metabolism. A graphical depiction of a healthy HSC located within the hypoxic BM niche, which creates an ideal environment for stabilizing HIF-1α.[1,2,21] The increased expression of HIF-1α reinforces the preference of quiescent HSCs for anaerobic glycolysis and minimizes the use of mitochondrial OXPHOS and the production of destructive ROS.[1,2,21] Decreased ROS production provides protection from genetic instability, metabolic stress, and functional decline of the HSC induced by ROS, while helping maintain cell quiescence.[1,2] Alongside increased glucose uptake for anaerobic glycolysis, quiescent HSCs also rely on FAO, thereby reducing reliance on OXPHOS.[5,22] However, OXPHOS is not eliminated but instead maintained at low levels with the help of SIRT7-mediated inhibition of NRF1, which suppresses mitochondrial biogenesis, function, and OXPHOS to reduce ROS accumulation.[1,23,24] Although OXPHOS is largely suppressed, mitochondrial homeostasis in healthy quiescent HSCs is maintained through the balance of mitochondrial biogenesis, fusion, fission, and mitophagy, collectively termed "mitochondrial dynamics."[5,9,10]
Abbreviations: BM: Bone marrow; FAO: Fatty acid oxidation; HIF-1α: Hypoxia-inducible factor 1α; HSC: Hematopoietic stem cell; NRF1: Nuclear regulatory factor 1; OXPHOS: Oxidative phosphorylation; ROS: Reactive oxygen species; SIRT7: Sirtuin 7.

Table 1. Key metabolic themes across normal hematopoietic stem cells and leukemia stem cells

Metabolic theme	Quiescent normal HSCs	Active normal HSCs	LSCs	References
Glycolysis	Increased glycolysis	Moderate glycolysis to support OXPHOS	Aerobic glycolysis (i.e., Warburg effect), in oxygen-rich conditions	5,14
Mitochondrial respiration	Low respiratory flux, minimal OXPHOS	Increased respiratory flux, enhanced OXPHOS to support proliferation	Enlarged mitochondrial mass, respiratory flux, and OXPHOS; reliance on mitochondrial metabolism for survival	5,14,25
FAO	Enhanced FAO to support energy demand and maintain stemness	Sustained FAO to meet energy demands during activation	Increased FAO utilization, crucial for survival and proliferation	5,22,26
ROS levels and redox state	Low ROS levels, robust antioxidant systems (enzymatic/non-enzymatic)	Increased mitochondrial-derived ROS levels, balanced by antioxidant responses	Elevated ROS levels, dependence on antioxidant systems for survival (e.g., glutathione pathway)	5,6,10
Hypoxia and HIF-1α pathway	Hypoxic niche preference, stabilized HIF-1α to maintain quiescence	Reduced HIF-1α stabilization upon exiting the hypoxic niche and proliferation/differentiation	Increased stabilization of HIF-1α and downstream pathways supporting survival, drug resistance, and leukemia progression	6,21,27,28

Abbreviations: FAO: Fatty acid oxidation; HIF-1α: Hypoxia-inducible factor 1α; HSC: Hematopoietic stem cell; LSC: Leukemia stem cell; OXPHOS: Oxidative phosphorylation; ROS: Reactive oxygen species.

primarily by osteoblasts and enhances HSC quiescence by stabilizing stem cell interactions through the Tie2 and phosphatidylinositol 3-kinase/protein kinase B pathways.[33] The niche factor CXCL12 is produced by BM stromal and endothelial cells and binds the C-X-C motif chemokine receptor type 4 on HSCs to reinforce dormancy.[34,35] Secreted by osteoblasts and adipocytes, SCF supports HSC quiescence by regulating metabolic homeostasis and promoting survival.[36-38] Alternatively, TPO limits cell cycle entry by stimulating the expression of Tie2 on HSCs[42] and preserves stemness.[39,42] OPN, secreted by osteoblasts and stromal cells, interacts with integrin receptors to suppress cell cycle progression and enhance HSC anchoring in the BM niche.[40,41]

Moreover, cell cycle regulatory components are equally essential for governing HSC dormancy and quiescence by tightly controlling cell cycle entry and progression.[1,19,20] These include members of the retinoblastoma (Rb) family[43-45] and the Forkhead box class O proteins,[46,47] as well as the interaction between cyclin D and cyclin-dependent kinase (CDK) 4/6,[48,49] and the CDK interacting protein/kinase inhibitory protein family (e.g., p21, p27, p57, and p53).[50-52] In HSCs, the Rb family restricts the transition from G_0/G_1 into S phase by suppressing DNA replication and cell proliferation through regulation of TPO-mediated signaling, thus reinforcing a quiescent state.[43-45] The Forkhead box class O transcription factor proteins suppress HSC cell cycle progression and are involved in ROS resistance, further promoting HSC quiescence.[46,47] Alternatively, cyclin D and CDK4/6 form a complex as a fundamental step controlling progression of the cell cycle out of quiescence by inhibiting Rb and promoting transition from G_1 into S phase.[48,49] The CDK-interacting protein/kinase inhibitory protein family functions to suppress the activity of the cyclin D–CDK4/6 complex, thereby preserving HSC dormancy and quiescence.[50-52]

In addition, evolutionarily conserved pathways, including the Wnt,[53,54] Notch,[53,55,56] and Hedgehog (HH)[57] developmental pathways, play supportive roles in the maintenance of HSC quiescence, self-renewal, and inhibition of differentiation.[1,20,37] Wnt signaling promotes HSC self-renewal through downstream activation of β-catenin and transcriptional genes governing the cell cycle, while also suppressing lineage-specific transcription factors.[53,54] Interestingly, overexpression of β-catenin in HSCs increases HSC expansion and inhibits differentiation both *in vitro* and *in vivo*.[58] Alternatively, Notch signaling reinforces HSC quiescence and self-renewal in an undifferentiated state by upregulating transcriptional repressors that inhibit differentiation and enhance cell cycle repression.[53,55,56] Through the activation of the Gli transcription factor, HH signaling similarly supports both quiescence and repression of lineage commitment in HSCs.[57] Of interest, mice deficient in *Gli1* exhibit decreased HSC proliferation and enhanced short- and long-term HSC engraftment, supporting the role of HH signaling in HSC maintenance and quiescence.[59]

Collectively, these findings demonstrate the tight regulation of HSC quiescence and how the niche-derived factors, cell cycle regulatory components, and developmental pathways work independently or cooperatively to ensure long-term hematopoietic homeostasis and regenerative capacity.

2.2. Glycolysis versus OXPHOS in quiescent and active HSCs

Sustained quiescence in HSCs depends on maintaining low metabolic rates and minimal mitochondrial OXPHOS.[1,5,14] However, the transition out of dormancy involves rapid changes in cellular metabolism.[5,14] This transition from highly glycolytic metabolism to OXPHOS supports the heightened energetic demands required for rapid proliferation and differentiation.[5,14] Glycolysis is a central metabolic pathway through which cells can derive critical metabolites and metabolic precursors from a single glucose molecule.[60] In the quiescent state, HSCs rely on glycolysis independent of the presence of oxygen. This is important, since a primary characteristic of the BM niche is a highly hypoxic microenvironment.[1,6,21] In fact, *in vivo* measurements in the hypoxic BM niche showed a local oxygen tension (pO_2) of <32 mm Hg[61] compared with an average atrial pO_2 of 90 mmHg.[62] Such a hypoxic microenvironment creates ideal conditions to stabilize a key transcription factor and mediator of cellular hypoxia, hypoxia-inducible factor (HIF)-1α.[21,63] HSCs are known to express HIF-1α at high levels.[21] Previous studies have shown that HIF-1α–deficient mice exhibit a loss of HSC quiescence and decreased HSC abundance, highlighting the importance of HIF-1α stabilization in HSCs.[64] Alternatively, overexpression of HIF-1α maintains quiescence but decreases transplantation capacity, suggesting an intricate and tightly regulated balance of HIF-1α expression in the maintenance of HSC quiescence.[64] Nevertheless, the stabilization of HIF-1α reinforces the prioritization of glycolytic metabolism, thereby providing long-term protection of HSCs from oxidative damage by OXPHOS-derived ROS.[1,5,6,21]

Upon activation, HSCs increase glycolytic influx to meet the rising metabolic and biosynthetic demands required for proliferation and differentiation.[5,14,19] This compels HSCs to increase expression of glycolytic transporters (e.g., glucose transporter 1 [GLUT1]) downstream of TGF-β1 stimulation, thereby enhancing

glucose intake and providing the necessary metabolites for rapid expansion.[5,14,65] These include pyruvate, required for mitochondrial OXPHOS,[60] and other precursors required for the biosynthesis of nucleotides,[66] lipids,[60,67] proteins,[60] and carbohydrates.[52,60,68]

In quiescent HSCs, mitochondrial OXPHOS is active but maintained at relatively low levels, potentially to limit ROS generation, protect cells from oxidative damage, and preserve their long-term self-renewal capacity.[1,4,5] This suppression is maintained largely by the BM niche factors, cell dormancy, and several developmental pathways. Interestingly, quiescent HSC mitochondria are further repressed through the suppression of nuclear regulatory factor 1 (NRF1) by sirtuin 7 (SIRT7).[1,24,69] NRF1 is a transcription factor that regulates mitochondrial biogenesis and function, enhancing OXPHOS and ROS accumulation.[23] In contrast, SIRT7 acts as a metabolic checkpoint that maintains HSC quiescence through the inhibition of NRF1.[70,71] Several studies suggest that this interaction also suppresses the mitochondrial unfolded protein response and metabolic activation, thereby enabling HSCs to maintain high levels of impaired mitochondria while ensuring the rapid engagement of OXPHOS upon HSC stimulation.[1,69,70] However, it should be noted that quiescent HSC OXPHOS is not eliminated by these regulatory factors, as quiescent HSCs remain dependent on minimal mitochondrial activity for survival.[1,4,5,9,10]

Several studies suggest that once activated, HSCs may shift their metabolism to FAO and mitochondrial OXPHOS to support proliferation, differentiation, and the energetic and biosynthetic demands sustained by the increased concentration of mitochondria.[5,9,10,14] These demands include the expansion of mitochondrial mass, enhanced respiratory capacity, and increased ATP production in support of active cell cycle progression and lineage specification.[5,9,10,14] An increase in OXPHOS coincides with elevated ROS production, which represents a major step toward differentiation. ROS accumulation is known to drive HSCs out of quiescence by suppressing self-renewal through the activation of p38 downstream of the mitogen-activated protein kinase signaling pathway.[1,5,14] A study investigating the chemical uncoupling of mitochondrial OXPHOS demonstrated that *ex vivo* HSCs exhibited lower mitochondrial mass and reduced mitochondrial membrane potential while displaying increased self-renewal potential in cultures designed to induce differentiation.[72] This finding supports the role of limited mitochondrial activity as a key characteristic of HSC quiescence and heightened mitochondrial OXPHOS as a driver pushing HSCs out of quiescence.

2.2.1. Lipid metabolism

In addition to OXPHOS, mitochondria also play a major role in lipid metabolism, specifically FAO.[5,22] FAO is a metabolic process that breaks down fatty acids, supported by the presence of BM adipocytes, to generate the integral metabolite acetyl-coenzyme A (CoA), required for the tricarboxylic acid (TCA) cycle and downstream OXPHOS, as well as macromolecule biosynthesis.[22,73] In this process, fatty acids undergo a series of reactions that repeatedly shorten the fatty acid chain by two carbons while producing acetyl-CoA, nicotinamide adenine dinucleotide (NADH), and the reduced form of flavin adenine dinucleotide, which are needed for the TCA cycle and electron transport chain (ETC), respectively, in addition to generating precursors for macromolecule synthesis. In quiescent HSCs, FAO is the primary form of lipid metabolism. Here, FAO is sustained by the low levels of mitochondrial respiration and is regulated by the peroxisome proliferator-activated receptor-δ (PPARδ) transcription factor.[5,22] PPARδ promotes the expression of genes regulating fatty acid uptake, transportation, and oxidative catabolism to preserve HSC longevity and self-renewal capacity while in the hypoxic BM niche.[74] It then follows that loss of PPARδ results in a decline in HSCs with the ability to self-renew, thereby supporting the role of FAO in HSC dormancy.[74]

Upon activation, HSCs show a notable reconfiguration in their lipid metabolic profile. Specifically, activated HSCs are proposed to engage in a dynamic interplay between FAO and lipid macromolecule biosynthesis.[5,75,76] Sustained FAO assists in meeting the metabolic demands of activation[75] but also contributes to differentiation and fate determination through acetyl-CoA-dependent histone modifications.[76] Concurrently, lipid biosynthesis is upregulated in active HSCs to meet the increasing demand for building membranes required during rapid cell division.[77,78] Taken together, these findings further highlight the delicate balance of the metabolic profile required to either maintain HSC dormancy or promote differentiation.

2.3. Mitochondrial dynamics and ROS regulation

Mitochondrial dynamics enable mitochondria to adapt to changing metabolic demands and regulate ROS levels required to exit quiescence.[5,9,10] The term "mitochondrial dynamics" refers to the continuous process of balancing mitochondrial fusion, fission, mitophagy, and biogenesis to maintain mitochondrial function, shape, and distribution within healthy cells.[79] In HSCs, mitochondrial dynamics are critical for balancing fusion, fission, and mitophagy to preserve quiescence and self-renewal capacity, while also providing a structured means to enhance mitochondrial

function and biogenesis upon stimulation.[5,9,10] Disruption of this dynamic equilibrium can impair mitochondrial function and lead to stem cell exhaustion or aberrant differentiation.[10]

Fusion allows mitochondria to merge and share mitochondrial contents, including DNA, proteins, and metabolites, thereby compensating for damaged or inefficient mitochondrial function and maximizing the ratio of metabolically healthy mitochondria.[9,10] In contrast, mitochondrial biogenesis provides mitochondria with the ability to produce more mitochondria, while fission enables division during cell proliferation or isolates damaged mitochondrial segments for removal through mitophagy.[9,10] A key consequence of improper regulation of mitochondrial dynamics is the accumulation of excess ROS beyond the levels needed for HSC stimulation. The physiology and metabolic state of mitochondria influence their morphology, dynamics, and turnover rate, which directly affect ROS production.[9,10] Although moderate ROS levels are required to drive HSCs out of quiescence, elevated ROS levels can induce DNA damage, impair self-renewal, promote senescence,[80] or induce oncogenic transformation.[80,81]

To mitigate excessive oxidative stress, cells deploy a selective form of autophagy termed "mitophagy." Mitophagy is a process by which the cell selectively degrades damaged or dysfunctional mitochondria.[10,82] It also prevents the buildup of ROS and eliminates dysfunctional mitochondria, thereby enabling HSCs to maintain homeostasis during quiescence and preserve a healthy mitochondrial population in anticipation of activation.[10,82] In addition, redox buffering systems, largely fueled by nicotinamide adenine dinucleotide phosphate (NADPH) reducing power, help to buffer accumulated ROS and prevent oxidative damage,[83] alongside other enzymatic (e.g., superoxide dismutase and catalase) or non-enzymatic (e.g., glutathione) antioxidant systems (e.g., superoxide dismutase 1/2). Taken together, mitochondrial dynamics and regulatory networks ensure mitochondrial function, health, and integrity, as well as the redox balance required to maintain functional, long-term HSCs.

3. Metabolic rewiring in LSCs

Compared to the activation of quiescent HSCs, the oncogenic transformation of LSCs entails significant metabolic reprogramming, allowing them to self-renew, survive in the BM niche, and resist therapeutic intervention (Table 1; Figure 2). However, unlike quiescent HSCs, which rely primarily on glycolysis for energy production, LSCs exhibit enhanced mitochondrial respiration associated with increased mitochondrial density. These changes are linked to elevations in FAO and OXPHOS.[84,85] However, the intrinsic organization of mitochondria in LSCs and their contribution to chemoresistance remain to be fully understood.[85,86]

3.1. Shared and divergent pathways between HSCs and LSCs

Quiescent HSCs and oncogenic LSCs both rely primarily on core metabolic pathways, including glycolysis and FAO, to maintain self-renewal and survival.[5,14] However, they differ in how these pathways are regulated. In HSCs, metabolism is tightly regulated by numerous extrinsic and intrinsic niche factors to preserve quiescence and genomic integrity, as discussed in previous sections.[5,14] Moreover, these quiescent stem cells are predominantly glycolytic, express high levels of glycolytic enzymes, and suppress mitochondrial membrane potential to minimize ROS production and prevent premature HSC activation.[5,14,89]

In contrast, LSCs rewire these metabolic programs to enhance mitochondrial efficiency, thereby sustaining elevated rates of oxidative metabolism, which may ultimately facilitate resistance to metabolic or therapeutic stress.[25] For example, LSC mitochondria depend primarily on components of the ETC to facilitate the generation of ATP and regulate redox balance.[14,25] In chronic myeloid leukemia (CML), LSCs exhibit elevated OXPHOS activity and increased catabolism of TCA cycle metabolites. An increase in mitochondrial respiratory flux to generate ATP sensitizes these cells to Complex I inhibition by phenformin.[90] Complex I, or NADH dehydrogenase, is a central regulatory step within the mitochondrial respiratory chain and a primary site of electron entry into the ETC through oxidation of NADH to NAD^+.[87] Complex I plays a key role in maintaining redox balance by transferring electrons to coenzyme Q (ubiquinone) while simultaneously pumping protons across the inner mitochondrial membrane.[87] The resulting proton gradient is utilized by ATP synthase to drive ATP production, as well as NADPH synthesis and metabolite transport. In LSCs, where glycolytic flexibility is limited, this dependency on intact Complex I function highlights a critical metabolic vulnerability. One study supports the notion that CML LSCs are highly dependent on mitochondrial oxidative metabolism for survival.[25] This finding confirms that mitochondrial activity and increased TCA cycle catabolism in CML LSCs are not merely passive characteristics but represent a critical energetic pathway that can be therapeutically targeted.

In addition to enhanced oxidative metabolism and TCA cycle activity observed in CML, LSCs in acute myeloid leukemia (AML) also rewire upstream metabolic inputs to

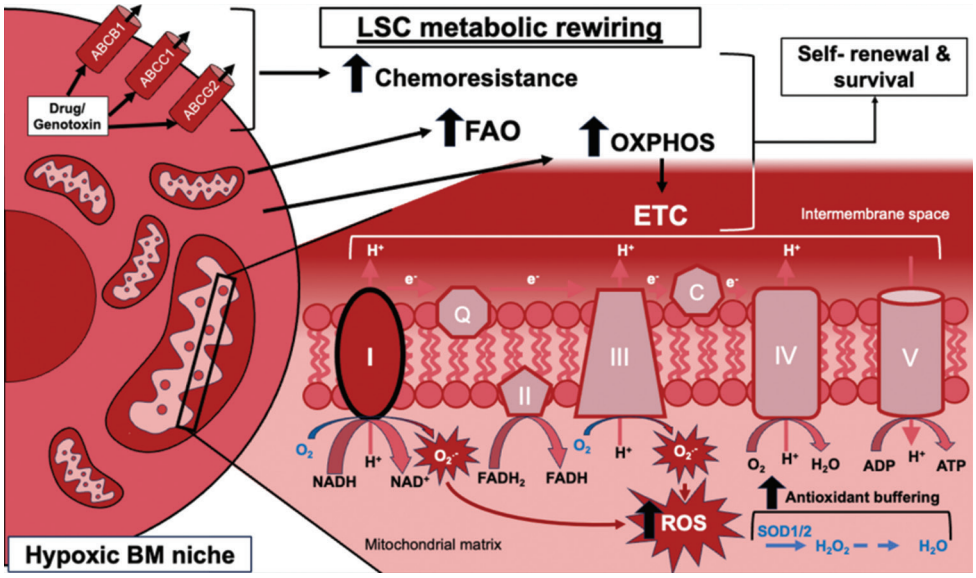

Figure 2. LSC metabolic rewiring. A graphical depiction of an LSC located within the hypoxic BM niche.[13,14] Here, LSCs rely heavily on upregulated FAO, OXPHOS, and ATP production for self-renewal and survival.[84,85] Complex I of the ETC plays an essential role in driving ATP production by acting as the primary site for NADH oxidation and electron (e^-) entry into the ETC.[87] From here, electrons are transferred to coenzyme ubiquinone (Q), while protons (H^+) are simultaneously pumped across the inner mitochondrial membrane, generating the proton gradient necessary for ATP synthesis.[87] During this process, LSCs also exhibit elevated OXPHOS activity and increased levels of ROS.[25] To enhance mitochondrial efficiency and sustain these elevated rates of OXPHOS, LSCs rewire conventional metabolic programs and mitigate elevated ROS levels through increased antioxidant buffering, which enables them to tolerate ROS at levels conductive to self-renewal while exhibiting oxidative resistance.[14,75,83,88] LSCs also remodel their microenvironment, leading to the increased expression of ABC transporters (e.g., ABCB1, ABCC1, and ABCG2), resulting in chemoresistance.[85,86]
Abbreviations: BM: Bone marrow; ETC: Electron transport chain; FADH: Flavin adenine dinucleotide; $FADH_2$: Reduced form of flavin adenine dinucleotide; FAO: Fatty acid oxidation; H_2O: Water; H_2O_2: Hydrogen peroxide; LSC: Leukemia stem cell; NADH: Nicotinamide adenine dinucleotide; OXPHOS: Oxidative phosphorylation; ROS: Reactive oxygen species; SOD1/2: Superoxide dismutase ½.

fuel mitochondrial respiration.[14,25] One such pathway that further differentiates HSCs from LSCs is FAO. In LSCs, FAO is upregulated to support mitochondrial respiration and promote chemoresistance.[14,26] To sustain this elevated FAO activity, LSCs highly express Cluster of Differentiation (CD)-36 for increased fatty acid transport and lipid scavenging and rely on carnitine palmitoyltransferase 1A-driven lipid oxidation.[85,86] AML LSCs exhibit increased carnitine palmitoyltransferase 1A expression to facilitate long-chain fatty acid breakdown and express CD36 when localized to adipocyte-rich niches, enabling the scavenging of extracellular lipids.[85,86] Notably, LSCs tolerate moderate levels of intracellular ROS by exhibiting enhanced oxidative resistance, which fuels proliferation without triggering oxidative damage, in contrast to the ROS sensitivity of normal quiescent HSCs.[5,14,88]

One mechanism that enables LSC tolerance to ROS is the integrated stress response (ISR).[91-93] The ISR is a conserved cellular pathway that balances innate biological processes, such as protein synthesis and gene expression, in response to stressors such as ROS-induced oxidative damage, nutrient deprivation, and mitochondrial dysfunction.[91,92] In LSCs, the ISR sustains cell survival under metabolic and therapeutic pressure. Phosphorylation and activation of components such as eukaryotic translation initiation factor 2α and the stress-adaptive activating transcription factor 4 reduce ROS-induced apoptosis, maintain redox balance, and support mitochondrial metabolism.[92-94] This process also enhances resistance to therapeutic intervention and promotes the long-term persistence of LSCs, serving as a critical mediator of survival.[92,93]

These fundamental differences in metabolic regulation demonstrate how LSCs are uniquely equipped for survival. Despite this, the notion that FAO is critical to LSC survival remains under scrutiny. AML LSCs exhibit a distinct metabolic phenotype compared with both normal HSCs and bulk leukemia blasts, characterized by a dependence on mitochondrial respiration rather than glycolysis.[95] Previous studies have demonstrated that AML LSCs maintain low ROS levels while displaying elevated oxidative metabolism and adenosine monophosphate-activated protein kinase (AMPK) activation.[95] These features are consistent with a mitochondria-centric bioenergetic program. Similarly, another study showed that the activation of the signal transducer and activator of transcription 3 (STAT3)–MYC axis enhances *SLC1A5*-mediated glutamine import, thereby reinforcing TCA cycle flux and mitochondrial metabolism.[96] While LSCs retain functional glycolysis

through GLUT1, its contribution to ATP production remains undefined. Moreover, other studies reported that dual inhibition of GLUT1 and OXPHOS is required to fully eliminate LSCs *in vivo*.[97] Although FAO remains a viable metabolic target, its precise contribution relative to other substrates, such as glutamine, remains an open question. Further investigation is needed to explore the specific fuel preferences of LSCs and to identify context-specific therapeutic vulnerabilities across leukemia subtypes.

3.2. Role of nutrient-sensing pathways in HSCs and LSCs

In addition to metabolic pathways, evidence suggests that nutrient-sensing pathways also play a role in regulating stem cell metabolism.[5,98,99] These include the AMPK and mechanistic target of rapamycin complex 1 (mTORC1) signaling pathways. In quiescent HSCs, suppression of mTORC1 activity is essential for maintaining dormancy and preventing premature differentiation by reducing protein synthesis and inhibiting cell growth.[5,100] Conversely, AMPK activation plays a pivotal role in preserving energy homeostasis during metabolic stress by promoting catabolic processes such as FAO and glycolysis to generate ATP, while inhibiting anabolic processes to preserve energy.[101] This balance supports both quiescence and the long-term self-renewal capacity of HSCs under physiological conditions.

In LSCs, however, this balance between nutrient-signaling pathways—namely, mTORC1 and AMPK—is reprogrammed. The mTORC1 pathway acts as a central regulator of anabolic metabolism, enhancing amino acid uptake, protein synthesis,[102] and RNA and DNA biosynthesis.[103] In contrast, AMPK inhibits fat and protein biosynthesis and promotes FAO and glycolysis to maintain energy balance.[104] Previous research has shown that chemical activation of AMPK in $CD34^+$ AML cells inhibits mTORC1 signaling and enhances sensitivity to cytarabine and idarubicin,[105] suggesting that therapeutic manipulation of these pathways can impair LSC maintenance and viability. Similar observations have been reported in solid tumor models, where AMPK activation reduces cancer stem cell survival.[106] Collectively, these findings underscore the conserved yet opposing roles of AMPK and mTOR1 signaling in regulating stemness. Moreover, as nutrient availability and metabolic signaling pathways are tightly linked to stemness and therapeutic resistance, targeting these pathways offers a promising strategy for selectively eliminating LSCs without compromising normal hematopoiesis.

3.3. Mechanisms of adaptation to hypoxia and chemoresistance

Previous reviews have highlighted that the hypoxic BM niche plays a significant role in maintaining HSC quiescence and regulating LSC localization.[2,27] As discussed in Section 2.2, quiescent HSCs downregulate mitochondrial function to minimize the negative effects of ROS accumulation. In contrast, LSCs maintain robust mitochondrial metabolism even under hypoxic conditions in the BM niche. In xenograft studies, AML LSCs that survive cytarabine treatment retain high mitochondrial activity and strong BM adhesion capacity, underscoring their ability to resist therapy through persistent mitochondrial function.[107] Unlike quiescent HSCs, LSCs maintain ROS at levels conducive to self-renewal and mitigate ROS-induced damage by upregulating antioxidant defenses, including non-enzymatic antioxidant systems similar to those of active HSCs.[14,75,83,88]

In addition to intrinsic metabolic adaptations, LSCs that exhibit resistance to chemotherapy exploit extrinsic cues from the microenvironment.[28] Adipocyte-rich BM provides LSCs with an exogenous source of fatty acids to support FAO and sustain mitochondrial respiration.[85] Furthermore, LSCs actively remodel their microenvironment by engaging in stromal interactions and altering chemokine gradients, thereby promoting niche retention and therapeutic evasion.[28] Through the increased expression of ABC transporters (e.g., ABCB1, ABCC1, and ABCG2), LSCs efflux chemotherapy drugs, reducing treatment efficacy and contributing to drug resistance across multiple cancers.[1,108] In LSCs, oncogenic drivers such as c-MYC enhance the activity of these transporters, enabling more efficient drug efflux and thereby increasing chemoresistance and relapse potential.[109] These strategies highlight the diverse resistance mechanisms by which LSCs resist hypoxia and chemotherapy, suggesting that targeting both intrinsic metabolic programs and extrinsic niche interactions may be necessary to overcome LSC-mediated relapse.

3.4. Genomic alterations in LSCs

Metabolic changes in LSCs do not occur passively but are instead typically driven by genetic mutations or modifications that actively influence cellular energy processing. In chronic lymphocytic leukemia, although genetic mutations do not directly alter the expression of genes involved in metabolic pathways, STAT3 becomes constitutively activated, with reduced levels of microRNA-125 playing a major role. This ultimately promotes the transition of LSCs toward more efficient fatty acid utilization for energy production.[110]

The HIF pathway helps healthy blood stem cells remain in a glycolysis-dependent resting state; however, its role in LSCs is more complex.[111,112] Although LSCs reside in hypoxic niches, elimination of HIF-1α accelerates disease

progression after chemotherapy-based treatment in mouse models of AML.[112,113] Thus, it remains unclear if HIF-1 is a feasible therapeutic target.

Other genomic alterations contribute to chemoresistance through well-established mechanisms. Activating mutations in metabolic enzymes such as isocitrate dehydrogenase (IDH) 1 and IDH2 lead to the accumulation of the oncometabolite 2-hydroxyglutarate (2-HG). The presence of 2-HG induces hypermethylation of specific genes, preventing cellular differentiation and triggering widespread epigenetic changes.[112] In addition, 2-HG upregulates B-cell lymphoma 2 (BCL-2), conferring resistance to apoptosis and increasing sensitivity to BCL-2 inhibitors.[112] Overall, elevated BCL-2 expression is crucial for LSC survival, supporting a resting, low-ROS state that depends on OXPHOS for energy production.[112]

4. Metabolomic approaches

Recent advances in metabolomic technologies have enabled more precise and comprehensive studies of metabolism in HSCs and LSCs. Metabolomics allows for highly precise measurements of small molecules and metabolites that reflect the metabolic state at the cellular or tissue level.[15,16,114] These measurements provide critical insights into processes that regulate energy production, biosynthesis, redox balance, and metabolism, thereby enabling thorough characterization of the metabolic states of quiescent and active HSCs as well as LSCs.[17] This is particularly important because both HSCs and LSCs rely on distinct metabolic patterns and pathways[14] (Table 1).

In addition, metabolic profiling can be used to identify diagnostic biomarkers, reveal alterations in metabolic pathways associated with treatment, monitor therapeutic responses, and uncover vulnerabilities that may be exploited for the development of novel therapies[17] (Figure 3). To better understand the metabolic profile of HSCs and LSCs, researchers employ technologies such as mass spectrometry (MS), single-cell and multi-omics approaches, and live-cell metabolic assays.

4.1. Targeted versus untargeted metabolomics

One widely used technology to study metabolism is MS, an analytical technique that ionizes chemical compounds and measures their mass-to-charge (m/z) ratios with high sensitivity and throughput.[115] Metabolomic analysis using MS typically employs two main approaches: targeted and untargeted metabolomics. Targeted metabolomics focuses on quantifying a specific set of metabolites using known standards for each compound under investigation, thereby providing a quantitative snapshot of the metabolome.[121,122] This approach enables profiling of metabolite concentrations, including glycolytic, FAO, or TCA cycle intermediates, as well as amino acids, nucleotides, lipids, and small molecules or drugs, at specific time points.

Techniques such as liquid chromatography–MS (LC-MS) and gas chromatography–MS (GC-MS) are commonly used for metabolite separation and detection[115,116] (Figure 3). LC-MS separates metabolites using a liquid mobile phase and a stationary phase based on properties such as charge, polarity, or hydrophilicity,[116] whereas GC-MS uses a gas mobile phase to separate compounds according to volatility and boiling point.[115] These targeted MS-based methods are especially valuable for examining the metabolic profiles of quiescent and active HSCs or LSCs, where subtle changes in glycolysis, OXPHOS, FAO, or amino acid metabolism can influence stemness, activation, proliferation, or therapeutic resistance.

However, a major limitation of these approaches is the low abundance of HSC and LSC populations within the BM.[123,124] This rarity makes it difficult to obtain sufficient cellular material for MS, often necessitating strategies such as pooling samples from multiple donors to achieve the required input for metabolomic analyses[125] or extensive cell sorting for metabolic flux measurements.[124] Consequently, the feasibility of single-sample untargeted workflows in these rare cell populations remains a significant challenge.

Untargeted metabolomics is a bottom-up approach that focuses on identifying and quantifying potentially 100 or 1000 metabolites along with novel metabolic features.[126] This technique allows researchers to identify and analyze shifts in the metabolic profile and metabolic pathways, as well as the effects of different treatments on the entire system.[15] This technique enabled the identification of chemotherapy-induced metabolic shifts in osteosarcoma stem cells using untargeted metabolomics by LC-MS that would not have been detected by targeted metabolomics.[127] A prominent example is the identification of 2-HG, which accumulates in IDH1/2-mutant AML and serves as both a key biomarker and a direct therapeutic target.[88] Similarly, analyses of CML stem cells have identified distinct metabolic enzyme signatures compared to normal HSCs, highlighting novel vulnerabilities for therapeutic exploitation.[112] It should be noted that choosing between targeted and untargeted approaches ultimately relies on the biological question, as both are suitable for testing hypotheses.[15] In the context of exploring the metabolic profile of HSCs and LSCs, either method can be used to map metabolic changes associated with HSC activation or oncogenic transformation. Furthermore, these technologies provide foundational data for enhancing our understanding of HSC and LSC metabolism.

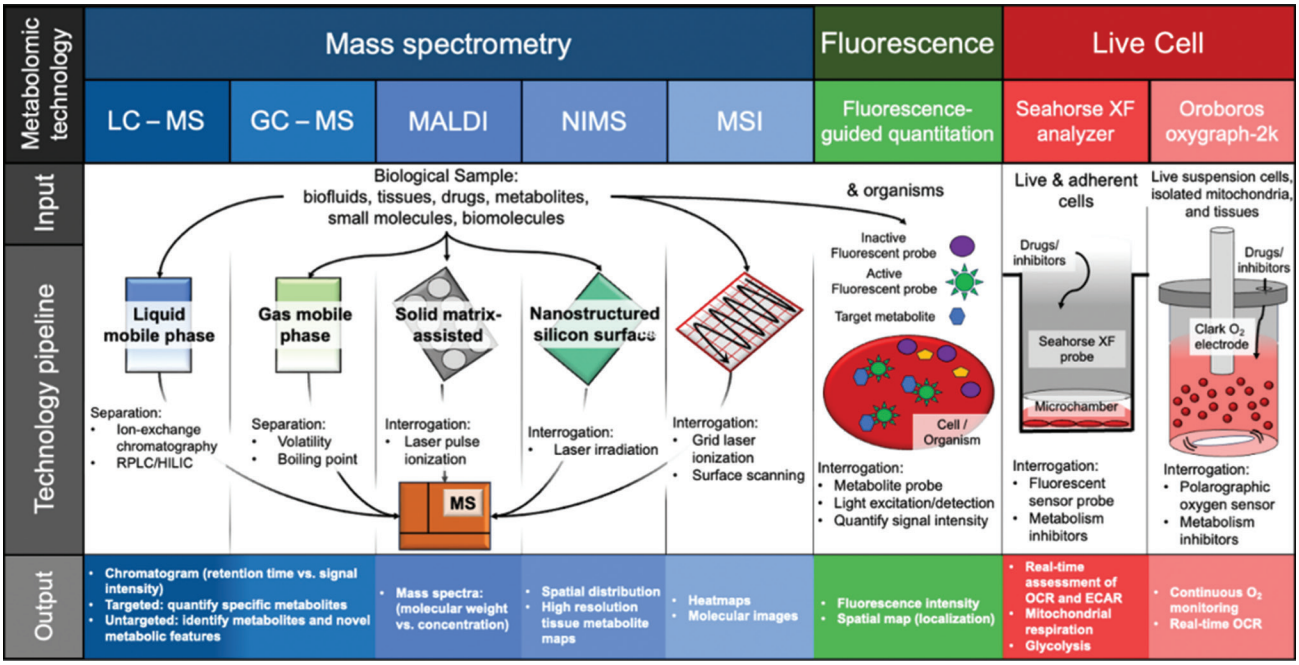

Figure 3. Overview of metabolomic technologies used to characterize metabolic features in HSCs and LSCs. This schematic overview shows the metabolomic platforms used to resolve the metabolic states of HSCs and LSCs, organized by detection modality: MS, fluorescence-guided quantitation, and live-cell metabolic assays. Under MS, LC–MS and GC–MS utilize liquid or gas mobile phases, respectively, for targeted or untargeted metabolite separation based on polarity, charge, or volatility.[115,116] Matrix-assisted technologies, such as MALDI and NIMS, enable surface-based ionization with spatial resolution of metabolite distributions.[117,118] MSI combines ionization with spatial scanning to generate molecular images.[117,118] Fluorescence-based techniques leverage metabolite-specific dyes and optical detection to produce intensity maps, while the Seahorse XF Analyzer and Oroboros Oxygraph-2k platforms provide real-time, high-resolution assessments of mitochondrial function, OCR, and ECAR in live cells or tissues.[119,120] Inputs and outputs for each platform are shown to further illustrate how these technologies enable comprehensive profiling of metabolism.
Abbreviations: ECAR: Extracellular acidification rate; GC: Gas chromatography; HSC: Hematopoietic stem cell; LC: Liquid chromatography; LSC: Leukemia stem cell; MALDI: Matrix-assisted laser desorption/ionization; MS: Mass spectrometry; MSI: Mass spectrometry imaging; NIMS: Nanostructure-initiator mass spectrometry; OCR: Oxygen consumption rate.

4.2. Single-cell and multi-omics platforms

Other useful techniques to explore metabolism include single-cell profiling and multi-omics integration. Single-cell metabolomic profiling allows for analysis at the single-cell level to better define the metabolic state, function, and interactions within the microenvironment.[128] This technique is a useful addition to targeted and untargeted metabolomics that helps resolve the metabolic heterogeneity within cells of a microenvironment down to a single-cell type, providing higher resolution and specificity.[129,130] Alternative techniques, such as matrix-assisted laser desorption/ionization, MS imaging (MSI), and nanostructure-initiator MS, enable the direct probing of low-abundance metabolites at subcellular resolution[117,118] (Figure 3). The feasibility of spatially mapping key metabolites within the hypoxic BM niche is supported by complementary approaches. For instance, high-resolution imaging has been used to correlate the localization of HSCs with specific hypoxic zones,[131] while MSI can be adapted to visualize how oncometabolites are distributed within BM tissue, allowing researchers to observe how LSCs metabolically reprogram their microenvironment.[132] These technologies also enable the identification of activation markers and metabolic rewiring associated with oncogenic transformation.[133,134] Furthermore, fluorescence-guided quantitation is an additional single-cell metabolomic technique designed to enhance specific metabolite detection through the use of spatial biology and fluorescence labeling strategies[119] (Figure 3). This technique enables researchers to improve the measurement accuracy of metabolite quantification in complex tissues.

Although metabolomics alone can provide highly sensitive and quantitative information on the metabolic state, it is important to consider the interplay between metabolomics and other omics processes to further decode specific metabolic phenotypes. Multi-omics platforms include the integration of transcriptomics, proteomics, and epigenomics, allowing for unprecedented resolution of the entire -omics profile within a given cell or tissue.[135] While each multi-omics platform provides unique data on its respective targets, integrating them with machine learning algorithms and computational workflows

can provide a comprehensive metabolic map to better understand the heterogeneity within HSCs and LSCs in the BM niche microenvironment, discern key metabolites and metabolic pathways, and develop novel therapeutic interventions.[136-138]

4.3. Technologies for live cells in mitochondria and metabolism

Live-cell technologies provide real-time functional insights into cellular processes and mitochondrial activity, making them powerful tools for studying metabolically dynamic populations like LSCs.[120] Instruments such as the Oroboros Oxygraph-2k (O2k) and the Seahorse XF Analyzer are widely used to assess bioenergetic function in intact or permeabilized cells, tissues, and isolated mitochondria under physiologically relevant conditions[120] (Figure 3). These platforms allow for the determination of oxygen consumption rate, enabling detailed profiling of ATP-linked respiration, proton leak, and spare respiratory capacity. The Seahorse technology is also capable of measuring the extracellular acidification rate to determine glycolytic activity.

The Agilent Seahorse XFe96 Analyzer was recently employed in CML LSCs, unveiling a deeply quiescent subset leukemia initiators (LI) characterized by suppressed Complex I activity yet enhanced FAO dependency.[139] Single-cell metabolomic profiling in parallel confirmed that this LI subset maintained low ROS levels despite high FAO flux, suggesting that mitochondrial complex I suppression is a protective adaptation within a functionally discrete LSC subpopulation.[139] Previous research has shown that cytarabine-resistant AML cells maintain a high oxygen consumption rate, indicating that persistent mitochondrial respiration is a hallmark of chemoresistant LSCs.[107]

Alternative to the Seahorse, the Oroboros O2k high-resolution respirometry provides a further depth of understanding by directly measuring Complex I–IV activity and coupling efficiency in primary AML samples.[140] In contrast, the Seahorse provides high throughput, dynamic stress testing. The Oroboros O2k offers extensive control over substrate and inhibitor addition, enabling precise dissection of respiratory chain function.[120,141] This makes Oroboros particularly valuable for evaluating subtle mitochondrial defects or drug-induced changes in electron transport that may underlie LSC persistence.[142,143] In the context of HSCs and LSCs, Oroboros O2k live-cell approaches not only reveal vulnerabilities in oxidative metabolism but also allow investigators to assess dynamic responses to metabolic inhibitors in real time. Today, the use of live-cell functional assays offers a critical readout for evaluating therapeutic efficacy and identifying bioenergetic escape mechanisms. Incorporating these technologies into HSC and LSC research helps bridge mechanistic insight with significant translational potential.

4.4. Problems and opportunities in therapeutic translation

Despite the advancements in metabolomics toward defining the metabolic profiles of HSCs or identifying LSC-specific vulnerabilities, translating these findings into effective therapies remains a challenge. The wide range and complexity of the metabolome can hide low-abundance but functionally critical metabolites. Furthermore, flux-based metabolic insights usually depend on stable isotope tracing, which is technically difficult to perform on limited clinical patient samples. Reproducibility also depends heavily on the standardization of sample preparation, instrumentation, and data analysis across platforms.

Nevertheless, recent technological advances are driving basic research toward clinical translation. An example of this innovation is the use of *in vivo* ^{13}C tracing to reveal metabolic circuits that are essential to LSC survival and to directly demonstrate the value of metabolomics in identifying targetable metabolic flux *in vivo.*[144] Additional research demonstrates the efficacy of co-targeting mitochondrial sirtuin 3 and cholesterol homeostasis to selectively disrupt mitochondrial function in AML LSCs,[145] while other studies demonstrate the efficacy of metformin for activating the AMPK pathway in AML to enhance chemosensitivity.[105] Together, these studies show a growing capacity to move from metabolomic discovery toward mechanistically driven interventions. As metabolomic technologies become more sensitive, especially in single-cell and live-cell contexts, they hold potential to define new biomarkers and therapies tailored specifically to LSCs, thereby advancing translational leukemia research.

5. Conclusion

The study of stem cell metabolism offers critical insight into the networks that govern HSCs and the precise regulation of quiescence and activation. Disruption of these tightly regulated metabolic programs leads HSCs to acquire abnormal metabolic profiles that contribute to oncogenic transformation. While LSCs maintain features of normal stemness, such as quiescence and drug resistance, they exhibit distinct metabolic characteristics that enable them to survive and resist therapeutic intervention. Recent advances in metabolic profiling, including single-cell analytics and live-cell functional assays, have enabled high-resolution assessment of these divergent metabolic states. Novel and emerging technologies supporting metabolic profiling not only reveal alterations in the biochemical pathways that contribute to HSC quiescence

and LSC survival but also highlight specific bioenergetic vulnerabilities within LSCs that may serve as therapeutic targets. By gaining a deeper understanding of the metabolic characteristics that distinguish healthy HSC activity from dysregulated LSC activity, researchers can better understand the mechanisms that sustain healthy function and identify those that lead to oncogenic transformation and other hematological disorders.

Collectively, these findings suggest that while LSCs are metabolically altered for survival, these unique adaptations may serve as targets for therapeutic intervention. Strategies that impair mitochondrial metabolism disrupt FAO or target nutrient-sensing pathways may offer promising approaches for selectively eliminating LSCs without compromising healthy hematopoiesis. As our understanding of stem cell metabolism deepens, these insights will guide the development of next-generation therapies to improve treatment durability and prevent relapse in hematologic malignancies such as AML and CML.

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Conflict of interest

Kathleen M. Sakamoto is an Editorial Board Member of this journal, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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ORIGINAL ARTICLE

The impact of fibromyalgia: A cross-sectional examination across different life domains

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Abstract

Background: Fibromyalgia is a complex, multifactorial chronic pain syndrome characterized by widespread musculoskeletal pain. Its symptoms significantly impact patients' quality of life, functional capacity, autonomy, and the ability to work or engage in leisure activities. **Aims:** Given the numerous hypotheses regarding the etiology of fibromyalgia, the difficulties faced by healthcare professionals in its diagnosis and management, and its substantial negative impact on the quality of life of those affected, this study aims to characterize the patient sample and assess the condition's impact across various life domains. **Methods:** A cross-sectional study was conducted with participants of both sexes, achieving a statistical power of 99%. **Results:** A higher prevalence of fibromyalgia was observed in individuals who reported being in a stable union (71.76%) and who possessed higher education (45.78%). The majority (56.47%) reported "very severe" pain. Significant differences were found in all evaluated domains: leisure, work, self-care, ability to exercise, functionality, and quality of life, indicating a significant deterioration following fibromyalgia diagnosis. **Conclusion**: The observed pattern of functional decline across various domains supports the allostatic load model of chronic pain and provides empirical evidence for the fear-avoidance model. **Relevance for patients:** Integrated treatments addressing physical and psychological aspects simultaneously may be, therefore, more effective.

Keywords: Epidemiology; Chronic pain; Quality of life; Public health

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1. Introduction

Fibromyalgia is a complex, multifactorial chronic pain syndrome primarily characterized by widespread musculoskeletal pain without evidence of inflammation in the painful areas.[1-3] It affects 2–10% of the global population, with a higher prevalence among women.[2,4,5] In Brazil, the general population prevalence is reported to be 2.5%.[1]

Classic nociplastic pain is a hallmark of fibromyalgia, manifesting as hyperalgesia, an exaggerated perception of pain in response to mildly painful stimuli, and allodynia, the perception of pain from normally non-painful stimuli.[4,6-8] Beyond pain, fibromyalgia is associated with fatigue, sleep disturbances, and cognitive dysfunction, all of which significantly impact patients' quality of life.[9-11] These symptoms often lead to a decline in self-care, functional capacity, autonomy, and the ability to work or engage in leisure activities, thereby exacerbating the overall burden on patients.[12-14] To date, no objective

test or specific biomarker with sufficient diagnostic accuracy has been identified; however, emerging findings from proteomic research and gene expression profiling show potential for developing novel diagnostic methods.[15]

As such, the onset of fibromyalgia symptoms marks a significant shift in individuals' functional, social, and emotional lives.[16] Research employing health questionnaires repeatedly shows that people with fibromyalgia suffer considerable disadvantages across multiple domains of health status (i.e., physical ability, social interaction, physical discomfort, overall well-being, energy levels, social functioning, and mental health) compared to the general population and other chronic pain conditions.[9] Fibromyalgia patients often struggle to fulfill their professional responsibilities, resulting in decreased output, higher rates of absenteeism, and presenteeism, where individuals are physically present but perform at a subpar level.[2,12,14] The intensification of symptoms directly correlates with decreased work productivity.[17] For example, a study in Australia found that among women with fibromyalgia, 54.2% worked full-time and 21.5% part-time at symptom onset, but 5 years later, only 15.6% worked full-time, and 44.8% were no longer engaged in paid employment.[18] Leisure activities are also adversely affected, as patients often lack the energy or physical capacity for recreational pursuits, leading to social and emotional isolation.[13,19] Research has found that the majority of women with fibromyalgia experience pain and fatigue for more than 90% of their waking hours, thereby reducing their enjoyment of leisure activities.[19]

Psychological factors, including anxiety, depression, and coping mechanisms, are significant contributors to the deterioration of quality of life and physical functioning in individuals with fibromyalgia.[13,14] Depression and anxiety are common comorbidities that worsen pain perception, fatigue, and sleep problems, thereby making it even more difficult for patients to participate in self-care and maintain their independence. Features such as pain catastrophizing and self-efficacy, one's perceived ability to cope with stressful situations, affect the impact of pain on daily activities, regardless of pain intensity. Research has found that catastrophizing about pain is linked to a greater detrimental effect on daily life tasks.[20]

Engaging in physical activity and exercise is widely acknowledged as a vital component for managing fibromyalgia's impact on health.[21,22] Individuals with fibromyalgia frequently exhibit avoidance behaviors, prioritizing pain prevention over achieving physical activity and exercise objectives.[23] Avoidance of movement (kinesiophobia) is negatively associated with self-efficacy, partly mediated by general fatigue and the functional impact of fibromyalgia.[23,24] Over time, this behavior can lead to impaired functionality, physical disability, and a rise in negative mood, contributing to a psychological feeling of helplessness that, if prolonged, may result in depression.[25,26] Conversely, maintaining physical activity is associated with a lower perception of functional limitation despite pain.[24] Despite these obstacles, physical activity is a recommended treatment option for individuals with fibromyalgia, even though pain, fatigue, and decreased mobility frequently limit their participation.[11,27]

Despite the south of Brazil being a significant hub for healthcare development, the state of Paraná still shows a scarcity of research in this area.[28] Understanding diseases and dispelling misconceptions about them requires a comprehensive epidemiological profile, which in turn enables the development of informed public policy.[29] Although fibromyalgia is not a contagious condition necessitating public health control measures, it has a significant impact on individuals with the syndrome as well as their family members.[30] Given the numerous hypotheses about the cause of fibromyalgia, the challenges healthcare professionals face in diagnosing and treating it, and the significant negative effect on the quality of life of those affected, this study aims to gather epidemiological data about fibromyalgia patients in a specific health region in Brazil. The objective is to characterize the sample and its effects across numerous life domains, which can be justified by the scientific and social significance of producing information about the local reality of fibromyalgia, raising awareness of this condition, and contributing to the enhancement of healthcare and quality of life for those affected.

2. Materials and methods

2.1. Study design and setting

This cross-sectional study was conducted as part of a broader project titled "Biopsychosocial Aspects of Individuals Diagnosed with Fibromyalgia." The study population was drawn from the 8th Health Region of the State of Paraná, a region with approximately 350,000 inhabitants. Following a sample size calculation, as detailed in the data analysis section, the study included 85 participants of both sexes. Of these, 84.71% were from the city of Francisco Beltrão, 8.24% from Barracão, and 7.06% from other municipalities. The average monthly income was Brazilian Real (R$) 4,439.10 (standard deviation [SD] = R$ 3,695.77).

2.2. Procedures

The study rigorously adhered to all ethical principles recommended by relevant regulatory bodies. Data collection

commenced only after participants signed the Free and Informed Consent Form, which was included in the study approved by the Research Ethics Committee of the Western Paraná State University (CAAE: 73259023.6.0000.0107). Participants were thoroughly informed about the study's objectives, potential risks, and benefits, as stipulated by current legislation. Each participant was also aware of the option to request individual feedback on their results. Furthermore, it was explained that participants could, at any time, withdraw from participation or request the removal of their information from the study. The sample was selected by convenience through contacts with institutions serving the target population. Data were collected between 2023 and 2025.

Inclusion criteria required participants to report a medical diagnosis of fibromyalgia, with the majority (78.8%) diagnosed by a rheumatologist. Exclusion criteria included being younger than 18 years and non-residence within the jurisdiction of the 8th Regional Health Department of Paraná, Brazil. Moreover, participants who were illiterate were excluded from the investigation, as the study relied on self-reported measures.

To achieve the objectives of this study, participants completed a series of instruments using a digital, individual platform. For this specific investigation, data were extracted from the "Sociodemographic, Health, and Occupational Forms" section. This questionnaire gathered information such as age, sex, non-communicable chronic diseases diagnosis and treatment, fibromyalgia diagnosis, and the duration of living with the condition. In addition, data on profession and field of work, working hours, marital status, and income were collected. These data points were selected based on their relevance to previous investigations with similar objectives.[31,32] To facilitate interpretation and comparison with international studies, participants' responses to these measures were recorded on a 0–10 Likert scale, where higher scores indicated higher agreement. Psychometric properties revealed that participants' understanding of the questions was deemed excellent for both the "perceived impact of fibromyalgia on different life domains" scale and the "satisfaction of individuals on different life domains before and after fibromyalgia diagnosis" scale ($\alpha = 0.90$).

2.3. Statistical analysis

Responses were extracted into Microsoft Excel spreadsheets and carefully checked for potential data entry errors. Jeffrey's Amazing Statistical Program (JASP) (JASP, version 0.19) was utilized for all statistical analyses. For descriptive purposes—the primary objective of this study, which aimed to outline the epidemiological profile—variables were expressed as frequencies, percentages, means, and SDs. Normality tests were conducted to determine the appropriateness of basic inferential statistics, revealing a non-normal distribution of the data. Consequently, non-parametric techniques were employed and are detailed in each respective table. Regarding the study's statistical power, the sample size calculation was performed using G*Power software (version 3.1.9). Inputting the smallest observed effect size along with the achieved sample size yielded a statistical power of 99.36% at an alpha level (α) of 0.05 (Figure 1).

3. Results and discussion

3.1. Sample profile

Fibromyalgia is marked by significant features that affect individuals, encompassing both physical limitations and psychosocial factors. Understanding the characteristics and epidemiological profile of individuals with fibromyalgia can significantly facilitate clinical reasoning and decision-making among multidisciplinary teams. Thus, the current study reports that participants had an average age of 49.71 years (range = 26–69; SD = 9.50), with a notable majority (96.47%) being female, and only 2.70% were older than 65 years. This demographic aligns with prior research in other areas of Brazil, such as a study in the northern region that reported 97% of patients were female.[28] Regarding chronic pain, participants reported, on average, living with pain for 14.82 years (SD = 9.81).

The time since fibromyalgia diagnosis varied widely from 7 months to 40 years (SD = 9.35), with an average of 11.13 years. The prolonged period required to receive a diagnosis is crucial, particularly concerning the associated economic burden of fibromyalgia. Studies indicate that the average interval between initial symptom onset and accurate diagnosis typically ranges from 4 to 10 years. A lengthy diagnostic process, complicated by the complex and subjective presentation of fibromyalgia symptoms, frequently increases the strain on healthcare resources.[33-35]

Table 1 provides an overview of the frequencies and percentages for each category within the analyzed variables. A higher prevalence of fibromyalgia was observed in individuals who reported being married or in a stable union (71.76%) and who possessed higher education levels, with 45.78% having completed higher education. The proportion of individuals with other comorbidities was rather high, with the most common being hypertension (19.72%), respiratory diseases (9.86%), and obesity (9.86%).

These results, depicted in Table 1, show some differences when compared to previous research by Rezende *et al.*,[1]

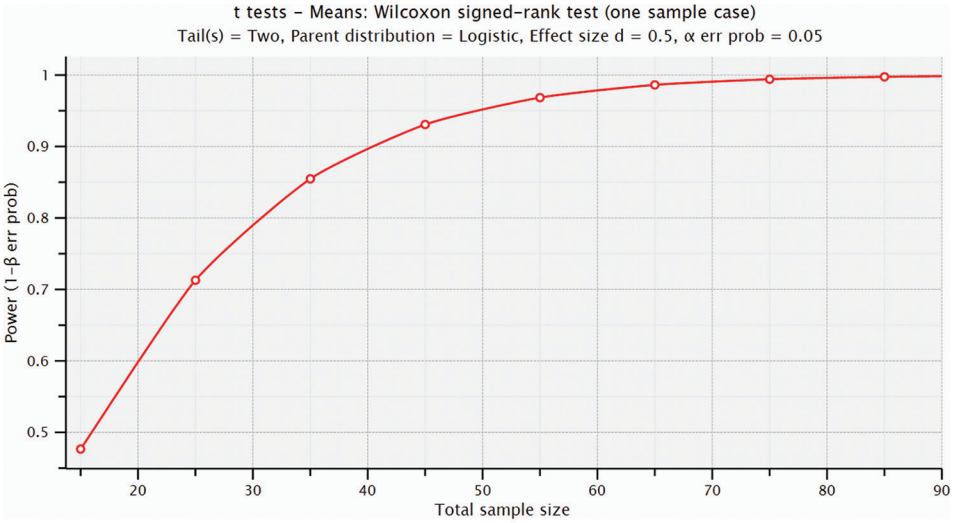

Figure 1. Power calculation plot

which analyzed 500 women diagnosed with fibromyalgia. In their study, 59.4% of women reported being married, a finding consistent with the sample in the current study. However, unlike the present study, they found a higher prevalence of women with complete elementary education, totaling one-third of their sample (37%), with only 8% having completed higher education. This discrepancy in educational attainment might be attributed to differences in sample size, methodology, and demographic characteristics between the studies.

3.2. Fibromyalgia and its impacts

Regarding the severity of pain experienced by participants in the last 30 days, the majority (56.47%) reported "very severe" pain, while 28.24% classified their pain as "moderately severe." Approximately 13% considered the pain "a little severe," and only 2.35% reported "not severe at all." Furthermore, nearly half of the participants (47.62%) reported that pain had a "very great" impact on their lives, and an additional 28.57% considered the impact "extremely great." Another 11.9% felt a "moderate" impact, and 10.71% stated that pain had "a little" impact. Only 1.19% of participants reported no impact from pain on their lives. These findings align with previous research demonstrating links between pain severity and quality of life.[36] In that study, 69.6% of participants rated their pain between 8 and 10 on a subjective scale, and the Fibromyalgia Impact Questionnaire score was 82.46 ± 2.9, collectively indicating a poor quality of life associated with the symptomatic profile of the sample.

Tables 2 and 3 provide data on the "perceived impact of fibromyalgia on different life domains" and "satisfaction of individuals on different life domains before and after fibromyalgia diagnosis." As shown in Table 4, inferential statistics are presented. Significant differences were found in all evaluated domains. Across all assessed dimensions—leisure, work, self-care, ability to exercise, functionality, and quality of life—there was a significant difference between the period "before" and "after" the fibromyalgia diagnosis, with $p<0.001$ in all cases. This indicates that all observed changes have strong statistical evidence and are highly unlikely to have occurred by chance. The *W*-test value, related to the non-parametric Wilcoxon analysis, further confirmed the existence of these differences in each domain, especially in conjunction with effect size analysis. Indeed, the magnitude of the effect can be considered high for all comparisons (point-biserial correlation coefficients >0.60). The Hodges-Lehmann estimate indicates the median change in participants' evaluations between the pre- and post-diagnosis periods.

Decline in scores across leisure activities, work functioning, exercise capacity, and functional capacity indicates a significant deterioration in physical functioning domains. The consistency across domains suggests a systemic rather than domain-specific pattern of deterioration, as previously reported.[37] The effect sizes in all domains (ranging from 0.60 to 0.70) are particularly noteworthy, as they exceeded what is considered clinically significant changes in fibromyalgia-related functioning measures.[38] The changes likely indicate genuine reductions in participants' everyday functioning abilities, with both clinical and statistical significance. The largest effect size was observed for the quality of life and overall functionality domains, suggesting that overall life satisfaction is remarkably susceptible to decline in this population. These findings are in line with previous

Table 1. Sociodemographic characteristics of study participants

Variables	*n*	Percentage
Gender		
Male	3	3.53
Female	82	96.47
Single		
No	61	71.76
Yes	24	28.24
Physically active (>150 min per week)		
No	40	47.62
Yes	44	52.38
Education		
Complete primary education	16	19.28
Complete secondary education	25	30.12
Complete higher education	38	45.78
Incomplete higher education	3	3.61
Prefer not to respond	1	1.20
Children		
No	7	8.24
Yes	78	91.76
Family history of psychiatric disorder		
No	37	54.41
Yes	31	45.59
Comorbidities		
No	7	8.24
Yes	75	97.76
Diagnosis psychiatric disorder		
No	43	63.24
Yes	25	36.76
Psychiatric treatment in the past year		
No	51	75.00
Yes	17	25.00
Psychological treatment in the past year		
No	41	60.29
Yes	27	39.71
Use of psychiatric medication		
No	43	63.24
Yes	25	36.76

Table 2. Perceived impact of fibromyalgia on different life domains

Parameters	Mean	SD	Minimum	Maximum
Leisure	8.26	1.64	1.00	10.00
Ability to work	8.32	1.55	3.00	10.00
Self-care	8.82	1.37	5.00	10.00
Overall functionality	8.43	1.35	5.00	10.00
Ability to exercise	8.40	1.53	4.00	10.00
Quality of life	8.83	1.54	4.00	10.00

Abbreviation: SD: Standard deviation.

Table 3. Satisfaction of individuals in different life domains before and after fibromyalgia diagnosis

Parameters	Mean	SD	Minimum	Maximum
Before fibromyalgia diagnosis				
Leisure	8.27	2.55	0.00	10.00
Ability to work	8.50	2.39	0.00	10.00
Self-care	8.46	2.48	0.00	10.00
Overall functionality	8.59	2.45	0.00	10.00
Ability to exercise	8.40	2.64	0.00	10.00
Quality of life	8.27	2.67	0.00	10.00
After fibromyalgia diagnosis				
Leisure	6.48	3.05	0.00	10.00
Ability to work	6.55	3.10	0.00	10.00
Self-care	6.09	3.38	0.00	10.00
Overall functionality	6.38	3.14	0.00	10.00
Ability to exercise	6.06	3.35	0.00	10.00
Quality of life	5.96	3.26	0.00	10.00

Abbreviation: SD: Standard deviation.

research highlighting the pervasive effects of chronic pain on quality-of-life outcomes.[39] Reduced physical activity aligns with the deconditioning cycle model, leading to physiological deconditioning and further limiting function in a self-perpetuating cycle that involves corresponding declines in exercise capacity and functional ability.[7,40] In addition, Macfarlane *et al.*,[41] found similar patterns of activity restriction and functional decline in their study of fibromyalgia patients, attributing these changes to both biological processes and psychological factors, particularly fear-avoidant behaviors.

The current findings, however, reveal significant declines in several areas. Thus, despite the long-standing nature of participants' pain conditions (lasting, on average, 14.82 years), adaptation mechanisms may have been inadequate to sustain psychological well-being over prolonged periods. Indeed, fibromyalgia patients experience a worsening psychological distress trajectory, which aligns with the observations by Clauw *et al.*,[42] even for those with long-standing diagnoses. Declines in work functioning and other areas appear to occur in parallel, suggesting a myriad of interconnected factors influencing multiple aspects of life at the same time. The pattern supports an integrated biopsychosocial model proposed by Edwards

Table 4. Comparisons of domains assessed by participants before and after fibromyalgia diagnosis

Domains	*W*	*p*-value	Hodges-Lehmann	Effect[a]	95% CI	
					Lower	Upper
Leisure	1,230.50	<0.001	3.00	0.60	0.37	0.76
Ability to work	1,336.50	<0.001	3.50	0.67	0.48	0.81
Self-care	1,529.50	<0.001	3.50	0.67	0.48	0.80
Overall functionality	1,356.00	<0.001	4.00	0.70	0.51	0.82
Ability to exercise	1,550.00	<0.001	3.50	0.69	0.51	0.82
Quality of life	1,263.00	<0.001	4.00	0.70	0.51	0.83

Notes: [a]Point-biserial correlation; *W* derived from the Wilcoxon test.
Abbreviations: SD: Standard deviation; CI: Confidence interval.

et al.,[43] which stresses the interconnected relationships among physical symptoms, psychological well-being, self-care, and social/occupational functioning in chronic pain conditions. Therefore, a significant reduction in self-care scores indicates deterioration in patients' ability to maintain personal care routines. This finding corresponds with research documenting progressive limitations in activities of daily living among fibromyalgia patients.[44] Indeed, evidence suggests that self-care activities are often compromised as pain conditions progress, partly due to increased fatigue, reduced physical capacity, and cognitive difficulties.[45-47]

3.3. Limitations and implications for theory and practice

The pattern of functional decline across various domains lends robust support to the allostatic load model of chronic pain, as proposed by Borsook *et al.*,[48] in which persistent pain imposes progressively heavier physiological and psychological loads on adaptive systems, ultimately resulting in accelerated deterioration across multiple functional areas. The current results showed substantial effect sizes across all functional domains, even among participants with long-standing pain conditions, consistent with this theoretical framework.

The present results also offer empirical evidence for the fear-avoidance model. This concept may be of paramount interest to society, healthcare professionals, and the broader healthcare system because it suggests that fear of pain causes individuals to shun physical activity, resulting in physical deconditioning and subsequent functional deterioration.[49,50] The observed declines in physical activity and functioning across various domains (e.g., leisure and exercise) and aspects (e.g., work and self-care) are consistent with the model's forecasted cyclical pattern of deterioration. Moreover, the findings also strengthen the theoretical understanding of the connection between physical capabilities and mental health in chronic pain situations. Physical and psychological deterioration, as proposed by Edwards *et al.*,[43] are interdependent, rather than one domain being the primary driver of changes in the other. This interdependence highlights the importance of preventive and intervention programs that target multiple domains simultaneously. Specifically, because declines in both physical and psychological domains often occur together, integrated treatments may yield more effective outcomes than treatments focusing on one area alone. Studies have shown that multimodal treatment programs, which combine physical and psychological interventions, achieve better results than single-modal approaches in fibromyalgia treatment.[21,44,51] Notably, studies have repeatedly demonstrated that physical activity is a vital element in the management of fibromyalgia. A review of 18 studies involving 1,184 participants found that physical exercise, especially when tailored to an individual's requirements, has positive effects on pain, depression, and quality of life.[52]

Research has consistently demonstrated that customized exercise plans, comprising aerobic exercises, strength training, and mind–body disciplines such as yoga and tai chi, can boost functional capacity, alleviate symptoms, and enhance quality of life.[11,27,36] Typically, an optimal exercise routine comprises moderate-intensity, tailored plans that balance physical activity with periods of rest to prevent the worsening of symptoms.[11,45] In addition to physical activity, psychological treatments also play a crucial role in fibromyalgia management. Approaches such as cognitive-behavioral therapy, mindfulness-based stress reduction, and acceptance and commitment therapy have shown effectiveness in enhancing psychological well-being and quality of life in fibromyalgia patients.[53] These interventions work by empowering patients with coping strategies to control pain and enhance their capacity for daily tasks, thereby promoting self-care and independence.[10] Moreover, group-based interventions have shown promising results in diverse settings, including primary care. A notable example is the *Amigos de Fibro* (Fibro Friends) program developed in the State of São

Paulo, which may serve as a model for similar initiatives in other regions.[54]

In clinical practice, the current results highlight the importance of establishing realistic expectations about disease progression and implementing measures to slow functional decline. Preventive approaches should particularly target domains that showed the largest effect sizes in this study, namely quality of life and self-care, by integrating both psychological and physical treatment protocols.[41]

Finally, several methodological limitations should be considered when interpreting these findings. First, the absence of a control group limits causal inferences about the natural progression of functional decline versus potential intervention effects or other confounding factors. Second, reliance on participants' self-reported diagnoses may introduce bias. Third, the study sample was predominantly female, which, although consistent with the epidemiology of fibromyalgia, may limit the generalizability of findings to more diverse populations. Finally, the reliance on self-reported measures without complementary objective functional assessments represents an additional limitation. Future research employing controlled, longitudinal designs would provide stronger evidence for disease progression patterns in chronic pain conditions.

4. Conclusion

This study indicates that fibromyalgia is a syndrome primarily affecting women of productive age, with a higher prevalence among those who are married, in a stable union, and with higher levels of education. A significant deterioration of fibromyalgia was found across multiple functional domains, including leisure, work, self-care, physical ability, and overall quality of life, underscoring the pervasive burden of this condition. The consistent pattern of decline across domains suggests a systemic rather than domain-specific deterioration. Moreover, the simultaneous deterioration in both physical and psychological domains supports integrated biopsychosocial models of chronic pain, suggesting that multimodal treatment approaches may be more effective for preserving functioning. Particular attention should be directed to domains showing the largest declines (i.e., quality of life and self-care) through integrated physical and psychological interventions.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: All authors
Investigation: All authors
Methodology: All authors
Writing–original draft: All authors
Writing–review & editing: All authors

Ethics approval and consent to participate

This study was approved by the Western Paraná State University Research Ethics Committee, under opinion number: 73259023.6.0000.0107. Written informed consent was obtained from all participants prior to their inclusion in the study.

Consent for publication

Participants provided their written consent for publication.

Availability of data

Data are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

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ORIGINAL ARTICLE

Comparative transcriptomic analysis of macrophages treated with a combination of ROCK pathway inhibitors

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Abstract

Background: At present, there is no therapy for the long-term (chronic) rejection of transplanted organs. This condition leads to tissue fibrosis and occlusion of the blood vessels. **Aim:** The overall goal of the current research is to identify a clinically applicable therapy for chronic rejection in transplanted organs. Our previous study showed that inhibitors of the RhoA/Rock pathway, such as Rezurock and fingolimod, prevent chronic rejection in rodent transplantation models, with Rezurock being superior in reducing fibrosis. **Materials and methods:** In this study, we analyzed the effect of a Rezurock and fingolimod combination on the transcriptome of mouse peritoneal macrophages and protein expression in both mouse and human macrophages. **Results:** The Rezurock/fingolimod combination resulted in the differential expression of 4,855 genes (2,477 downregulated and 2378 upregulated). Downregulated genes were related to fibrotic pathways, extracellular matrix, blood vessel development, cell adhesion, and cytokine production. Protein expression analysis showed that Rezurock/fingolimod treatment had a significantly stronger effect on the expression of pentraxin 3, chemokine (C-C motif) ligand 2, C-C motif chemokine receptor 2, and transforming growth factor beta 1 in mouse macrophages, and was much more effective in reducing the expression of Notch1 and Rho-associated coiled-coil kinase 2 in human macrophages compared to individual treatments. **Conclusion:** Rezurock/fingolimod treatment not only affects fibrotic pathways but also downregulates genes related to cell cycle progression and cytokine production and disrupts macrophage recruitment signaling. These findings indicate that Rezurock, alone or in combination with other immunomodulators, may be a promising candidate for clinical therapy targeting chronic rejection.

Keywords: Rezurock; Fibrosis; Chronic rejection; Macrophage; Ras homolog family member A/Rho-associated coiled-coil kinase pathway

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1. Introduction

Although short-term survival rates of transplanted organs have reached satisfactory levels, long-term survival remains a major post-transplantation challenge. According to the International Report on Organ Donation and Transplantation Activities from Global Observatory on Donation and Transplantation for 2022,[1] and published data,[2] approximately 157,000 solid organ transplants were performed worldwide, including kidney (~102,090); liver (~37,436), heart (~8,988), lung (~6,784), pancreas (~2,026), and small bowel (~170). However, around 70% percent of transplant recipients experienced symptoms of organ rejection within 10 years post-transplantation. Current treatment approaches depend on organ type; however, the most common treatments include immunosuppressants and corticosteroids, which primarily target the immune response and reduce inflammation.[3,4] The primary histological features in biopsies of chronically rejected organs are macrophage-driven narrowing or occlusion of the vessels and tissue fibrosis.[3,5] Because fibrosis is an important hallmark of chronic rejection, we hypothesize that targeting macrophages could become a new, efficient approach in post-transplant treatment. In our quest for clinically applicable anti-chronic rejection therapy, we showed that in a rodent cardiac transplantation model, the pharmacologic inhibition of the Ras homolog family member A (RhoA)/Rho-associated coiled-coil kinase (ROCK) pathway or macrophage-specific RhoA knockout eliminates, through changes in the actin cytoskeleton, macrophage infiltration of the allograft and inhibits chronic rejection.[6,7] After testing several commercially available RhoA/ROCK inhibitors, we found that Rezurock—Food and Drug Administration (FDA)-approved for the treatment of chronic graft-versus-host disease—is superior in inhibiting fibrosis in the mouse cardiac transplantation model.[8] Molecularly, RhoA/ROCK signaling mediates cytoskeletal remodeling during cell migration and polarization of macrophages and additionally affects the proliferation rates of immune cells.[9] Therefore, we believe that inhibitors of the RhoA/ROCK pathway could represent a novel approach for post-transplant treatment.

Here, we studied the effects of Rezurock and fingolimod combination on gene and protein expression in mouse and human macrophages. Fingolimod, primarily recognized as a nonselective functional antagonist of sphingosine-1-phosphate receptors, is FDA-approved for multiple sclerosis treatment. However, our previous work demonstrated that fingolimod also inhibits the RhoA/ROCK pathway, although it is much less effective in inhibiting fibrosis than Rezurock.[9] Our transcriptomic analysis reveals that combined Rezurock/fingolimod treatment downregulates genes associated with cell cycle progression, metabolic and cytokine production pathways, and dysregulates signaling pathways involved in macrophage recruitment. Overall, our findings suggest that Rezurock/fingolimod, in combination, holds therapeutic potential in preventing the chronic rejection-related functions of macrophages in organ transplantation.

2. Materials and methods

2.1. Mice handling

All experiments were performed according to The Methodist Hospital Research Institute's animal care and use standards, based on the National Institute of Health (NIH) guidelines outlined in the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 85-23 Revised 1985). The Institute also mandates compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*.

2.2. Isolation and culture of peritoneal macrophages

Mouse peritoneal macrophages were isolated from the peritoneal cavity of C57BL/6J mice (n = 25) obtained from Jackson Laboratory, Bar Harbor, USA and cultured as previously described.[10]

2.3. RAW 264.7 macrophage culture

RAW 264.7 cells (ATCC, Manassas, USA) were maintained and cultured as previously described.[10]

2.4. Isolation and culture of peripheral blood mononuclear cells from human blood and differentiation of monocytes into macrophages (human monocyte-derived macrophages)

Blood (500 mL) was purchased from the blood bank. Fifty-milliliter stem cell tubes (SepMate™ Peripheral Blood Mononuclear Cell [PBMC] Isolation Tubes, STEMCELL Technologies, Vancouver, BC, Canada) were filled to the 15 mL mark with Ficoll-Paque Plus (Cytiva, Marlborough, MA 01752, USA). After slowly adding blood to the 40 mL mark, the tubes were centrifuged at 700 × g for 15 min at room temperature (RT). The white PBMC layer was transferred to the 50 mL tubes, and after adding phosphate-buffered saline, centrifuged at 500 × g for 10 min at RT. Cells were cultured in RPMI 1640 medium (11875-093, Gibco, Waltham, Massachusetts, USA. Supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 units/mL), and macrophage colony-stimulating factor (Peprotech 300-25, Cranbury, New Jersey 08512,

USA) at 50 ng/mL, at 37°C and 5% carbon dioxide. A total of 2×10^7 cells were seeded in each T75 flask. After 24 h, the medium was changed to remove non-adherent cells, leaving only monocytes (adherent cells). Cells were differentiated into macrophages (human monocyte-derived macrophages [HMDM]) over 6 days. On day 6, the HMDM were treated with inhibitors.

2.5. Treatment with inhibitors

At 70% confluency, cells were treated for 24 h with Rezurock (HY-15307, MedChem Express, United States of America [USA]) at a concentration of 10 μM, fingolimod (S5002, Selleckchem, USA) at 300 nM, or Rezurock and fingolimod in combination. For HMDM, Rezurock alone or in combination with fingolimod was used at a concentration of 5 μM. Dimethyl sulfoxide (DMSO; Sigma, USA) was used as a control, and its volume matched the volume used for the highest drug concentration to ensure consistent DMSO levels across all samples.

2.6. RNA isolation

Mouse peritoneal macrophages were pelleted and sent to Active Motif, Inc. (USA) for RNA isolation, library preparation, and sequencing analysis.

2.7. RNA sequencing and analysis

Next-generation sequencing was performed using the Illumina platform, and Venn diagrams were generated as described previously.[10] Genes selected for the Metascape Gene Ontology (GO) analysis had a $p \leq 0.05$ and $\log_2$ fold change‖ > 0. All genes considered differentially expressed in the treated samples, compared to the control, had a $p \leq 0.05$ and ‖$\log_2$ fold change‖ > 0. For RNA sequencing (RNA-seq) analysis, two biological replicates were used for each treatment condition. In our gene expression analysis, sequencing quality was ensured by filtering raw reads to obtain clean reads. This was followed by alignment to the reference genome using HISAT2 and STAR tools. Gene expression levels were normalized using the "Fragments Per Kilobase of exon per Million mapped reads" method, and principal component analysis plots were used to visualize sample clustering and assess potential batch effects. All samples were processed under identical conditions to minimize technical variability.

2.8. Cell lysis and western blots

Control, Rezurock-, fingolimod-, and Rezurock/fingolimod-treated RAW 264.7 and HMDM macrophages were lysed in radioimmunoprecipitation assay cell lysis buffer (#9806, Cell Signaling, USA) supplemented with 1× protease inhibitor (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, #11836170001, Roche, USA) and 1× phenylmethanesulfonyl fluoride (#8553, Cell Signaling, USA), and prepared for Western blotting as described previously.[10] Western blot bands were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as a loading control (protein of interest vs. GAPDH), and each experiment was repeated three times to reduce technical noise. Statistical analysis was performed to ensure data significance.

2.9. Antibodies

The following primary antibodies were used: ROCK2 (8236s, Cell Signaling, USA), Notch1 (3608s, Cell Signaling, USA), pentraxin 3 (PTX3; PA5-36156, Invitrogen, China), collagen Type1 (14695-1-AP, Proteintech, USA), chemokine (C-C motif) ligand 2 (CCL2; MA5-17040, Invitrogen, China), C-C motif chemokine receptor 2 (CCR2; MA5-42780, Invitrogen, China), and transforming growth factor beta 1 (TGF-β1; 21898-1-AP, Proteintech, USA), all at 1:1,000 dilution. ROCK1 antibody (ab199899, Abcam, USA; currently discontinued; https://www.abcam.com/en-us/products/unavailable/rock1-antibody-c-terminal-ab199899) was used at 1:2,000 dilution, and GAPDH (14C10, Cell Signaling, USA) at 1:3,000 dilution. For secondary antibodies, anti-rabbit immunoglobulin G (IgG; 7074P2, Cell Signaling, USA) and anti-mouse IgG (7076, Cell Signaling, USA) were used at 1:5,000 dilutions.

2.10. Statistics

For western blotting (protein expression) analysis, three biological replicates were used to ensure statistical power, as described previously.[10] Differentially expressed genes (DEGs) were selected using a p-value cutoff of < 0.05, and a $\log_2$ fold-change > 0. These thresholds were applied when generating volcano plots for each treatment group.

The protein expression changes after drug treatment were consistent with the transcriptomic findings, supporting the reliability of the DEG selection. Pathway enrichment and GO analyses were carried out using the Metascape platform based on DEGs with $p \leq 0.05$ and a $\log_2$ fold change > 0. Results were interpreted using bar plots and clustering analyses to identify significantly enriched biological processes. RNA-seq data were analyzed using DESeq2, which includes internal normalization and applies the Wald test followed by Benjamini–Hochberg correction to control the false discovery rate (FDR). All pathway enrichment analyses (e.g., GO and Kyoto Encyclopedia of Genes and Genomes [KEGG]) were likewise corrected for multiple hypothesis testing using FDR-adjusted p-values. Statistical significance thresholds (adjusted p<0.05) were applied consistently across all analyses.

3. Results

In all our analyses, we purposely used non-activated (M0) macrophages. M0 (or naïve) macrophages are in a resting state and serve as precursors to polarized macrophages. It is known that the transcription and protein expression profiles of activated macrophages depend on the type (direction) of polarization (M1 vs. M2), and particularly on the specific type of activator. In addition, our previous research showed that the response of M0 macrophages to RhoA/ROCK inhibition is very similar to that of M2 macrophages but differs from the response of M1 macrophages. Both M0 and M2 macrophages exhibit high levels of RhoA messenger RNA (mRNA), whereas M1 macrophages express RhoA mRNA at approximately three times lower levels.[11] Thus, to obtain "basic" or naïve transcriptomic and proteomic data, we chose to use non-activated (M0) macrophages.

3.1. RNA sequencing

We performed RNA sequencing of mouse peritoneal macrophages after combined treatment with Rezurock and fingolimod. The data were then compared with our previously published results from individual treatments with Rezurock or fingolimod in mouse peritoneal macrophages.[10] A schematic representation of the workflow is shown in Figure 1. Figure S1A displays the distribution of control (DMSO-treated), Rezurock-only, and Rezurock/fingolimod-treated macrophages. Figure S1B shows a box plot representing gene expression distribution in control (DMSO-treated) and Rezurock/fingolimod-treated macrophages. Some of the data presented in the Supplements were already published in our previous publication.[10] All genes considered differentially expressed compared to the control (DMSO-treated) cells had a $p \leq 0.05$ and $\|\log_2 \text{fold change}\| > 0$. Pathways associated with these DEGs were identified using Metascape.

3.2. Effect of Rezurock/fingolimod combination on mouse peritoneal macrophages

The combination treatment of macrophages with Rezurock and fingolimod resulted in the differential expression of 4,855 genes (2,477 downregulated and 2,378 upregulated) compared to the DMSO control (Figure 2A). In the volcano plot, green dots represent upregulated genes, whereas red dots represent downregulated genes. The distribution of the DEGs is depicted in the heatmap (Figure 2B). The downregulated genes were related to the cell cycle, DNA metabolic processes, neutrophil degranulation, chromosome organization, leukocyte proliferation, and cytokine production (Figure 2C; Tables S1 and S2). The upregulated genes were associated with protein processing, membrane trafficking, Rho GTPase signaling, autophagy, Golgi organization, neuron projection development, and histone modification (Figure 2D; Tables S3 and S4).

Our previously published work showed that Rezurock is superior to fingolimod in modulating the transcriptome profile of mouse peritoneal macrophages and in regulating fibrosis pathway-related proteins in both mouse macrophages and human monocyte-derived macrophages.[10] Here, we show that 1,751 genes were shared between Rezurock-treated macrophages and the combination of Rezurock/fingolimod, 726 genes were downregulated only after the combination treatment,

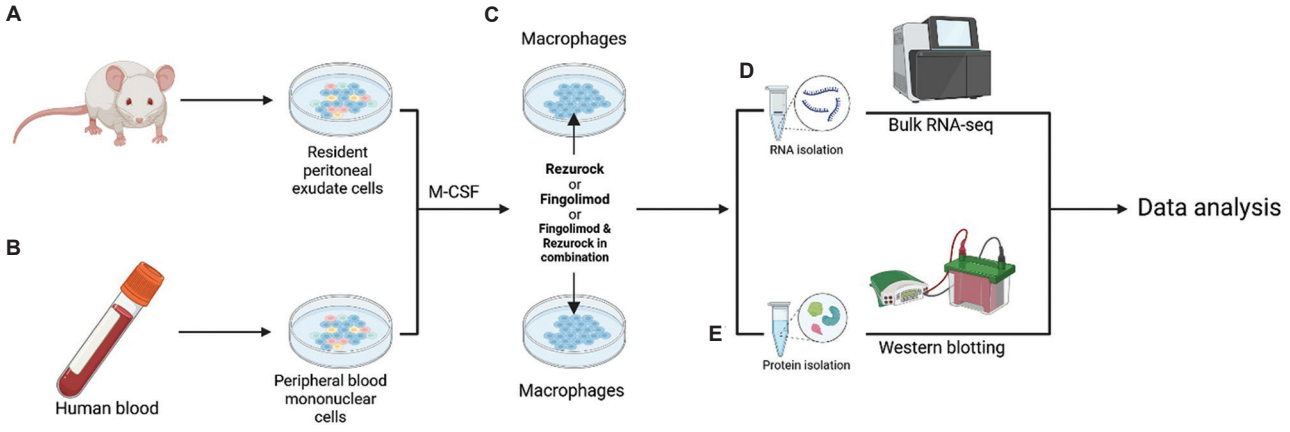

Figure 1. Experimental workflow for macrophage isolation, drug treatment, and downstream analysis. Resident peritoneal exudate cells were harvested from the peritoneal cavity of mice (A), while mononuclear cells were isolated from human blood samples (B). In both cases, cells were cultured in the presence of M-CSF to induce macrophage differentiation. Once differentiated, macrophages were treated with Rezurock, fingolimod, or a combination of both drugs to assess their individual and combined effects (C). Following treatment, RNA was isolated for bulk RNA sequencing to evaluate gene expression changes (D), and protein was extracted for western blotting to assess alterations in protein levels (E).
Abbreviation: M-CSF: Macrophage colony-stimulating factor.

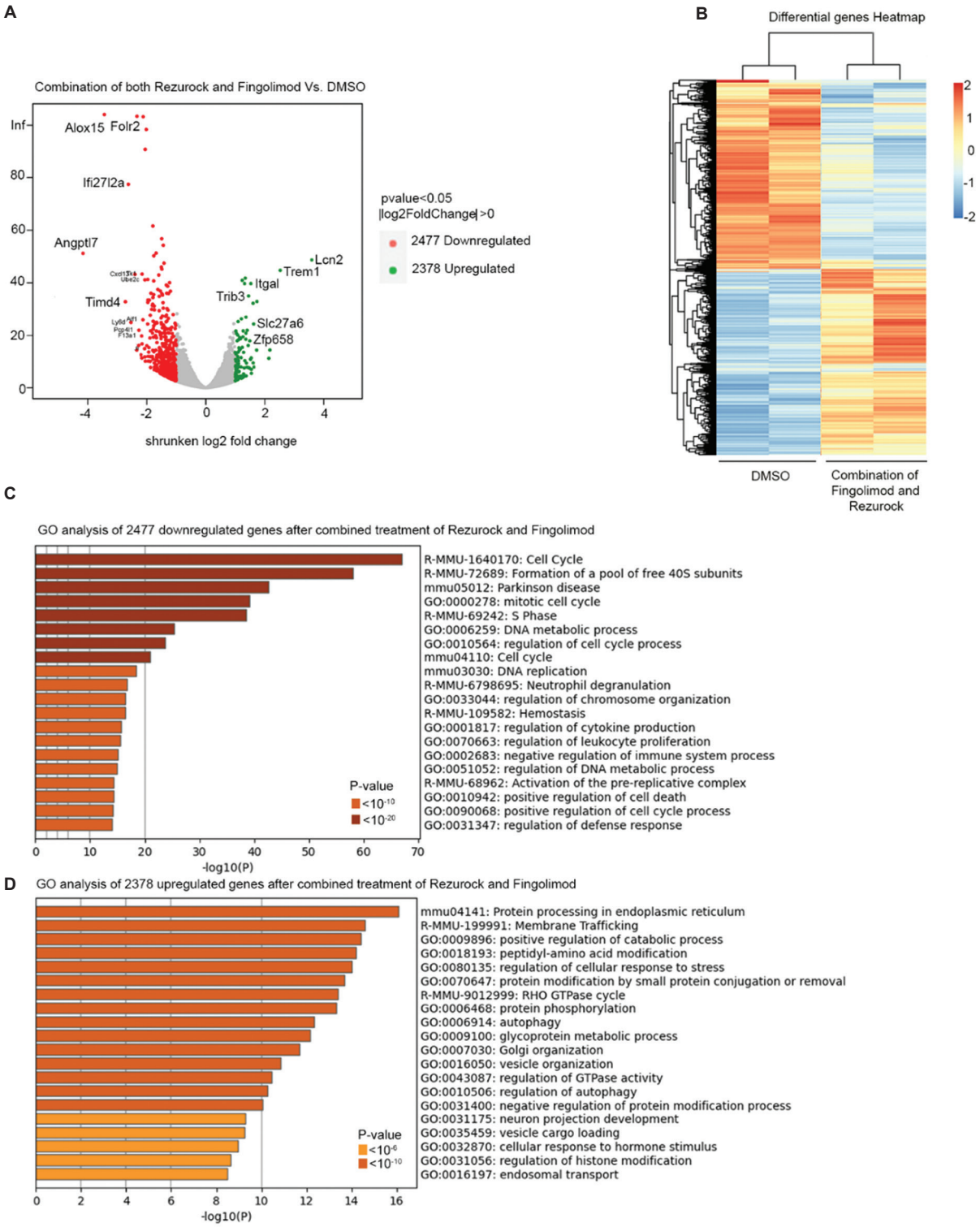

Figure 2. Distribution of DEGs and associated biological pathways in mouse macrophages treated with the Rezurock/fingolimod combination compared to control (DMSO-treated) macrophages. (A) Volcano plot showing DEGs in Rezurock/fingolimod-treated macrophages versus DMSO-treated controls (p<0.05 and $\|\log_2$ fold change$\|$ > 0). (B) Heatmap illustrating the distribution of DEGs between DMSO- and Rezurock/fingolimod-treated mouse macrophages. (C) GO analysis of downregulated genes in the Rezurock/fingolimod combination treatment. (D) GO analysis of upregulated genes in the Rezurock/Fingolimod combination treatment.

Abbreviations: DEGS: Differentially expressed genes; DMSO: Dimethyl sulfoxide; GO: Gene ontology; Rho: Ras homolog family member.

whereas 310 genes were downregulated exclusively in Rezurock-treated macrophages (Figure 3A and B). The genes downregulated after combination treatment were related to extracellular matrix organization, blood vessel development, neutrophil degranulation, cell–cell adhesion, and cytokine production (Figure 3C; Table S5). The downregulated genes specific to Rezurock treatment were involved in lipid metabolism, atherosclerosis, hemostasis, cytokine signaling, neutrophil signaling, lipid localization, and regulation of the actin cytoskeleton (Figure 3D; Table S6). The genes downregulated in both Rezurock and combination treatments were related to the cell cycle, translation, chromosome organization, regulation of cellular stress, adaptive immune response, and chromosome segregation (Figure 3E; Table S7). Among the upregulated genes, 357 were unique to Rezurock treatment (Figure 4A and B), 821 were uniquely upregulated after Rezurock/fingolimod treatment, and 1,554 genes were shared between the two treatments. The genes upregulated only after combination treatment were related to Golgi vesicle transport, histone modification, the Rho GTPase pathway, protein modification, DNA methylation, and the mitogen-activated protein kinase signaling pathway (Figure 4C; Table S8). The genes shared between Rezurock and combination treatments were associated with protein processing in the endoplasmic reticulum (ER), metabolic processes, autophagy, GTPase activity, and membrane trafficking (Figure 4D; Table S9). The genes upregulated only in Rezurock-treated macrophages were related to ER-to-Golgi transport, lysozyme pathway, fatty acyl-coenzyme A synthesis, membrane lipid metabolism, protein localization to the ER, neutrophil degranulation, and apoptotic signaling (Figure 4E; Table S10).

In summary, treatment with Rezurock/fingolimod had a more pronounced effect on the mouse macrophage transcriptome than individual treatment with either Rezurock or fingolimod.[10] The shared 87 downregulated genes were mostly related to antigen processing, cytokine production, chemokine signaling, regulation of leukocyte differentiation, cell activation, macrophage markers, G alpha signaling, and interleukin (IL)-2 production (Figure 5A-C; Table S11). Among the upregulated genes, 35 were shared by all three treatment groups (Figure 5D and E). These 35 genes were associated with protein folding, adipogenesis, transcription, immunoglobulin production, and cell–cell adhesion (Figure 5F; Table S12).

3.3. Effect of Rezurock/fingolimod combination on the fibrosis pathway-related proteins

Rezurock prevents fibrosis more effectively than other RhoA/ROCK inhibitors. It downregulates genes involved in fibrosis and collagen deposition pathways. Table 1 lists some of the genes downregulated by Rezurock and the Rezurock/fingolimod combination, but not by fingolimod alone. We previously published the expression data of these fibrosis-related proteins (some selected from Table 1 and others from independent fibrosis studies) following treatment with Rezurock or fingolimod separately.[10] Here, we compared the expression of fibrotic pathway-related proteins in control (DMSO-treated), Rezurock-only, fingolimod-only, and Rezurock/fingolimod combination-treated treatment.

We evaluated the expression levels of ROCK1, ROCK2, Notch1, PTX3, collagen Type I, CCL2, CCR2, and TGF-β1 in RAW 264.7 mouse macrophages (Figure 6). We found that the expression of ROCK1, ROCK2, Notch1, and collagen Type I was similar across Rezurock alone, fingolimod alone, and the Rezurock/fingolimod combination treatments. However, for PTX3, CCL2, CCR2, and TGF-β1, the combination treatment had a much stronger inhibitory effect compared to either drug alone (Figure 6). We also analyzed the protein expression of ROCK1, ROCK2, and Notch1 following Rezurock, fingolimod, and combination treatment in HMDMs (Figure 7). All three treatments reduced the levels of these proteins. However, the Rezurock/fingolimod combination was significantly more effective at reducing Notch1 and ROCK2 expression in HMDMs than either individual treatment.

In addition, we performed GO enrichment analysis of *Stat3*/*Stat5*-related pathways among DEGs in macrophages treated with Rezurock, fingolimod, or their combination (Figure 8; Tables S13-S15). Macrophages treated with either drug, or the combination, exhibited differential modulation of immune-related pathways. GO enrichment analysis revealed significant changes in cytokine signaling and *Stat3*/*Stat5*-associated processes, with the strongest enrichment observed following combination treatment (Figure 8A-C). Heatmap analysis showed more pronounced transcriptional changes following Rezurock treatment compared to fingolimod alone, while the combination treatment induced the most extensive modulation, suggesting enhanced macrophage reprogramming (Figure 8D-F).

4. Discussion

Chronic rejection of transplanted organs remains incurable, and long-term organ survival rates continue to be unsatisfactory.[3,4] Existing therapies primarily focus on inhibiting T-cell proliferation and activation to prevent graft rejection.[3,4] However, we and others hypothesize that targeting macrophages could offer additional therapeutic benefits, as they are well-established contributors to

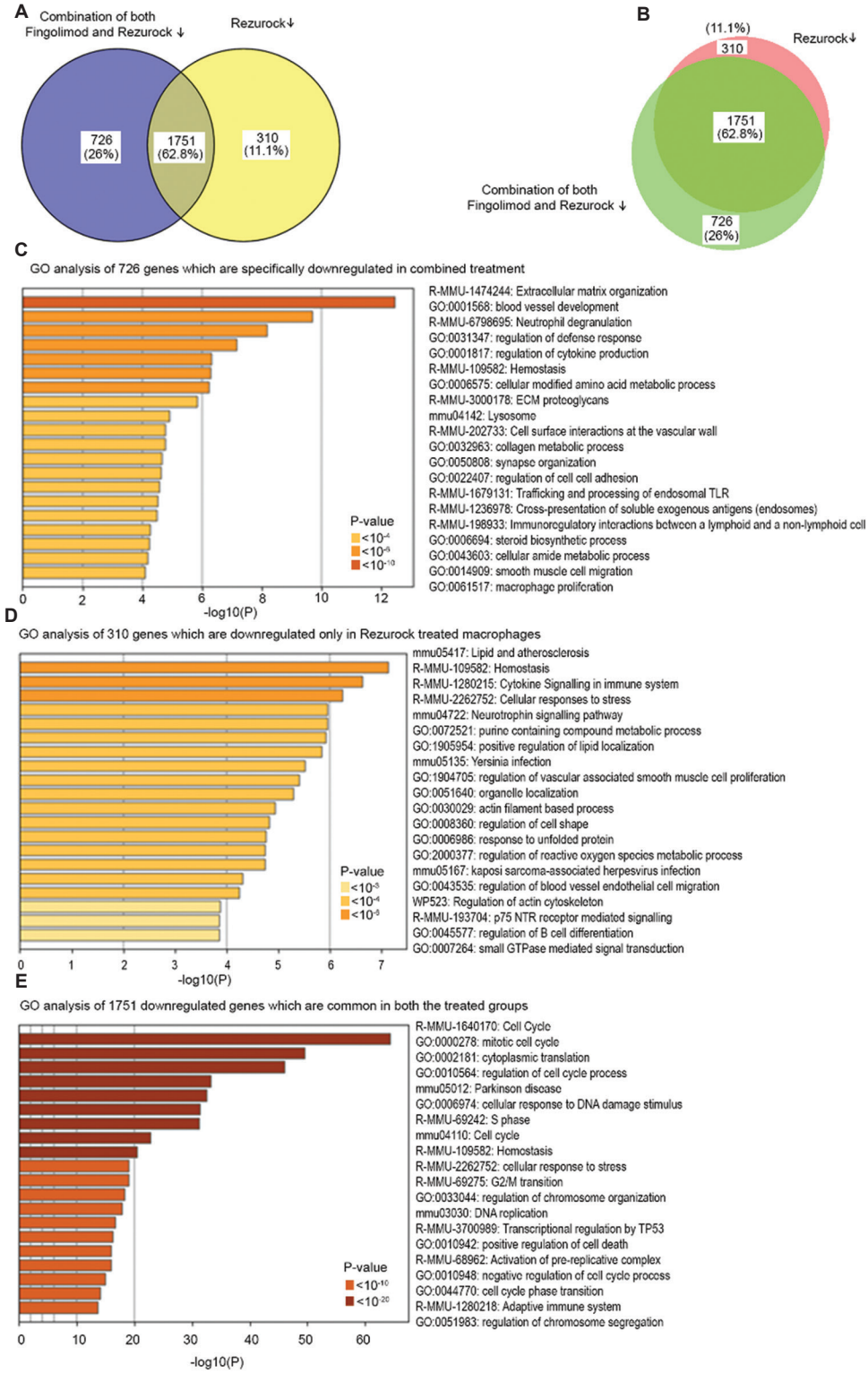

Figure 3. Comparison of downregulated genes between Rezurock/fingolimod combination treatment and Rezurock-only treatment in mouse macrophages. (A and B) Venn diagrams showing the overlap of downregulated genes in Rezurock/fingolimod combination treatment and Rezurock-only treatment. (C) GO analysis of downregulated genes specific to the Rezurock/fingolimod combination treatment. (D) GO analysis of downregulated genes specific to the Rezurock-only treatment. (E) GO analysis of downregulated genes common to both Rezurock/fingolimod combination and Rezurock-only treatments. Abbreviations: GO: Gene ontology; ECM: Extracellular matrix; NTR: Non-catalytic tyrosine-phosphorylated receptor; TP53: Tumor protein 53.

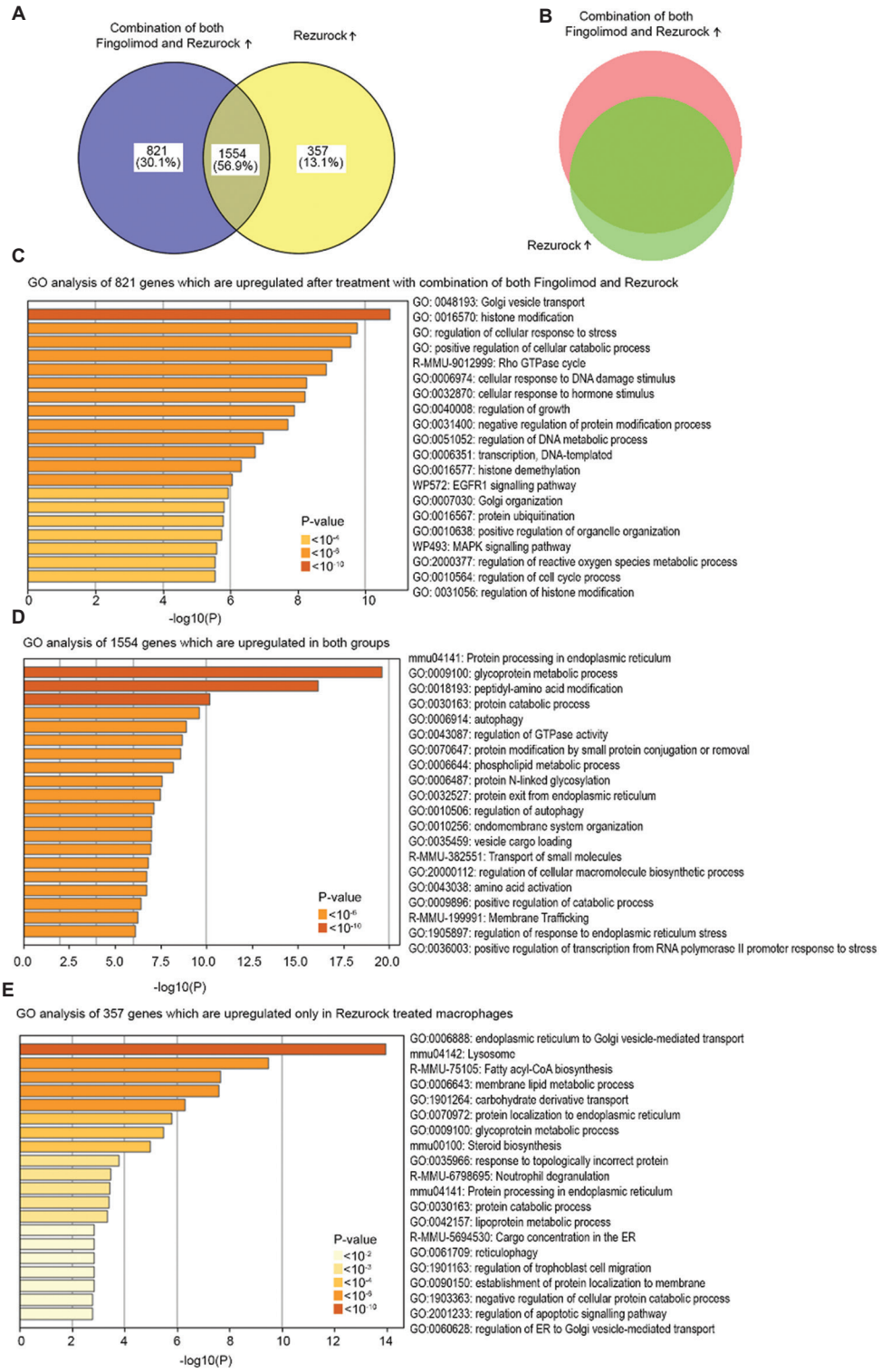

Figure 4. Comparison of upregulated genes between Rezurock/fingolimod combination treatment and Rezurock-only treatment in mouse macrophages. (A and B) Venn diagrams comparing upregulated genes between the Rezurock/fingolimod combination treatment and Rezurock-only treatment. (C) GO analysis of upregulated genes specific to the Rezurock/fingolimod combination treatment. (D) GO analysis of upregulated genes common to both the Rezurock/fingolimod combination and Rezurock-only treatments. (E) GO analysis of upregulated genes specific to the Rezurock-only treatment.
Abbreviations: GO: Gene ontology; CoA: Coenzyme A; ER: Endoplasmic reticulum; MAPK: Mitogen-activated protein kinase; Rho: Ras homolog family member.

Table 1. Fibrotic pathway-related genes downregulated by Rezurock treatment, either alone or in combination with fingolimod, in mouse macrophages

Gene name	Role in fibrosis	Fold change in fingolimod-treated macrophages	Fold change in Rezurock-treated macrophages[a]	Fold change in Rezurock/ fingolimod combined treatment in macrophages	Protein expression level (relative to control sample=1)		
					Fingolimod treatment	Rezurock treatment	Rezurock/ fingolimod combined treatment
Cxcl13	Prognostic biomarker of idiopathic pulmonary fibrosis	-	−2.23	−2.58	-	-	-
Cxcl10	A profibrotic factor	-	−2.02	−2.14	-	-	-
Ptx3	Associated with fibrotic lesions and collagen deposition	-	−1.92	−2.68	0.51	0.15	0.08
Wnt4	Mainly associated with renal fibrosis	-	−1.52	−2.72	-	-	-
Tgfb3	Regulates cytokine-stimulating fibrosis	-	−1.18	−1.62	-	-	-
Tcf19	Associated with pulmonary fibrosis	-	−1.07	−1.97	-	-	-
Ccr2	Associated with hepatic and pulmonary fibrosis	-	−1.04	−1.19	0.36	0.19	0.17
Notch1	Enhances protein expression in pulmonary fibrosis	−1.03	-	-	0.54	0.38	0.29
Cxcl12	CXCR4 drives tissue fibrosis through binding its specific ligand of CXCL12	-	−0.63	−1.94	-	-	-
Cxcl14	Recruits fibroblasts to the sites of fibrosis	-	−0.61	−0.84	-	-	-
Mapkapk2	Plays an essential role in the cell migration of neutrophil, macrophage, leading to fibrosis	-	−0.50	−0.35	-	-	-
Pak1	A profibrotic factor	-	−0.42	−0.52	-	-	-
Smad3	Promotes renal fibrosis by binding to the promoter region of collagens to trigger their production	-	−0.40	−0.37	-	-	-
Ccl2	Associated with monocyte/macrophage inflammatory response, angiogenesis, collagen synthesis, myofibroblast differentiation, and fibroblast recruitment	-	−0.32	−0.48	0.96	0.29	0.17
Pdgfb	Plays a key role in the expansion of myofibroblasts by stimulating their proliferation, migration, and survival	-	−0.31	-	-	-	-
Pdgfbra	Associated with connective tissue growth, leading to a progressive fibrosis phenotype	-	-	−1.49	-	-	-
Timp3	Localized to fibroblastic foci, extracellular matrix, and important mediator of lung fibrogenesis	-	−0.72	-	-	-	-
Rock1	Associated with pulmonary fibrosis	-	-	-	0.49	0.42	0.47
Rock2	Associated with pulmonary fibrosis	-	-	-	0.53	0.35	0.4
Col1	Activated in fibrosis	-	-	-	0.38	0.44	0.41
Tgfb1	Activated and upregulated in fibrosis	-	-	-	0.67	0.42	0.25

Note: [a]Some information presented in Table 1 was previously published.[10]
Abbreviations: CXCL12: C-X-C motif chemokine ligand 12; CXCR4: C-X-C chemokine receptor Type 4.

vessel occlusion and fibrosis progression after organ transplantation.[5,6] In response to chemoattractants released by damaged or inflamed allograft tissues, macrophages infiltrate the transplanted organ and secrete various profibrotic factors, which promote fibroblast activation and differentiation into myofibroblasts. This interaction contributes to extracellular matrix accumulation, potentiating fibrosis and eventually leading to graft dysfunction and chronic rejection over time.[5,6] In our previous studies, pharmacological inhibition of the RhoA/ROCK pathway effectively abrogated chronic rejection after heart transplantation by preventing macrophage

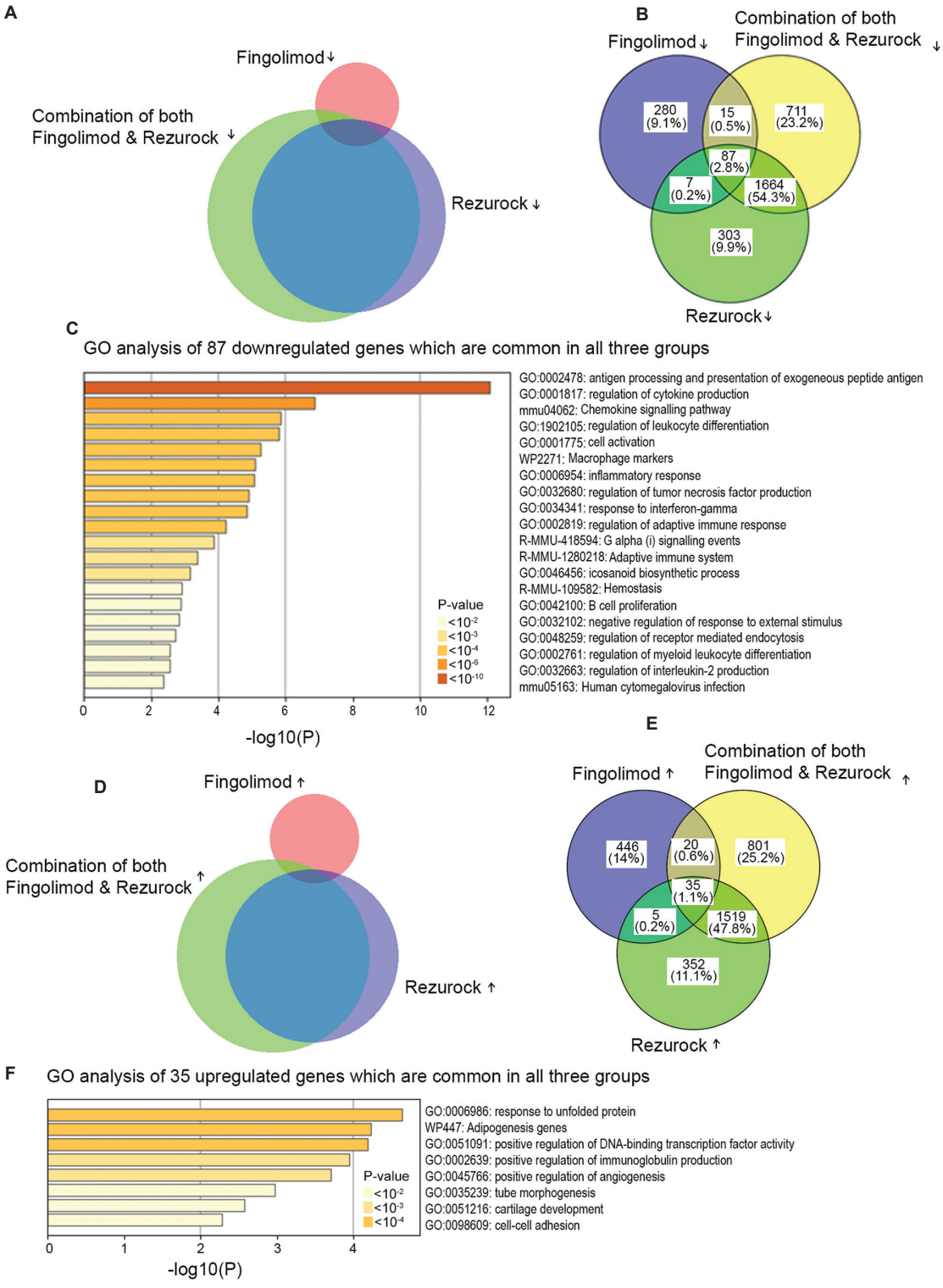

Figure 5. Comparison of differentially expressed genes in Rezurock/fingolimod combination treatment and Rezurock- or fingolimod-only treatments in mouse macrophages. (A and B) Venn diagrams comparing downregulated genes among Rezurock-only, fingolimod-only, and Rezurock/fingolimod combination treatments. (C) GO analysis of downregulated genes common to all three treatment groups. (D and E) Venn diagrams comparing upregulated genes among Rezurock-only, fingolimod-only, and Rezurock/fingolimod combination treatments. (F) GO analysis of upregulated genes common to all three treatment groups.
Abbreviation: GO: Gene ontology.

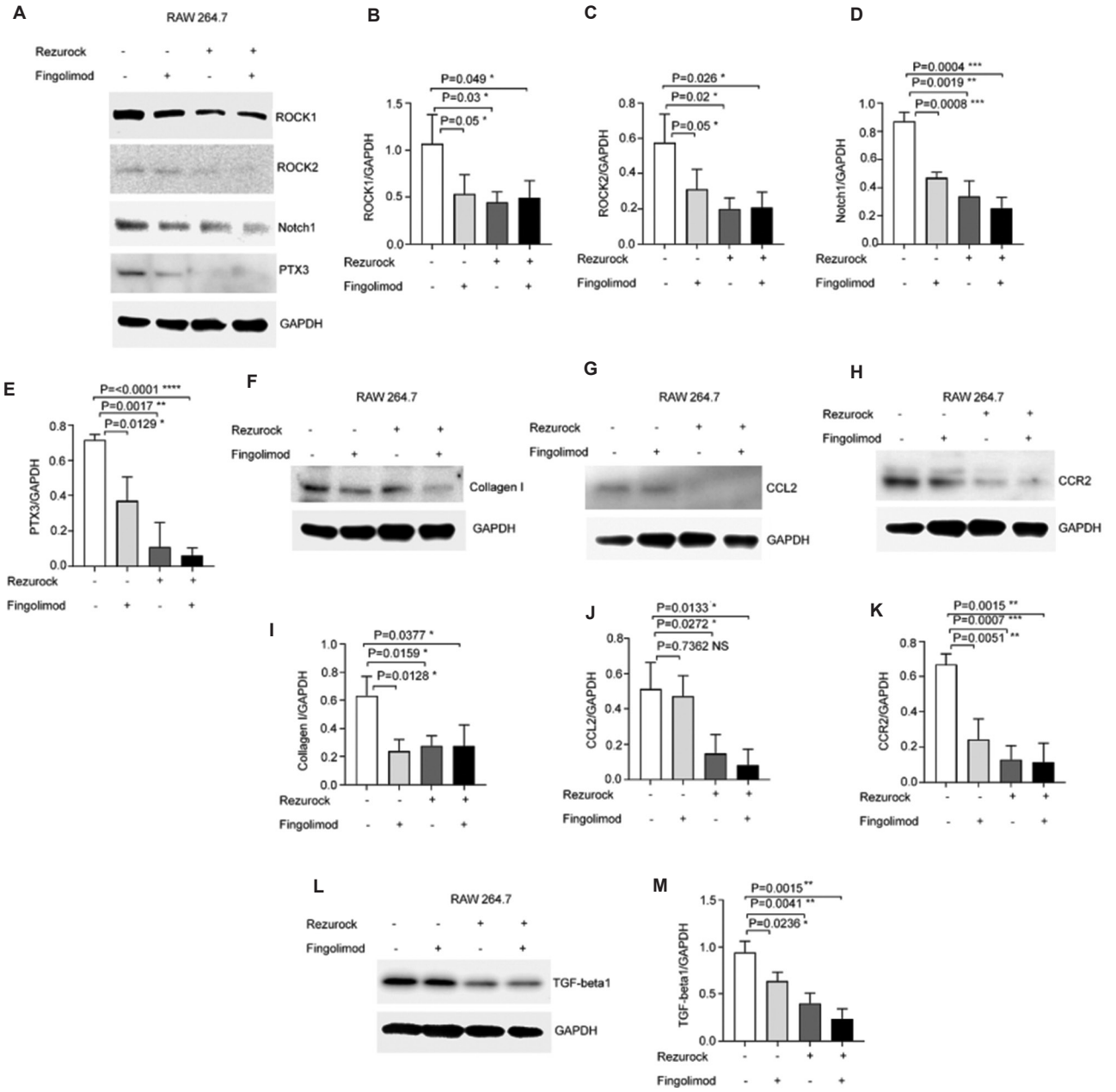

Figure 6. Effect of Rezurock and fingolimod treatment alone or in combination on fibrosis pathway-related protein expression in RAW 264.7 cells. (A, F-H, L) Western blot analysis of ROCK1, ROCK2, Notch1, PTX3, collagen Type I, CCL2, CCR2 and TGF-β1, respectively. GAPDH was used as a loading control. (B-E, I-K) Graphical representations of three independent western blot experiments corresponding to each protein.
Notes: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.
Abbreviations: CCL2: Chemokine (C-C motif) ligand 2; CCR2: C-C motif chemokine receptor 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; PTX3: Pentraxin 3; ROCK: Rho-associated coiled-coil kinase; TGF-β1: Transforming growth factor beta 1.

infiltration into the allografts and reducing collagen deposition.[11,12] In line with our findings, other studies have shown that pharmacological inhibition of ROCKs attenuates bleomycin- and radiation-induced pulmonary fibrosis by regulating macrophage polarization.[13]

Our current research aimed to investigate changes in gene expression patterns in macrophages following combined treatment with the RhoA/ROCK inhibitors Rezurock and fingolimod. We recently demonstrated that fingolimod, when administered alongside an early T-cell

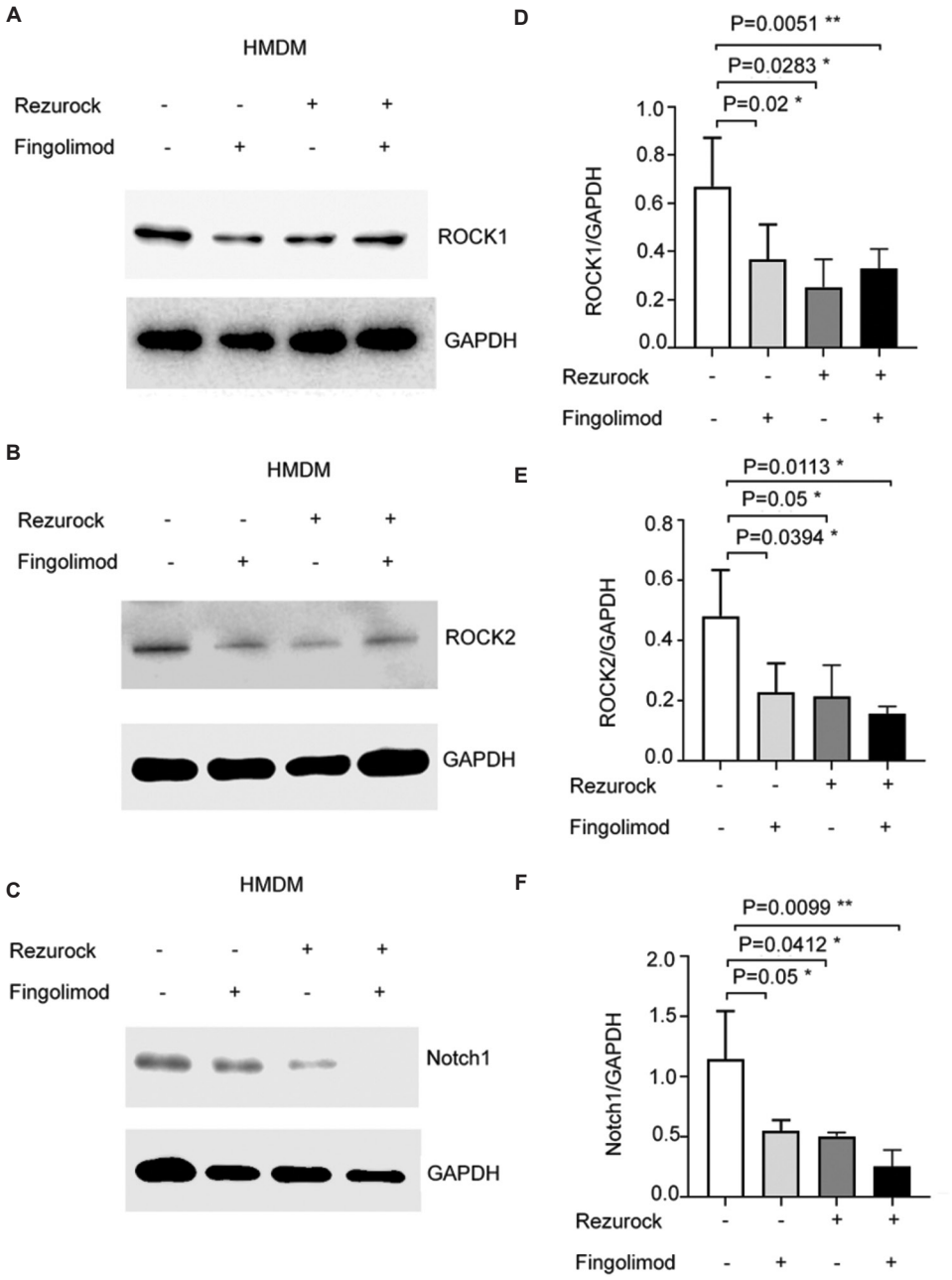

Figure 7. Effect of fingolimod and Rezurock treatment alone or in combination on fibrosis pathway-related protein expression in HMDM. (A-C) Western blots showing the expression of ROCK1, ROCK2, and Notch1, respectively. GAPDH was used as a loading control. (D-F) Graphical representations of three independent Western blot experiments corresponding to each protein.
Notes: *p≤0.05; **p≤0.01; ***p≤0.001.

response inhibitor, prevented macrophage infiltration into allografts, reducing vessel occlusion and fibrosis.[8] In addition, peritoneal macrophages treated with fingolimod exhibited downregulation of pathways involved in cell–cell adhesion and cellular defense mechanisms.[10,14] Rezurock, a selective ROCK2 inhibitor, was superior in inhibiting allograft fibrosis and suppressed pathways related to cell cycle progression, DNA replication, adaptive immune responses, and organelle assembly. Both drugs also shared commonly downregulated pathways associated with cytokine production and chemokine signaling.[10,12]

In our present and previous studies,[10] GO analysis revealed that Rezurock and fingolimod alone downregulated cell cycle- and immune-related pathways in macrophages.

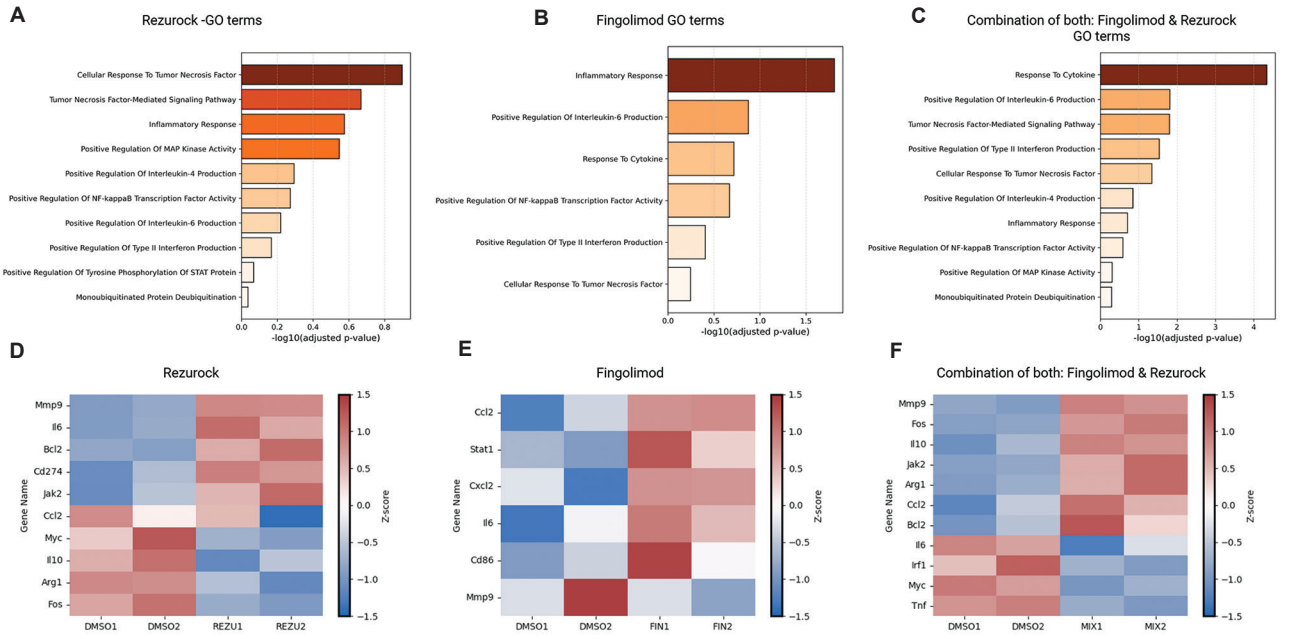

Figure 8. Stat3/Stat5-driven immune modulation by Rezurock, fingolimod, and their combination in macrophages. Gene Ontology enrichment analysis of Stat3/Stat5-related pathways among differentially expressed genes in macrophages treated with Rezurock (A), fingolimod (B), or their combination (C). Heatmaps showing the expression of selected Stat3/Stat5 downstream genes and inflammatory markers in macrophages treated with Rezurock (D), fingolimod (E), and the combined treatment (F).
Abbreviation: Stat: Signal transducer and activator of transcription.

Notably, Rezurock also downregulated actin assembly pathways, suggesting that it affects macrophage migratory properties, which are crucial for allograft infiltration.[10] These findings are supported by another research showing that ROCK2 downregulation reduces macrophage motility.[15] In addition, Rezurock was shown to inhibit TNF secretion and macrophage migration, thereby impeding liver fibrosis in mice.[16] The combination of Rezurock and fingolimod further downregulated genes related to cell cycle progression. Transcriptome analyses of patients with fibrotic lungs have shown that the most upregulated pathways in alveolar macrophages are associated with the mitotic cell cycle and migration.[17,18] Thus, by inhibiting macrophage proliferation, the combined treatment may contribute to reduced fibrosis. We also observed that the combined treatment upregulated signaling pathways broadly related to cellular homeostasis and protein quality control, such as protein processing in the ER and membrane trafficking. In addition to the significant downregulation of cell cycle-related genes, the combination treatment also downregulated profibrotic pathways such as extracellular matrix organization and collagen metabolic processes, as well as immune-related pathways involved in defense responses and cytokine production.

We validated our transcriptomic findings through western blot analysis. As expected, we observed downregulation of ROCK1 and ROCK2 after both single and combined treatments in RAW 264.7 cells and human monocyte-derived macrophages. In addition, we found reduced Notch1 expression following Rezurock or fingolimod treatment, with the combined Rezurock/fingolimod treatment further enhancing this effect in both cell types. The downregulation of Notch1 attenuates hepatic fibrosis by preventing M2 macrophage polarization, thus reducing the profibrotic activity of these cells.[19] Consequently, the combined treatment may slow fibrosis progression. We also observed significant downregulation of inflammation-related proteins, particularly PTX3, CCL2, and its receptor CCR2, after individual treatments. However, CCL2 expression was not significantly affected by fingolimod alone. The combined treatment further enhanced the downregulation of these proteins in RAW 264.7 cells. PTX3, primarily produced by macrophages in atherosclerotic lesions, is associated with chronic rejection characteristics such as inflammation, endothelial dysfunction, and vascular remodeling.[20,21] Elevated PTX3 expression has also been detected in bleomycin-induced fibrotic lungs, highlighting its involvement in fibrosis progression.[21]

CCL2 acts as a chemoattractant for monocytes and macrophages, recruiting them from the bone marrow to sites of inflammation. CCR2, predominantly expressed in leukocytes, monocytes, and macrophages, serves as

the primary receptor for CCL2.[22-24] Elevated CCL2 levels have been observed in lung biopsies from patients with idiopathic pulmonary fibrosis, while CCR2 has been implicated in promoting hepatic fibrosis in mice.[25,26] Our data suggest that the combined treatment effectively blocks the CCL2/CCR2 axis, preventing macrophage infiltration into the transplanted organ by reducing both the recruitment signals from macrophages within the allograft and the responsiveness of bone marrow-derived macrophages to these signals.

Finally, we found that both drugs, when administered individually, significantly reduced the expression of key profibrotic proteins, including collagen I and TGF-β, in RAW 264.7 macrophages. While the combined treatment downregulated collagen I to a similar extent as the individual treatments, it further enhanced the downregulation of TGF-β. Extensive collagen deposition is a well-established marker of fibrotic tissues,[27,28] whereas TGF-β secreted by macrophages promotes the fibroblast-to-myofibroblast transition.[29,30]

Current immunosuppressive regimens in solid organ transplantation are largely similar, with only minor adjustments based on organ-specific immune tolerance and risk of rejection.[31-34] The cornerstone therapy typically includes a calcineurin inhibitor (CNI), an antiproliferative agent, and corticosteroids, with some variation in induction therapy.[31-34] CNIs block IL-2 transcription by inhibiting calcineurin, thereby disrupting T-cell proliferation.[35] Antimetabolites suppress lymphocyte proliferation by inhibiting purine synthesis and thus DNA replication.[36] Corticosteroids exert broader effects by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cell activation, leading to reduced cytokine production, adhesion molecule expression, and antigen presentation.[37] However, because these regimens do not target the fibrotic activity of macrophages, they are effective for acute rejection but do not reduce or prevent chronic rejection.

It is worth noting that, beyond pharmacological interventions aimed at preventing transplant rejection, non-pharmacologic methods, such as the application of magnetic nanoparticles or magnetic devices, have emerged as promising novel approaches.[38-40] A recent study showed that functionalized magnetic nanoparticles could eliminate donor-specific antibodies (DSAs) from saline, blood, and plasma of healthy donors and sensitized patients. DSAs, such as antibodies directed against donor class I human leukocyte antigens (e.g., HLA-A), remain a major barrier to kidney transplant success.[41,42] Another possible approach is the magnetic manipulation of the macrophage actin cytoskeleton to prevent their infiltration into transplanted organs.[43-46]

5. Conclusion

We showed that combined treatment with FDA-approved ROCK2 inhibitors—Rezurock and fingolimod—reprograms macrophages isolated from mice and humans, shifting them from a highly proliferative, profibrotic state toward a less fibrotic phenotype with reduced activity. Our data also indicate that Rezurock, by being superior in preventing fibrosis and enhancing the effect of another ROCK inhibitor, represents a promising strategy for preventing chronic rejection, as it targets critical profibrotic pathways in macrophages. Furthermore, transcriptomic analysis revealed significant downregulation of cell cycle-related pathways, indicating that this combination therapy could benefit not only post-transplant patients but also individuals with autoimmune and chronic inflammatory disorders, where reducing macrophage proliferation is crucial for disease management.

We must recognize that in the future, pharmacologic intervention will probably not be the only option for alleviating organ rejection. With the advent of new technologies, such as magnetic nanoparticles that remove harmful antibodies or magnetic devices that affect immune cell trajectories, novel approaches may emerge for managing both acute and chronic rejection of transplanted organs.

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Conflict of interest

Malgorzata Kloc is Editor-in-Chief of this journal, but was not involved in any way, directly or indirectly, in the editorial and peer-review process for this paper. All other authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

All experiments were performed according to The Methodist Hospital Research Institute animal care and use standards, as outlined in the *Guide for the Care and Use of Laboratory Animals* (DHHS publication No. [NIH] 85-23 Revised 1985). The Institute also mandates concordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institute of Health *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*. The use of mice was approved by The Houston Methodist Research Institute Institutional Animal Care and Use Committee (IACUC; Protocol number #S00007095).

Consent for publication

Not applicable.

Availability of data

Data from this study are available from the corresponding author on reasonable request.

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ORIGINAL ARTICLE

Association between serum uric acid and prostate cancer risk: The modifying role of *CTGF* genotype

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Abstract

Background: The role of uric acid in prostate cancer risk remains uncertain, with evidence suggesting both carcinogenic and protective effects. Genetic factors may be key modifiers of this association. **Objective:** This study aimed to determine whether the relationship between uric acid and prostate cancer risk differs by the *rs9399005* genotype of connective tissue growth factor (*CTGF*). **Methods:** We examined 6,259 Japanese-American men in Hawaii, cancer-free at baseline (1965–1968, ages 45–68), who were followed for incident prostate cancer until 1999. Hyperuricemia was defined as serum uric acid ≥7.0 mg/dL. *CTGF* genotypes were classified as common allele homozygotes (CC) or minor allele carriers (T). Cox proportional hazards models estimated hazard ratios (HRs), adjusting for age and potential confounders. **Results:** During a median follow-up of 29.7 years, 285 prostate cancer cases were identified. A significant interaction between *CTGF* and hyperuricemia was observed. Among men with the *CTGF–T* genotype, hyperuricemia was not associated with risk (HR = 0.77, 95% confidence interval [CI]: 0.51–1.17). In contrast, among *CTGF–CC* homozygotes, hyperuricemia was linked to a higher risk (HR = 1.91, 95% CI: 1.21–2.99). Men with both the *CTGF–CC* genotype and hyperuricemia had a higher risk (HR = 1.72, 95% CI: 1.17–2.54) compared with all other subjects. **Conclusion:** The association between uric acid and prostate cancer varied by *CTGF* genotype. Hyperuricemia increased risk among *CTGF–CC* homozygotes, whereas a nonsignificant protective effect was seen among *T* allele carriers. **Relevance to patients:** Monitoring and lowering serum uric acid may help reduce prostate cancer risk in men with the *CTGF–CC* genotype.

Keywords: CTGF; Connective tissue growth factor; Uric acid; Hyperuricemia; Gene-environment interaction; Prostate cancer

1. Introduction

Prostate cancer ranks as the second most frequently diagnosed cancer and the fifth leading cause of cancer-related deaths among men worldwide, with an estimated 1.46 million new cases and 396,000 deaths reported in 2022.[1] Despite its high prevalence and substantial impact on health and quality of life, the underlying causes of prostate cancer remain largely unclear. Established risk factors include advancing age, family history, race or genetic predisposition, a Western diet, and alcohol consumption.[2] Identifying new, and particularly modifiable, risk factors and biomarkers is crucial for improving strategies for prevention, early detection, and treatment.

Uric acid, a by-product of purine metabolism, is a known biomarker of inflammation and can be modified by lifestyle changes.[3-5] It has been studied for its potential role in prostate cancer development.[6-15] However, its association with prostate cancer risk remains inconclusive. Some research suggests that elevated uric acid promotes chronic inflammation and oxidative damage, thereby facilitating tumorigenesis, and finds a positive association with prostate cancer risk.[6,7] Gout, a condition associated with hyperuricemia, has been reported to elevate the risk of prostate cancer.[8] Other studies propose that uric acid functions as an antioxidant, reducing oxidative stress and inflammation, both of which contribute to carcinogenesis, and report an inverse relationship between uric acid levels and prostate cancer incidence.[9-11] Yet other investigations found no significant correlation between uric acid levels and prostate cancer risk, implying that uric acid may not play a critical role in the pathogenesis of prostate cancer.[12-15]

One reason these studies have observed conflicting associations between uric acid and prostate cancer is their reliance on conventional epidemiological frameworks, which typically treat exposures and outcomes as having simple, linear, and independent relationships. Such frameworks typically overlook nonlinear dynamics, especially the interactive effects that contribute to the characterization of biological systems. To address this limitation, we propose employing an integrative modeling approach that simultaneously incorporates genetic susceptibility, uric acid levels, and their gene–environment interactions. This approach would capture more of the underlying complexity and potentially reveal subtler associations between uric acid and prostate cancer risk.

Connective tissue growth factor (CTGF), also known as cellular communication network factor 2 (CCN2), is a secreted protein associated with the extracellular matrix (ECM). CTGF interacts with multiple cell surface receptors, ECM components, and cytokines.[16] Transforming growth factorβ (TGFβ), a pleiotropic cytokine with contextdependent tumorsuppressive and tumorpromoting roles, is implicated in prostate cancer initiation and progression.[17,18] CTGF has been shown to modulate TGFβ signaling, thereby influencing prostate cancer pathogenesis.[19] Notably, TGFβ levels are elevated in hyperuricemic individuals and correlate positively with uric acid concentration.[20] Taken together, these observations suggest that CTGF may modulate the relationship between uric acid and prostate cancer development.

The aim of the present study was to determine whether the relationship between serum uric acid and prostate cancer incidence differs by *CTGF* genotype.

2. Methods

2.1. Study cohort

The Kuakini Japan-Hawaii Cancer Study (Kuakini-JHCS) is based on the Kuakini Honolulu Heart Program (Kuakini-HHP) cohort. The Kuakini-HHP Examination 1 was conducted between 1965 and 1968, recruiting 8,006 American men of Japanese ancestry aged 45–68 years, all of whom were residents of the Hawaiian island of Oahu. The Kuakini-JHCS was initiated during the third examination of the Kuakini-HHP cohort, conducted between 1971 and 1974 (n = 6,860; age range 51–75 years), when the cancer surveillance program was established.[21,22]

2.2. Definition of risk factors and potential confounders

All variables in the present study were measured during the Kuakini-HHP Examination 1.[23] The assay for serum uric acid (non-fasting) was performed using an automatic colorimetric method (Technicon AutoAnalyzer Methodology N-13b) with a phosphotungstic acid reagent. Further details can be found in our earlier publication.[24] Hyperuricemia was defined as a serum uric acid level ≥7.0 mg/dL (≥0.416 mmol/L) at baseline, while levels below this threshold were classified as normouricemia. To assess the association of uric acid concentrations with prostate cancer risk, we also categorized participants into quartiles (Q1-Q4) based on their serum uric acid concentrations. Gout, a condition associated with hyperuricemia, was self-reported at baseline.

Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared. Physical activity index (PAI) was quantified as metabolic output during a typical 24-h period by multiplying a weighting factor by the reported number of hours spent in five activity levels (no activity = 1.0, sedentary = 1.1, slight = 1.5, moderate = 2.4, and heavy = 5.0).[25] Smoking was categorized as either a never smoker or a smoker (including past or current cigarette smoking). Pack-years of cigarette smoking were computed for past and current smokers.

Alcohol intake was calculated based on self-reported usual monthly consumption of beer, wine (including Japanese saké [15% alcohol] and fortified wines [17–20% alcohol]), and spirits (including whiskey, gin, brandy, or other liquor) among current drinkers. The factors used to obtain estimates of alcohol content in all beverages consumed were 3.7% for beer, 10% for wine, and 38% for spirits.

Hypertension was defined as systolic blood pressure (BP) ≥140 mm Hg, diastolic BP ≥90 mm Hg, or the self-reported use of antihypertensive medications. Normal BP (normotension) was defined as systolic BP <140 mm Hg, diastolic BP <90 mm Hg, and not taking antihypertensive medication. The percentage of calories from animal protein was calculated using a 24-h dietary recall by dividing the calories from animal protein by the total calories. More detailed information can be found elsewhere.[26]

2.3. Genotyping

Among the 12 tagging single-nucleotide polymorphisms (SNPs) in *CTGF* that we tested in a previous case–control study for association with longevity, carriers of the minor (*T*) allele of rs9399005 had a significantly longer lifespan.[27] Therefore, rs9399005 was chosen as the SNP of interest for the present study.

Genotyping was performed using DNA extracted from the buffy coat of blood samples collected at Kuakini-HHP Examination 4 (1991–1993), and the samples were kept at –70°C. For participants who did not attend the Kuakini-HHP Examination 4, we used serum samples available from Examination 3. For the latter, DNA was amplified using a combination of QIAmp cell-free DNA isolation followed by REPLI-g Single-Cell WGA and WTA amplification (QIAGEN Sciences, Germantown, MD, USA). Genotyping was performed using TaqMan on an Applied Biosystems QuantStudio 12K Flex system (ThermoFisher Scientific, Waltham, MA, USA).

2.4. Ascertainment of prostate cancer

All incident cancer cases diagnosed between 1965 and 1999 were captured by the Kuakini-JHCS surveillance program. For cancer incidence among subjects who died before or did not participate in the Kuakini-JHCS examination (1971–1974), ascertainment was conducted retrospectively according to the criteria of the Kuakini-JHCS surveillance program when the cancer surveillance program began.[22] Prostate cancer incidence was determined by a physician consensus group using hospital records, tumor registry data, and confirmation through histological evidence. The Kuakini-JHCS surveillance program concluded on December 31, 1999.

2.5. Statistical analysis

Baseline characteristics were compared between subjects with and without hyperuricemia, and with different *CTGF* genotypes: common allele (*C*) homozygotes (genotype *CC*: termed *CTGF–CC*) and minor allele (*T*) carriers (genotypes *CT* or *TT*: termed *CTGF–T*), the latter having been found to be associated with longevity in our previous study.[27] Continuous variables were analyzed using Student's *t*-test, while categorical variables were compared using the χ^2 test.

Cox proportional hazard models were used to assess the association of hyperuricemia and *CTGF* genotype with prostate cancer. The main effects, hazard ratio (HR), and 95% confidence intervals (95% CI) of hyperuricemia and *CTGF* genotype on prostate cancer incidence were estimated using a multivariate Cox proportional hazard model that included hyperuricemia and/or *CTGF* genotype while adjusting for confounders. This model was referred to as the main effect model. The interaction effect of hyperuricemia and *CTGF* genotype was tested using a "full model," which extended the main effect model by including an interaction term between hyperuricemia and *CTGF* genotype. The goodness of fit between the full model and the main effect model was compared using likelihood ratio tests. Stratified analyses were conducted to assess the association between hyperuricemia and prostate cancer within each *CTGF* genotype. The Cox proportional hazard assumption was tested for the stratified Cox models. All tests were two-sided, and a $p<0.05$ was considered statistically significant. All statistical analyses were performed using the Statistical Analysis System version 9.4 (Cary, NC, USA).

3. Results

3.1. Baseline characteristics

From the 8,006 middle-aged men who participated in Kuakini-HHP Examination 1, we excluded 82 men with any type of cancer at baseline, 35 men without uric acid measurements, 1,399 men without *CTGF* rs399005 genotype data, and 231 men who self-reported having a history of gout at baseline. As a result, our analytical sample included 6,259 subjects. Over a median follow-up period of 29.7 years, 285 prostate cancer cases were identified from baseline to December 1999. Table 1 presents the baseline characteristics of subjects by hyperuricemia status and *CTGF* rs399005 genotype. Subjects with hyperuricemia were younger, less physically active, had a higher BMI, had a higher prevalence of hypertension, smoked more cigarettes, drank more alcohol, and had a higher dietary percentage of calories from animal protein intake. However, no baseline variables were associated with the *CTGF* genotype.

Table 1. Baseline characteristics by hyperuricemia status and *CTGF* rs9399005 genotype

Variables	Hyperuricemia status			*CTGF* rs9399005 genotype		
	Yes	No	*p*	*CTGF–CC*	*CTGF–T*	*p*
n	1410	4849		2245	4014	
Continuous variables, mean±SD						
Age (year)	53.5±5.3	54.0±5.4	0.0014*	54.0±5.4	53.9±5.4	0.53
BMI (kg/m^2)	25.0±3.1	23.5±2.9	<0.0001	23.9±3.1	23.8±3.0	0.19
Smoking (pack-years)	23.9±24.3	22.3±23.5	0.031	22.9±23.6	22.5±23.8	0.52
Alcohol intake (oz/month)	20.7±29.7	10.9±19.9	<0.0001	13.4±24.3	12.9±22.1	0.38
Physical activity index	32.4±4.5	33.0±4.5	<0.0001	32.8±4.6	32.9±4.5	0.84
Percentage of calories from animal protein	12.7±4.4	12.4±4.2	0.018	12.4±4.3	12.4±4.2	0.84
Uric acid (mg/dL)	8.0±0.9	5.4±1.0	<0.0001	5.9±1.4	6.0±1.5	0.14
Categorical variables, n (%)						
Smoking status						
Never smoker	431 (30.6)	1530 (31.6)	0.49†	694 (30.9)	1267 (31.6)	0.59
Ever smoker	978 (69.4)	3319 (68.4)		1551 (69.1)	2746 (68.4)	
Missing	1	0		0	1	
Alcohol drinking status						
Non-drinker	391 (27.8)	1886 (38.9)	<0.0001	805 (35.9)	1472 (36.7)	0.51
Drinker	1014 (72.2)	2958 (61.1)		1437 (64.1)	2535 (63.3)	
Missing	5	5		3	7	
Hypertension status						
Normotensive	700 (49.6)	3118 (64.3)	<0.0001	1343 (59.8)	2475 (61.7)	0.15
Hypertensive	710 (50.4)	1731 (35.7)		902 (40.2)	1539 (38.3)	
Missing	0	0		0	0	

Notes: *p-value from t-test for continuous variables; †p-value from χ^2 test for categorical variables.
Abbreviations: BMI: Body mass index; CTGF: Connective tissue growth factor.

3.2. Descriptive data for prostate cancer

Table 2 shows the age-adjusted incidence rates of prostate cancer by hyperuricemia status for the whole cohort and by *CTGF* genotypes. Among men having a *CTGF–T* genotype, hyperuricemia was associated with a lower incidence of prostate cancer compared to normouricemia (12.7 vs. 18.0 cases/10,000 person-years). In contrast, among men with the *CTGF–CC* genotype, hyperuricemia showed an increased prostate cancer incidence compared to normouricemia (25.5 vs. 17.5 cases/10,000 person-years). These data suggest that the association between hyperuricemia and prostate cancer incidence may vary depending on *CTGF* genotype.

Table 2. Age-adjusted incidence rates of prostate cancer (per 10,000 person-years) by hyperuricemia status in the overall cohort and stratified by *CTGF* genotype

Overall/Sub-cohort	Hyperuricemia	Normouricemia	*p*
Overall cohort			
n (cases)	1410 (60)	4849 (225)	
Incidence rate	17.2	17.8	0.99
CTGF–T			
n (cases)	922 (29)	3092 (146)	
Incidence rate	12.7	18.0	0.11
CTGF–CC			
n (cases)	488 (31)	1757 (79)	
Incidence rate	25.5	17.5	0.036

Abbreviation: CTGF: Connective tissue growth factor.

3.3. Main and interaction effects of hyperuricemia and *CTGF* genotype on prostate cancer

Table 3 presents the main and interaction effects of hyperuricemia and *CTGF* genotype on prostate cancer, estimated by: (i) Cox models adjusted for age only and (ii) multivariate Cox models further adjusted for BMI, smoking (pack-years), alcohol intake (oz/month), hypertension, PAI, and percentage of calories from animal protein. At the population level,

neither hyperuricemia (HR = 1.11, 95% CI: 0.82–1.51, p=0.49) nor *CTGF–CC* genotype (HR = 1.11, 95% CI: 0.87–1.42, p=0.39) was significantly associated with prostate cancer in the main effect models. However, the "full model" showed that interaction effects between hyperuricemia and *CTGF* genotype were statistically significant in both the age-adjusted model (p=0.0082) and the fully adjusted model (p=0.010), indicating that the association between hyperuricemia and prostate cancer varies across the *CTGF–CC* and *CTGF–T* genotypes. A significant likelihood-ratio test confirmed that including this interaction substantially improved model fit, making the "full model" the preferred basis for interpretation.

3.4. Association of hyperuricemia and uric acid levels with prostate cancer incidence stratified by *CTGF* genotypes

To illustrate the differing relationships between hyperuricemia, serum uric acid concentration, and prostate cancer risk according to *CTGF* genotype, we conducted genotype-stratified analyses. Table 4 presents HRs and 95% CIs for prostate cancer incidence, comparing: (i) individuals with hyperuricemia versus those with normouricemia, and (ii) participants in the higher serum uric acid quartiles (Q2-Q4) versus those in the lowest quartile (Q1), within each *CTGF* genotype. All estimates were obtained using Cox proportional hazards models adjusted for potential confounders.

Table 3. Main and interaction effects of hyperuricemia and *CTGF* genotype on prostate cancer incidence estimated using Cox models

Model	Main-effect model				Full model		Likelihood ratio test*
	Effect of hyperuricemia		Effect of *CTGF–CC* versus *CTGF–T*		Interaction		
	HR (95% CI)	*p*	HR (95% CI)	*p*	Coefficient	*p*	*p*
1	1.00 (0.75–1.33)	0.99	–	–	–	–	–
2	–	–	1.14 (0.90–1.44)	0.29	–	–	–
3	1.00 (0.75–1.33)	0.98	1.14 (0.90–1.44)	0.29	0.78	0.0082	0.0083
4	1.11 (0.82–1.50)	0.50	–	–	–	–	–
5	–	–	1.11 (0.87–1.42)	0.40	–	–	–
6	1.11 (0.82–1.51)	0.49	1.11 (0.87–1.42)	0.39	0.76	0.010	0.011

Notes: Model 1: Hyperuricemia, adjusted for age; Model 2: *CTGF* genotype, adjusted for age; Model 3: Hyperuricemia and *CTGF* genotype, adjusted for age; Model 4: Hyperuricemia, adjusted for age and confounders (BMI, smoking, alcohol intake, hypertension, physical activity, percentage of calories from animal protein); Model 5: *CTGF* genotype, adjusted for age and confounders; Model 6: Hyperuricemia and *CTGF* genotype, adjusted for age and confounders. *p-value of the test for goodness of fit between the full model and the main-effect model. The terms "main effect" and "interaction effect" refer to statistical associations and are not intended to imply causality.
Abbreviation: CTGF: Connective tissue growth factor.

Table 4. Association of hyperuricemia and quartiles of uric acid with prostate cancer incidence in the whole cohort and each *CTGF* genotype

Uric acid	Whole cohort		*CC* (*n*=2,245)		*CT* (*n*=2,977)		*TT* (*n*=1,037)		*T* (*n*=4,014)	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Hyperuricemia status										
Normouricemia (ref)	1		1		1		1		1	
Hyperuricemia (≥0.416 mmol/L)	1.11 (0.82–1.50)	0.50	1.91 (1.21–2.99)	0.005	0.69 (0.42–1.12)	0.13	1.06 (0.47–2.42)	0.88	0.77 (0.51–1.17)	0.22
Quartiles of uric acid (mg/dL)										
Q1 (0.7–5.0) (ref)	1		1		1		1		1	
Q2 (5.1–5.8)	1.05 (0.75–1.46)	0.79	0.99 (0.56–1.76)	0.97	1.17 (0.74–1.84)	0.51	0.83 (0.31–2.19)	0.70	1.08 (0.72–1.63)	0.72
Q3 (5.9–6.8)	1.08 (0.77–1.50)	0.66	1.05 (0.59–1.85)	0.87	1.10 (0.69–1.75)	0.69	1.12 (0.47–2.70)	0.80	1.08 (0.72–1.63)	0.71
Q4 (6.9–14.8)	1.11 (0.77–1.59)	0.57	2.01 (1.16–3.50)	0.014	0.66 (0.38–1.16)	0.15	0.97 (0.36–2.58)	0.95	0.73 (0.45–1.18)	0.20
p-value for homogeneity	0.94		0.025		0.21		0.94		0.34	

Notes: HRs and 95% CIs were estimated from multivariate Cox models, adjusted for age, BMI, smoking (pack-years), alcohol intake (oz/month), hypertension, physical activity index, and percentage of calories from animal protein.

Among men with the *CTGF–CC* genotype, hyperuricemia was significantly associated with an increased risk of prostate cancer compared to normouricemic individuals (HR = 1.91, 95% CI: 1.21–2.99, p=0.005). Within this genotype group, only those in the highest serum uric acid quartile (Q4) had a significantly elevated risk relative to Q1 (HR = 2.01, 95% CI: 1.16–3.50, p=0.014). In contrast, participants with the *CTGF–T* genotype showed nonsignificant inverse associations for hyperuricemia (HR = 0.77, 95% CI: 0.51–1.17, p=0.22) and for Q4 versus Q1 (HR = 0.73, 95% CI: 0.45–1.18, p=0.20).

3.5. Relative risk (RR) of prostate cancer for exposure groups defined by the *CTGF* genotype and hyperuricemia

Figure 1 illustrates the adjusted RR of prostate cancer among four exposure groups defined by *CTGF* genotype (*CTGF–CC* and *CTGF–T*) and hyperuricemia status. RRs were estimated using a multivariate Cox model adjusted for potential confounders, with men carrying the *CTGF–T* genotype and normouricemia (normal) serving as the reference group. Notably, individuals with both the *CTGF–CC* genotype and hyperuricemia exhibited the highest prostate cancer risk among all other exposure groups. Specifically, these individuals had a significantly increased risk (HR = 1.72, 95% CI: 1.17–2.54) compared to all other subjects.

4. Discussion

In this study, we first examined whether hyperuricemia (defined as uric acid ≥7.0 mg/dL) and the *CTGF* genotype were independently associated with prostate cancer incidence. Next, we assessed whether the *CTGF* genotype modified the association between hyperuricemia and prostate cancer by testing an interaction between the *CTGF* genotype and hyperuricemia. We found no overall association between hyperuricemia and prostate cancer incidence, consistent with previous studies using simple causeandeffect models.[12-15] The *CTGF* genotype alone likewise showed no independent effect on risk. However, a significant interaction between *CTGF* genotype and hyperuricemia (p=0.010), estimated from the multivariate "full model," indicated that the impact of elevated uric acid on prostate cancer varies by *CTGF2* genotype. Genotype-specific analyses revealed that among men homozygous for the common allele (*C*), hyperuricemia was associated with a 1.91fold increased prostate cancer risk compared to men with normouricemia. In contrast, among carriers of the minor allele (*T*), hyperuricemia exhibited a nonsignificant inverse association. These findings underscore the importance of stratifying analyses by *CTGF* genotype in future studies of uric acid and prostate cancer to avoid obscuring associations within specific subpopulations.

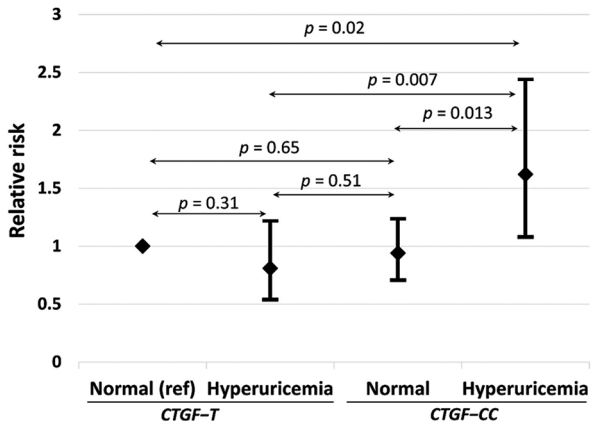

Figure 1. Relative risk of prostate cancer for the exposure groups defined by *CTGF* genotype and hyperuricemia status. The relative risks were estimated from Cox models adjusted for age, BMI, smoking (pack-years), alcohol intake (oz/month), physical activity index, and percentage of calories from animal protein; *CTGF*–T and normouricemia (Normal) were treated as the reference group.
Abbreviations: BMI: Body mass index; CTGF: Connective tissue growth factor.

Notably, neither hyperuricemia nor the *CTGF–CC* genotype alone was associated with prostate cancer risk (Table 3). However, individuals with both hyperuricemia and the *CTGF–CC* genotype exhibited an increased risk (Figure 1). This finding aligns with one of the gene-environment interaction scenarios described by Ottman,[28] where the simultaneous presence of a genetic variant and an environmental factor is required to raise disease susceptibility.

An earlier publication from our group indicated that uric acid was associated with the risk of prostate cancer during the first 10 years of follow-up, but not thereafter.[24] In contrast, the present study's tests of the Cox proportional hazards assumption for hyperuricemia, conducted within each *CTGF* genotype, indicated that the HR for prostate cancer associated with hyperuricemia remained constant throughout the follow-up period.

Our findings have significant clinical relevance. We recommend that clinicians consider genetic testing for *CTGF* in men with hyperuricemia and provide personalized recommendations to mitigate prostate cancer risk. For instance, in men homozygous for the *CTGF* rs9399005 common allele (*C*), lifestyle modifications (such as reducing red meat and sugar consumption, quitting smoking, limiting alcohol intake, and increasing physical activity) should be strongly encouraged, alongside pharmacological interventions such as urate-lowering therapy.

Uric acid is known to exert both antioxidative and pro-inflammatory effects in cancer development. Based on our

observations, we propose a new hypothesis: the impact of uric acid on prostate cancer may be modulated by *CTGF* genotype. In men homozygous for the *CTGF* common allele (*C*), uric acid may act as a pro-inflammatory agent, potentially increasing the risk of prostate cancer. In contrast, among carriers of the *CTGF* minor allele (*T*), uric acid may exert antioxidative effects, serving as a protective factor, or have no significant impact on prostate cancer risk. Antioxidants that can react with molecular oxygen and are reducing agents can act as prooxidants in the event that they become overloaded.

Figure 2 illustrates our interpretation of the effects of the *CTGF* gene on the tumorigenesis of prostate cancer. Soluble uric acid (a metabolic damage-associated molecular pattern, DAMP) activates the NLRP3 inflammasome,[29] causing interleukin-1 beta (IL-1β) release and reactive oxygen species (ROS) generation, which in turn converts latent TGF-β to its active form. Active TGF-β signals through SMAD2/3 to induce *CTGF* (*CCN2*) transcription.[30,31] CTGF is a matricellular factor that strongly induces fibroblast activation (cancer-associated fibroblast, CAF) and ECM deposition and has been implicated in promoting tumor angiogenesis.[32] These stromal changes create a reactive tumor microenvironment (with CAFs, dense ECM, and new vessels) that fosters prostate cancer cell motility and metastatic spread.

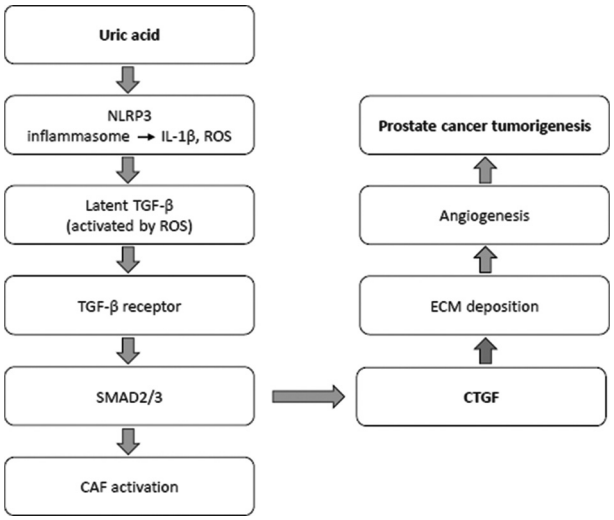

Figure 2. Proposed pathway diagram linking uric acid to *CTGF* expression and prostate cancer risk through TGF-β/SMAD signaling in the tumor microenvironment. Soluble uric acid (a metabolic DAMP) activates the NLRP3 inflammasome, causing IL-1β release and ROS generation, which in turn converts latent TGF-β to its active form. Active TGF-β signals through SMAD2/3 to induce *CTGF* (*CCN2*) transcription. CTGF is a matricellular factor that strongly induces fibroblast (CAF) activation and ECM deposition and has been implicated in promoting tumor angiogenesis. These stromal changes create a reactive tumor microenvironment (with CAFs, dense ECM, and new vessels) that fosters prostate cancer cell motility and metastatic spread. Experimental studies and reviews describe UA as an inflammasome-activating DAMP, TGF-β/SMAD-driven induction of CTGF, and CTGF's roles in fibrosis and angiogenesis. The pro-metastatic effects of CTGF in prostate cancer are supported by reports of enhanced prostate cancer cell migration and bone metastasis. Preclinical efficacy of TGF-βR inhibitors in prostate cancer models is documented. All pathways and interventions shown are grounded in these references.
Abbreviations: CAF: Cancer-associated fibroblast; CTGF: Connective tissue growth factor; DAMP: Damage-associated molecular pattern; ECM: Extracellular matrix; IL-1β: Interleukin-1 beta; ROS: Reactive oxygen species; TGF-β: Transforming growth factor beta.

CTGF plays an integral part in maintaining stem cell niches for hematopoietic stem cells,[33] osteoblasts,[34] and mesenchymal stem cells.[35] We propose that the longevity variant of *CTGF*, rs9399005 (*T*), maintains a healthy stem-cell niche without the toxic environment that would otherwise promote carcinogenesis and malignancy. The longevity variant (*T*) of rs9399005 is predicted to increase the binding of the transcription factor SRF that stimulates both cell proliferation and differentiation.[36] This enhanced binding may contribute to healthier tissue renewal and resistance to oncogenic stress. There should be further research, including studies using cell lines and animal models, to validate this hypothesis and establish a theoretical foundation for developing targeted prevention and treatment strategies.

Several limitations of this study should be acknowledged. The current study was restricted to American men of Japanese ancestry, necessitating replication in other racial groups to validate our findings. In addition, as with other cohort and longitudinal studies involving consecutive examinations, participants who completed the Kuakini-HHP Examinations 3 and 4 (where blood samples were collected for genotyping) were generally healthier than those who did not participate, as noted in a previous publication.[37] Another limitation was the availability of only a single uric acid measurement in this cohort, preventing us from assessing the longitudinal impact of uric acid on prostate cancer incidence.

A key strength of our study is that all participants underwent the same risk factor assessments and were monitored using a standardized surveillance protocol for outcomes. In addition, the large cohort size and long follow-up period further strengthen our findings. The American men of Japanese descent studied were particularly unique, as the genetic homogeneity of Japanese populations is higher than that of most other racial groups.[38] In general, Asian populations exhibit a greater degree of linkage disequilibrium between SNPs, which enhances the identification of genotype-disease associations.[39] Moreover, to the best of our knowledge, our hypothesis has not been tested previously. Furthermore,

our surveillance system was highly comprehensive, supported by the fact that our study was conducted in an island population, which allowed for meticulous follow-up.

5. Conclusion

In this population-based cohort study, we found that the effect of serum uric acid on prostate cancer incidence, whether protective or harmful, varies according to an individual's *CTGF* rs9399005 genotype. Men who were homozygous for the *CTGF* common allele (*C*) and had hyperuricemia exhibited the highest risk of prostate cancer compared to other exposure groups. These findings suggest that lowering uric acid levels in this subgroup may help reduce prostate cancer risk. Further research is needed to confirm these results.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Randi Chen
Investigation: Randi Chen, Timothy A. Donlon
Methodology: Randi Chen, Kamal H. Masaki
Writing–original draft: Randi Chen
Writing–review & editing: All authors

Ethics approval and consent to participate

This prospective study was conducted in the Department of Research, Kuakini Medical Center, and was approved by the Institutional Review Board of Kuakini Medical Center (#18-02). Written informed consent was obtained from participants or, when they were unable, from their families or caregivers, for clinical examinations, procedures, and access to medical records for disease surveillance.

Consent for publication

All participants provided written consent for publication of their de-identified data at each Kuakini-HHP examination.

Availability of data

Data used in this work are available from the corresponding author or Dr. Kamal H. Masaki (km1@hawaii.rr.com) upon reasonable request.

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SHORT COMMUNICATION

Prospective evaluation of the adapted Ontario Protocol Assessment Level score for predicting clinical research coordinator workload: An internal validation study

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Abstract

Background: The escalating complexity of clinical trial protocols has considerably increased the workload for research coordinators, exacerbating staffing shortages and contributing to operational inefficiencies. These challenges are particularly pronounced at under-resourced and minority-serving research institutions, where limited capacity may hinder the implementation of trials. Early and accurate estimation of research coordinator effort is essential for effective planning, resource management, and successful clinical trial conduct. **Aim:** This study assesses the accuracy of an adopted Ontario Protocol Assessment Level (OPAL) score in predicting coordinator workload to improve operational planning in clinical research. **Methods:** A prospective observational study was conducted over a 12-month period at a Historically Black College and University medical school. Seven coordinators recorded hours for seven actively enrolling interventional trials. Estimated workloads were calculated using a published, adapted OPAL reference table, and were compared with actual hours using descriptive statistics and paired *t*-tests. To ensure consistent benchmarking, workday equivalencies (7.5 h for institutional standards and 8 h for industry standards) were applied. **Results:** There was no statistically significant difference between estimated and actual hours, with an average difference of 24.1 h (p=0.761). The mean absolute error was 167.0 h, equivalent to roughly 1 month of full-time work. **Conclusion:** The adapted OPAL score provides a practical tool for estimating coordinator workload and aligning staffing with protocol complexity, including in under-resourced settings. However, broader multi-site validation is required to confirm its generalizability and to support its integration into feasibility planning. **Relevance for patients**: Accurate workload forecasting enhances trial efficiency, supporting timely, high-quality studies, and accelerating access to new treatments.

Keywords: Workload estimation; Ontario Protocol Assessment Level score; Clinical trial operations; Research coordinator workload; Protocol complexity; Implementation science; Workforce planning; Coordinator staffing models

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1. Introduction

The increasing complexity of clinical trial protocols has significantly amplified the demands placed on research coordinators, who serve as the operational backbone of study implementation. These professionals are responsible for a wide range of critical tasks, including regulatory compliance, patient engagement, data collection, adverse event reporting, and visit schedule adherence, all of which have become more time-consuming and resource-intensive. As protocols become more intricate, workload imbalances among coordinators are becoming increasingly common, contributing to elevated stress, burnout, and staff turnover.[1-5] These challenges are further exacerbated by staffing shortages and funding limitations at many academic and community-based research sites.

Accurately estimating research coordinator effort is essential for informed decision-making around staffing, resource allocation, and study feasibility. Effective planning depends on the ability to forecast the operational and administrative complexity of a study before it begins.[6,7] Without reliable workload prediction models, sites risk under- or over-allocating personnel, potentially compromising compliance and performance.

The Ontario Protocol Assessment Level (OPAL) score was previously developed to quantify protocol complexity by assigning numerical values to objective trial characteristics such as intervention type, number of study procedures, and frequency of patient visits.[8] While the OPAL score has gained broad adoption as a baseline tool, it has limitations when used in isolation. Specifically, it does not account for site-level operational variables that meaningfully influence workload, such as brief recruitment windows, complex specimen handling requirements, language barriers, or high-intensity data queries and monitoring activities. To address these limitations, it is recommended that the score be adapted by reweighing existing elements and incorporating additional workload drivers.[8,9]

Tyson *et al.*[9] developed an adapted OPAL score that integrates supplemental complexity indicators and links the score to observed coordinator effort using retrospective data from a diverse portfolio of clinical trials. Their analysis revealed a strong linear relationship between the adapted OPAL score and actual hours worked by coordinators ($\beta = 77.22$; $p=0.01$; $R^2 = 0.78$), resulting in a practical reference table for estimating staff effort during trial planning. However, this tool has not yet been validated.

Validation of workload estimation tools is critical in confirming their utility, accuracy, and generalizability across research settings. A validated tool provides measurable confidence that its estimates reliably reflect the construct being assessed—in this case, the Clinical Research Coordinator (CRC) effort. According to Streiner *et al.*,[10] validation requires demonstrating that tool-derived predictions align with observed outcomes, ideally across multiple contexts and study designs. For tools predicting operational metrics, prospective validation enhances external validity by assessing real-time workflows rather than relying solely on retrospective analyses.[11,12]

In this study, prospective validation methods are applied to evaluate the adapted OPAL score by comparing its predicted coordinator workload against actual hours logged across seven interventional trials at a single site. This approach aligns with best practices in validating prediction models and workload estimation frameworks.[13-15]

2. Materials and methods

This prospective observational study was conducted at an academic clinical research site to evaluate the accuracy of the adapted OPAL score in predicting research coordinator workload. Between January 01 and December 31, 2024, seven CRCs tracked the hours they spent managing seven actively enrolling interventional trials. The selected studies varied in sponsor type (industry-sponsored vs. federally funded) and intervention type (drug vs. behavioral).

The adapted OPAL score was calculated for each trial based on predefined criteria, including procedural volume, visit intensity, monitoring requirements, and biospecimen complexity. Estimated coordinator workload hours were then derived using a previously published reference table developed by Tyson *et al.*,[9] which was constructed from retrospective coordinator time-tracking data collected across a range of experience levels. This approach was intended to produce weights representative of overall coordinator effort rather than a single experience stratum. The reference table maps OPAL score tiers to predicted effort (Table 1).

Each CRC prospectively logged actual hours worked per protocol using a standardized digital time-tracking system. Data were reconciled weekly to ensure completeness and accuracy. Estimated workload hours were also converted into workday equivalents using both a 7.5-h academic standard and an 8-h industry standard. This conversion was performed solely to facilitate cross-sector benchmarking. Such conversions are common in resource planning models, allowing institutions to interpret workload estimates in the context of their operational norms.

2.1. Statistical analysis

To assess the agreement between the estimated and actual workload, descriptive statistics–including mean absolute

error (MAE) and mean difference–were calculated, consistent with early-stage predictive model validation practices.[16] A paired Student's *t*-test was used to assess differences between estimated and actual hours, as both values were generated from the same coordinator-study pairing. Unpaired Student's *t*-tests were used for subgroup analyses (i.e., sponsor type and intervention type) to compare mean values between independent groups.[17]

These methods enable both absolute and relative evaluations of prediction accuracy, highlighting areas for potential refinements of the adapted OPAL score. Workday equivalencies were computed using 7.5-h institutional and 8-h industry standards. This adjustment ensures consistency when comparing internal workloads to external benchmarks. *p*-values were not calculated for individual trials because each trial's estimated and actual values represent a single paired observation for the entire 12-month period, making statistical significance testing at the trial level mathematically inappropriate. The reported *p*-value for the "estimated versus actual comparison" was calculated using the aggregated paired dataset across all trials, allowing for appropriate variance estimation.

This study did not require Institutional Review Board approval, as it was classified as a quality improvement and operational research initiative aimed at enhancing internal clinical trial management processes. No identifiable private information was collected, and the project was not considered human subjects research.

3. Results

The seven interventional trials included in this study consisted of five Phase 3 trials, one Phase 2/3 hybrid trial, and one Phase 2 trial, spanning a range of therapeutic areas.

Table 1. Reference table for the adapted Ontario Protocol Assessment Level score

Adapted Ontario Protocol Assessment Level score	Estimated hours (6-month period)	Estimated hours per month
5.5	30.7	5.1
6.0	69.3	11.5
6.5	107.9	18.0
7.0	146.5	24.4
7.5	185.1	30.9
8.0	223.7	37.3
8.5	262.3	43.7
9.0	301.0	50.2
9.5	339.6	56.6

Note: Estimated coordinator workload hours were calculated using the adapted Ontario Protocol Assessment Level reference table previously published by Tyson *et al.*[9]

The MAE between the adapted OPAL-based estimated workload and the actual coordinator hours logged for the 12-month study period was 167.0 h, equivalent to approximately 22.3 workdays (4.5 weeks or 1.0 month) using a 7.5-h institutional workday and 20.9 workdays (4.2 weeks or 1.0 month) using an 8-h industry-standard workday. Despite this variability, the average difference between estimated and actual hours across all trials was relatively modest at 24.1 h and was not statistically significant (t = 0.32, p=0.761). This difference represents approximately 7–8% of a full-time coordinator's annual effort. A detailed summary is provided in Table 2 and Figure 1.

When analyzed by sponsor type, industry-sponsored trials required more coordinator time, with an average of 422.8 actual hours compared to 246.0 h for federally funded trials. This difference indicated a trend toward significance (t = −2.06, p=0.095), suggesting that sponsor type may be influential in predicting coordinator burden. Although industry trials demonstrated slightly higher average adapted OPAL scores, the difference was not statistically significant (Figure 2). No substantial differences between drug and behavioral intervention trials were observed

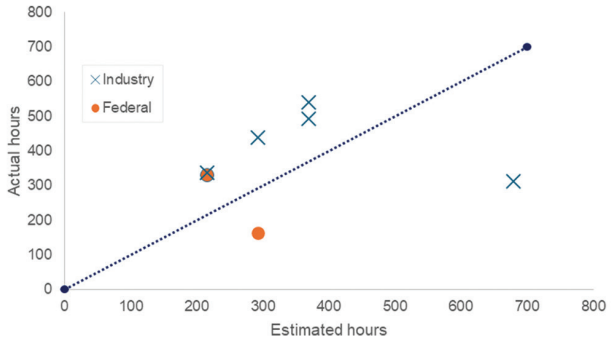

Figure 1. Estimated versus actual hours over a 12-month period

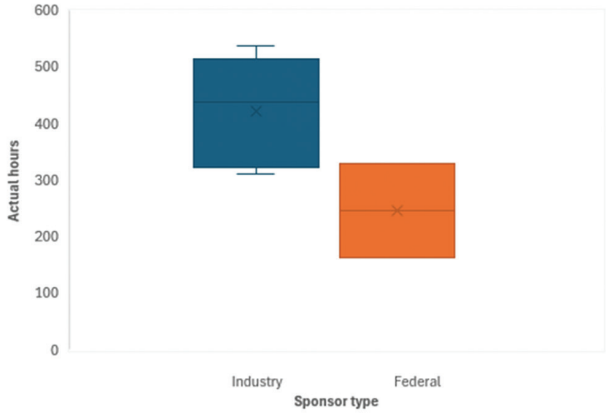

Figure 2. Comparison of actual coordinator hours by sponsor type

Table 2. Summary of study characteristics and results by protocol

Trial number	Adapted OPAL score	Trial phase	Sponsor type	Intervention	Estimated hours[a]	Actual hours	Difference
1	7.5	3	Industry	Drug	370.2	538	167.8
2	6.5	3	Industry	Drug	370.2	492	121.8
3	7.0	2/3	Industry	Drug	293.0	438	145.0
4	9.5	3	Industry	Drug	679.2	310	−369.2
5	6.5	3	Federal	Behavioral	215.8	330	114.2
6	6.5	3	Industry	Drug	215.8	336	120.2
7	7.0	2	Federal	Drug	293.0	162	−131.0

Note: [a]Hours were estimated for 12 months.
Abbreviation: OPAL: Ontario Protocol Assessment Level.

in estimated or actual coordinator hours. However, interpretation of these comparisons is limited by the small number of trials and the limited behavioral studies in the dataset.

These findings suggest that the adapted OPAL score is a promising tool for estimating workload, particularly in later-phase trials characterized by diverse and complex operational requirements. No statistically significant difference was observed between the estimated and actual hours, which may be due to the small sample size. Because the MAE reflects a 12-month study period, even if the difference was robust in larger samples, it would remain relatively small, representing merely 7–8% of the annual CRC workload. Variability in prediction accuracy across trials suggests the tool may benefit from further refinement to account for study-specific factors.

4. Discussion

This study builds on the foundational work of Tyson *et al.*,[9] who introduced the adapted OPAL score as a tool for estimating CRC effort. Unlike the original retrospective analysis, the present study utilizes the tool prospectively, demonstrating its real-time utility for operational planning and staffing allocation. Future multi-center validation studies should consider stratifying results by coordinator experience or including experience as a covariate in predictive modeling to further enhance predictive accuracy.

The findings indicate that the adapted OPAL score can predict coordinator effort with a high degree of accuracy. No statistically significant difference was observed between the estimated and actual hours, and the MAE reflected a manageable variance of approximately 8%, equivalent to less than one month of full-time work. Although absolute differences in hours were sometimes large for individual studies, the percentage variance was modest, thereby supporting the adapted OPAL's utility for budget and staffing estimates. Future multi-site, prospective time-tracking could be used to refine the weighting system further and reduce variability, particularly for protocols with high operational complexity or atypical team structures. These findings highlight the value of the tool in helping research sites anticipate and manage staffing needs, potentially reducing the risk of understaffing, missed milestones, and staff burnout. As pressure mounts for research operations to become more efficient, particularly in light of proposed reductions in administrative cost allowances for grants, accurate workload estimation and budgeting will become increasingly critical for maintaining operational sustainability.

By providing workload estimates in both 7.5-h (academic) and 8-h (industry) workday equivalents, the score enhances its practical relevance for a broad range of stakeholders, including academic health centers, contract research organizations, and community-based research institutions. This flexibility ensures that operational estimates remain meaningful regardless of institutional norms, improving cross-site comparability and planning.

By quantifying protocol complexity and translating it into time-based estimates, the adapted OPAL score addresses a key limitation in traditional trial feasibility practices, which often rely on subjective judgment or historical precedent.[7,18,19] The adapted OPAL score allows for a more data-driven and scalable approach to workload forecasting and supports a more efficient staffing model for better resource alignment and enhanced financial sustainability. The adapted OPAL builds on a methodology designed to include both protocol-driven and ancillary activities; however, some unstructured tasks will inevitably remain unmeasured. Pairing OPAL estimates with periodic portfolio-level reviews and integration into a Clinical Trial Management System (CTMS) can help identify emerging or unaccounted workload, enabling mid-course staffing adjustments.

Further tool development could include integration with CTMS, allowing real-time updates to workload projections in response to evolving study demands. This dynamic approach would improve operational agility and allow research teams to respond proactively to mid-study shifts, such as protocol amendments and accelerated recruitment timelines.

In addition, the observed trend toward higher coordinator burden in industry-sponsored trials, though not statistically significant, aligns with findings from prior studies that suggest increased operational complexity in commercially funded studies.[20] These trials often include more rigorous documentation requirements, frequent monitoring visits, and a higher frequency of protocol amendments, all of which demand greater coordinator time. These findings highlight the importance of adapting workload estimations to sponsor characteristics during site-level planning.

Several limitations remain in this study, including the small sample size and single-site design, which limit generalizability. The limited number of federally funded trials in the sample (n = 2, one of which was behavioral) created an imbalance in sponsor representation, constraining the ability to assess whether the adapted OPAL tool's predictive accuracy differs meaningfully between sponsor types. Future multi-site studies across diverse trial portfolios are needed to further validate the tool and assess its impact on trial performance metrics. With broader validation, the adapted OPAL score could be integrated into feasibility review workflows, budget justification tools, and institutional staffing frameworks. Future research should explore whether improved workload forecasting correlates with enhanced study outcomes, including faster recruitment, fewer protocol deviations, and improved data quality. A multi-site evaluation would strengthen generalizability and enable the development of benchmarking tools to compare coordinator efforts across institutions.

Importantly, this study was conducted at a medical school of a historically Black College and University—a community-based, minority-serving, and under-resourced institution. As such, it provides critical insight into the operational realities of underrepresented research sites. These institutions often carry a disproportionate operational burden and systemic barriers to trial participation and sustainability.[21,22] The successful application of the adapted OPAL score in this context highlights its potential as an equitable, scalable tool for supporting workload planning and staffing decisions. These adaptations were intentionally designed to address the disproportionate operational burden and systemic barriers faced by similar sites, and future iterations could incorporate additional site-specific modifiers for broader applicability. Broader implementation of such models may help reduce disparities in site performance, build long-term research capacity, and promote workforce sustainability, particularly in settings vital to expanding clinical research access to underserved populations.

5. Conclusion

When applied at the outset of a clinical trial, the adapted OPAL score offers a reliable, evidence-based method for forecasting coordinator workload and aligning staffing needs with protocol complexity. By translating study requirements into projected full-time equivalent allocations, the tool supports more informed feasibility assessments, facilitates sponsor-site negotiations, and improves operational readiness. Early estimation of effort also enables proactive staffing and budget forecasting, which are critical elements of efficient and sustainable trial execution.

Importantly, no statistically significant difference was observed between the estimated and actual hours, thereby supporting the adapted OPAL score's accuracy. The MAE of 167.0 h (approximately 1 month of full-time work) provides a practical benchmark for staffing calibration based on institutional norms. However, these results should be interpreted in the context of the study's limited sample size and single-site design. Further validation across institutions, trial phases, and therapeutic areas is needed to strengthen the generalizability and support integration of the tool into feasibility planning workflows.

This study was conducted at a medical school of a historically Black College and University, a community-based, minority-serving, and under-resourced institution. The successful application of the adapted OPAL score in this setting underscores its practicality and relevance for research sites facing systemic barriers and operational constraints. Broader adoption of this tool could help reduce disparities in trial performance, improve infrastructure, and promote workforce sustainability in underserved settings.

With continued refinement and integration into CTMS, the adapted OPAL score could serve as a standard tool for feasibility reviews, budget planning, and staffing models. Its application may ultimately enhance trial efficiency, support research staff, and promote equity in clinical research.

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Conflict of interest

The authors declare that they have no competing interests.

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Writing–original draft: All authors
Writing–review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author on reasonable request.

Further disclosure

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