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Contents

Reviews

Establishing new boundaries for medical liability: The role of AI as a decision-maker

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Abstract

The introduction of artificial intelligence (AI) in healthcare has created novel challenges for the field of medical malpractice. As healthcare professionals increasingly rely on AI in their decision-making processes, traditional medicolegal assessments may struggle to adapt. It is essential to examine AI's role in clinical care – both its current applications and future advancements – to clarify accountability for diagnostic and therapeutic errors. Clinical decision support systems (CDSSs), in particular, unlike other traditional medical technologies, work as co-decision makers alongside physicians. They function through the elaboration of patient information, medical knowledge, learnt patterns, etc., to generate a decision output (e.g., the suggested diagnosis), which should then be evaluated by the physician. In light of the AI Act, CDSSs cannot function fully autonomously, but instead physicians are to be assigned an oversight role. It is questionable, however, whether it would always be appropriate to assign full responsibility, and consequently liability, to the physician. This would be especially true if oversight is limited to reviewing outputs generated by the CDSS in a manner that leaves no real control in the hands of the physician. Future research should aim to define clear liability allocation frameworks and design workflows that ensure effective oversight, thereby preventing unfair liability burdens.

Key words: medical malpractice, liability, legal medicine, lawsuit, artificial intelligence

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Highlights

- AI in healthcare upends traditional medicolegal liability frameworks, raising questions about fault attribution for AI-driven diagnostic and treatment errors.
- Clinical decision support systems (CDSSs) serve as co-decision makers, producing recommendations that – under the AI Act – require explicit physician oversight.
- Assigning sole liability to physicians is increasingly inappropriate, as CDSS involvement diminishes clinicians' direct control over care decisions.
- Clearly defining liability allocation and establishing oversight workflows is essential to enable effective clinician governance of AI tools and to avoid unfair liability exposures in AI-augmented clinical practice.

Introduction

The issue of medical malpractice has long been recognized and continues to proliferate across countries, imposing considerable costs and burdens on healthcare systems and personnel.[1] Medical liability is typically a fault-based system, where, in the event of harm, a healthcare professional may be held liable if their actions are found to deviate from the accepted standard of care. The primary focus is determining whether the provider's actions conformed to the established standard of care. In similar cases, courts generally rely on experts who, depending on the national system, may be either medical professionals from the relevant specialty and/or medicolegal experts. These experts are tasked with advising the court, often through written opinions or courtroom testimony, on whether the healthcare provider's actions aligned with or fell short of established medical norms and guidelines.

Indeed, legal medicine, as an expert branch of contemporary medicine, is dedicated to assessing cases of alleged professional liability among healthcare providers, according to codified and standardized methodologies.[2] This aligns with the evolution of several subdisciplines of legal medicine that address specific concerns, continually striving to adapt to the ever-evolving context in which they operate.[3,4] Nevertheless, this progress remains uneven, as evidenced by the absence of dedicated specialties that rigorously address urgent contemporary issues, such as the medicolegal implications of deploying artificial intelligence (AI), which have received scant attention to date.[3] This represents a critical aspect that will progressively develop. Historically, healthcare professionals have relied on guideline recommendations and updated article databases. This is motivated by the desire to comply with the "golden standard" of evidence-based medicine, which ensures a higher level of healthcare quality and consequently, better health outcomes for the patients.[5] Physicians are also further incentivized to follow such guidelines as this presents the opportunity to lower the risk of incurring possible medicolegal disputes. In the future, they are thus likely to increasingly rely on decision-making support facilitated by AI, potentially to distill the vast amount of evidence available on various topics within applied medicine, including guidelines from scientific societies.[6] However, this evolution entails an additional dimension of healthcare professional responsibility, which healthcare professionals may not be adequately equipped to acknowledge and address. The integration of AI into routine clinical practice is proving to be profoundly transformative, prompting a multitude of complex and nuanced inquiries. AI-based diagnostic tools offer numerous benefits for medical practices, including the potential to achieve a uniform standard of healthcare services,[7] to monitor the activities of less experienced physicians,[8] etc. At the same time, though, they also raise ethical and legal issues, concerning, e.g., the possible misuse of patient data.[9] Specifically, in instances where a diagnostic, or in future also therapeutic, error is committed by a physician, based on recommendations provided by AI, the determination of legal liability becomes a critical question. After identifying the error and establishing its causal link to quantified harm, the issue arises as to who bears legal responsibility.[2] The medicolegal assessment, grounded in established evaluation methodologies and criteria, traditionally focuses on the decision-making processes of the healthcare professional. A pertinent question emerges: Would reliance on AI decisions, which turn out to be erroneous and cause harm to the patient, lead to liability for the healthcare provider?[10] This issue is highly contentious, and scholarly debate on the topic is both extensive and varied.

As AI infiltrates more aspects of life, the issues surrounding its implementation in work processes loom over the general enthusiasm. This can be illustrated through the simple, fearsome, and popular question of: "What will be the role of AI?". In the field of healthcare, AI-powered clinical decision support systems (CDSSs) are meant to assist physicians.[11] The types of decisions that CDSSs can help with can vary vastly, but some of the most significant and crucial decisions are those that physicians perform in the context of clinical care in single-patient consultations. What CDSSs can provide support in, e.g., is deciding what diagnosis to give the patient and what treatment to prescribe.[12] While current CDSS uptake is low,

the latest developments in AI methodologies are likely to increase implementation. The first attempts at CDSSs were based on knowledge modeling (e.g. Mycin, developed in the 70s, was a rule-based system that aided in antibiotic selection),[13] but new machine learning (ML) applications, including large language model (LLM)-based systems, seem to hold greater potential for popularity.[14,15] As CDSSs become increasingly available and relied on for clinical decision-making, the specific perimeters of the physician's role, and consequently the precise boundaries of liability, become more and more blurry.

The differences with other non-AI medical technologies

At first glance, one might believe that CDSSs are just providing information, not unlike previously existing medical technologies, and that it is the physician who is the ultimate decision-maker, and, consequently, the party held liable. The CDSSs, though, cannot be fully compared to other medical technologies. For example, if we consider X-rays, ECGs, etc., the information they provide is limited to medical data on the patient. In this sense, these tools are just "capturing" the state of the patient. This knowledge is then entirely elaborated upon by the physician, and it helps inform decision-making among a variety of other information sources. The CDSSs go a step further: They do not capture patient information, they elaborate on it. More specifically, CDSSs combine medical information about the patient with medical knowledge and provide a recommendation to the physician, which could potentially be followed without any need for further reasoning.[16] In the case of machine-learning CDSSs (ML CDSSs), they might even follow logic that falls beyond existing medical knowledge, e.g., ML CDSSs could diagnose breast cancer with great accuracy, and follow an inferred logic that is not fully understandable to physicians.[17,18] In light of all these reasons, CDSSs are not just like any other medical technology. They shall be considered decision-makers that work alongside physicians and provide them with fully elaborated and potentially definitive recommendations for diagnosis and/or treatment.[10] When physicians use a CDSS, clinical decisions emerge from a collaboration between the physician and the AI system. It would seem unreasonable to place the entirety of the liability burden on the physician when they are not the only entity responsible for the decision.

It must also be considered that CDSSs can be categorized as either white-box or black-box AI, with the difference lying in the degrees of interpretability. White (or transparent) box systems are designed in a way that allows an understanding of the logic that was followed in the elaboration of the output. They are typically rule-based systems, meaning that they work through the combination of a symbolic representation of knowledge and a knowledge engine.[19,20] In contrast, black box models predominantly employ machine learning methodologies, with a particular emphasis on deep learning techniques. The process of deep learning is founded upon the training of artificial neural networks, and it requires large quantities of data to be processed through a variety of layers (the majority of which are "hidden").[19,21] Black box systems are designed in such a manner that even technical experts are unable to fully comprehend the reasoning behind the AI's specific output.[22,23]

In terms of the relationship with the physician, white box systems do not differ significantly from traditional information tools such as risk score charts and flowcharts. Black box systems, on the other hand, are a novelty in this respect, since they work as "opaque" advisors to physicians. The lack of understanding of the machine's processes can make it difficult to assess the meaning and reliability of the output, thus potentially resulting in lower trust levels.[24] To try and overcome these issues, a growing line of research is the development of explainable AI (XAI).[22] Interpretability refers to a model's built-in transparency, whereas explainability involves post hoc techniques that translate a model's outputs into human-understandable explanations.[20,25] The explanation provided by the explanatory algorithm, though, is by definition just an approximation of the actual prediction of the black box system, so it is bound to be imperfect and may at times even be misleading.[20]

The human oversight

It is also important to consider that liability rules must, in this instance, be read alongside market safety regulations. Liability norms function as ex post governance mechanisms, meaning that they intervene only after harm has occurred. On the other hand, market safety regulations operate through an ex ante approach, aiming to prevent harm by ensuring that products entering the market comply with specific safety standards.[26] While safety regulations reduce the risk that a certain product will produce harm, the liability norms ensure that if harm does occur, those affected will be compensated. In the case at hand, the AI Act (Regulation 2024/1689),[27] which is a safety regulation, does not directly regulate liability, but indirectly shapes liability allocation by imposing stringent safety obligations. Art. 14 of the AI Act, imposes a particular requirement for high-risk systems, including medical devices, which is that of human oversight.[28,29] The article does not provide detailed guidance on how this oversight should be exercised concretely to ensure its effectiveness. What is clear, though, is the EU's overall position of denying full automation for systems that are "too risky" and instead imposing the presence of humans to oversee AI's activities. This article further reinstates the role of the physician as a supervisor, which can have repercussions from a liability standpoint.[30] To provide a more comprehensive legal framework, it is essential to consider how

data protection laws such as the General Data Protection Regulation (GDPR)[31] and health privacy regulations like the Health Insurance Portability and Accountability Act (HIPAA)[32] intersect with AI liability. These frameworks regulate the lawful processing and protection of sensitive personal and health data, which are critical in AI deployment and oversight. Their intersection with AI liability shapes compliance requirements and the scope of responsibility, particularly regarding transparency, data governance, and protection of individual rights.

The human oversight requirement is particularly relevant in shaping how responsibility is distributed between AI systems and human operators. The degree of automation plays a crucial role in the distribution of task responsibility: The higher the level of human involvement, the greater their share of responsibility, and vice versa.[33] For example, if a diagnosis is formulated by a physician using a diagnostic decision support system and the process involves low levels of automation, the physician is more directly involved and would therefore likely bear greater responsibility for any errors.[34] In this sense, the oversight requirement not only mandates human participation but also implicitly assigns a degree of responsibility to the human operator. An increase in task responsibility generally increases the likelihood of liability being attributed to an individual as well, particularly where a failure arises within their domain of responsibility (though, of course, liability remains contingent upon the satisfaction of the relevant legal requirements).[33] It is crucial, in this context, to evaluate how oversight was concretely exercised. If the conditions necessary for meaningful oversight are lacking, it can be questioned whether the responsibility (and potential liability) assigned to the physician is proportionate to their actual contribution in the decision-making process.

Imagine a scenario where a new CDSS, such as one used for early sepsis detection or oncology treatment planning, which, according to research, performs better than humans and is particularly time-efficient, is introduced in a clinic. In light of this CDSS's characteristics, the clinic assigns physicians the role of mere "controllers", where they only have to review the outputs of the AI. Instead of carrying out full clinical assessments themselves, physicians are expected to validate or flag the system's suggestions. The clinic also decides that physicians must take on more patients than usual (since review tasks are less time-consuming), so their load is significantly increased compared to before. This means that the time at physicians' disposal for the evaluation of the outputs is cut quite short, and they are forced to make decisions very swiftly. In such a situation, it seems likely that most physicians would end up simply relying on the AI (so-called automation bias).[35,36] The control task would be reduced to blindly accepting the AI's suggestions, without any true critical thinking involved. This would be further aggravated by black-box AI, since it is challenging for physicians to understand and contextualize the reasoning behind its decisions.[37]

The principle of oversight is intended to mitigate the risks associated with automation by balancing the advantages and disadvantages of using AI to prevent adverse outcomes.[38] In healthcare, e.g., human oversight can ensure that the shared decision-making process between patient and physician is maintained.[39] Human oversight helps ensure that final decisions align with patients' preferences and values – elements that AI systems often overlook.[40,41] This integration of AI capabilities with physicians' expertise, experience, and emotional intelligence can be highly beneficial.[42,43] Moreover, human oversight is vital in identifying and mitigating AI errors and in preserving physician autonomy in clinical decision-making.[43] Overall, human oversight is essential in ensuring that AI is used safely and effectively in healthcare. If measures for proper oversight are not employed, though, the risk is that human oversight becomes a hollow requirement that primarily serves to identify someone to be held accountable. In this sense, Elish and Hwang's considerations on the risk of humans becoming a "liability sponge" for the mistakes of machines ring truer than ever.[37] While multiple parties may be considered liable in a single malpractice case, and liability may be apportioned among them or subject to claims for contribution under joint and several liability, the human actor nonetheless risks being assigned a portion that is disproportionate to their actual involvement in the decision-making.[44] As such, their liability may (at least partially) shield several other parties, including, e.g., the designers and manufacturers of the AI.[45] In the context of healthcare, the physician may also take part of the fall for some issues that should actually be blamed on the hospital, such as when the workflow is designed by the hospital itself.[46]

To ensure that the benefits of human oversight are achieved and that physicians do not become "liability sponges", it is crucial to implement measures to achieve effective human supervision. This can include, e.g., specific training for healthcare professionals.[47,48] Although explainability is often presented as a potential solution to automation bias, it has been shown that physicians may not always be able to use explanations effectively.[49] Consequently, their decisions may still be biased, even when they are presented with explanations. Allocating adequate time to critically evaluate a system's outputs can help mitigate the risk of automation bias.[48]

Conclusions

In certain contexts, CDSSs may function as collaborative decision-makers, highlighting the necessity of physician oversight calibrated to the specific system and clinical environment. Said oversight should be exercised through the adoption of appropriate measures to ensure the desired security level is achieved. It is equally important to avoid a possible scenario where physicians, while formally assigned a supervision role, are not given the necessary

conditions to effectively and concretely exercise control, thereby potentially facing an undue liability burden.[44] While the provision of human oversight shall be welcomed for ensuring that the physician/patient relationship is not lost, better overall health outcomes, etc., it is also essential to ensure that its concrete enactment is coherent with its inspiring principles, and that AI producers and hospitals do not take undue advantage.

A key issue is thus how to precisely define the allocation of liability among all involved parties: It is currently unclear what parameters should be considered relevant to this evaluation and how to properly assess and weigh them. The uncertainty around AI liability regimes and their concrete applicability further complicates the matter and calls for further dedicated research. On top of this, it must be questioned how to ensure that AI systems and workflow processes are both designed in a manner that enables physicians to correctly perform their oversight functions.

Forensic medicine has already found its proper role as a crucial discipline in the ascertainment and evaluation of medical malpractice cases, thus delineating individual liabilities among healthcare professionals. It remains to be seen whether forensic medicine – beyond defining standards of care in collaboration with clinical disciplines – will also be called upon to assess liability for AI systems that err or provide incorrect guidance to healthcare professionals. The stakes are significant, and the ex-ante and ex-post guarantor role that forensic medicine can play in this context is relevant.

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Family medicine: Discovering new fields for research and clinical care in the current world

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Abstract

Strengthening primary healthcare (PHC) research is vital to address the demands of a rapidly changing health landscape. Leadership, infrastructure, and sufficient funding have been discussed as key factors in expanding PHC research capacity. This editorial aims to highlight emerging research priorities in a world increasingly affected by crises such as war, conflict, and climate change. Research on suffering, meaning, hope, and compassion represents a promising and necessary new frontier in PHC. This field urgently needs the attention of academic institutions and funding bodies committed to strengthening primary care and family medicine.

Key words: family medicine, research, clinical care, fields

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Highlights

- PHC & UHC: PHC alone can't deliver UHC; system-level reform and political will are essential.
- Eudaimonia vs hedonism: Blending purpose-driven (eudaimonic) and pleasure-driven (hedonic) well-being can reduce burnout.
- Lifelong purpose: Support eudaimonic motivation throughout physicians' careers, not just in training.
- Sense of coherence: Comprehensibility, manageability, and meaningfulness predict better health and coping.
- Compassion's satisfaction: Compassion – for others and self – deepens care and guards against burnout.
- Research gaps: Study on suffering, hope, loneliness, conflict, and climate change to be addressed by the primary and public health researchers and practitioners.
- Six research priorities: Boost evidence, funding, leadership, team science, psychosocial and cultural determinants of health.

Introduction

A critical discussion has emerged regarding the current role of primary healthcare (PHC) within the framework of universal health coverage (UHC). Recent commentaries have challenged the longstanding view of PHC as foundational. As stated in a *Lancet* editorial: "…The stubborn persistence of this narrow approach to UHC utterly fails to recognise the transformation in disease profiles taking place in low-income and middle-income settings. Primary healthcare alone is insufficient to meet the demands of this new health landscape."[1]

In a letter to the editor of *The Lancet*, the authors agreed that primary care, especially is low-income and middle-income settings, is increasingly failing to meet the complex needs of populations. They emphasized that breaking this cycle would require sustained political will and visionary leadership – leadership capable of disrupting the status quo and creating an environment where governance, health financing, and incentive structures are realigned to support bold new action and collaboration for health for all.[2]

In this context, strengthening PHC research is vital to address the demands of a rapidly changing health landscape. Wright et al. identified leadership, funding, team science, and departmental culture as key factors in expanding PHC research capacity.[3] This editorial aims to highlight emerging research priorities in a world increasingly affected by crises such as war, conflict, and climate change. The transformation of PHC must address evolving disease profiles, rising emotional distress (especially among youth), compassion fatigue, and burnout – challenges that underscore the urgency of this discussion.

Eudaimonia: Position statements on family physicians and flourishing

In a world marked by crises, uncertainty, and suffering, the role of family physicians extends far beyond clinical expertise. It is essential to cultivate healthcare professionals who are not only competent and knowledgeable but also emotionally resilient, creative, and fulfilled – individuals who inspire hope and promote well-being. Realizing this vision calls for a renewed emphasis on the philosophical ideal of eudaimonia.

Eudaimonia, often translated as "human flourishing" or "well-being," derives from the Greek words *eu* (good) and *daimon* (spirit).[4] For Aristotle, eudaimonia denoted the highest human good – achieved through virtue, rational activity, and purposeful living – and today is understood as eudaimonic well-being, characterized by growth, authenticity, and meaning.

By contrast, Epicurus advocated a hedonistic view of happiness, emphasizing the pursuit of pleasure and the avoidance of pain.[5] This gave rise to hedonic well-being, oriented toward comfort and enjoyment.[6]

While traditionally seen as distinct, modern perspectives suggest a synthesis. For frontline healthcare workers, integrating eudaimonic and hedonic elements offers a robust framework for resilience and professional fulfillment. Eudaimonia can also be viewed as a dynamic balance between meaning and pleasure. As such, it can reduce emotional exhaustion and foster joy in clinical practice. When physicians align personal growth with meaningful patient relationships and broader societal impact, medicine transcends being a job – it becomes a calling. This path is not easy, but it is both possible and necessary to sustain a compassionate and resilient healthcare workforce. As Bauer et al. note, eudaimonia also serves as a motivational force for personal development.[7] This drive must be cultivated during medical training and throughout a physician's career. It demands systemic and individual commitment to holistic well-being. Indeed, this subject invites family physicians and primary care researchers to further contribute.

Additional and neglected areas of primary care research also need to be revisited. Ronald Epstein, in his seminal work, observes that "the world's suffering is strikingly absent in conversations among physicians and patients."[8] While clinicians often address pain, disability, and quality of life, many patients seek care simply because they suffer.

Epstein emphasizes that suffering can persist even after a disease is "cured," and that even asymptomatic conditions can provoke deep emotional distress. Suffering is intimately connected to hope and meaning – elements that must be reclaimed in medical practice.[8]

Coping with stress: Sense of coherence and compassionate care

Family physicians are routinely exposed to occupational and emotional stress, making the development of effective coping mechanisms essential. A potent source of resilience lies in the pursuit of meaning. When individuals lack a sense of purpose, suffering becomes unmanageable. Findings from a recently published study suggest that certain kinds of purpose are especially relevant in predicting people's well-being, and that these relationships are largely robust across cultures.[9] Research shows that a lack of meaning is closely associated with poor mental health outcomes,[10] underscoring the need to explore how professional suffering impacts physician's well-being.

A promising model that offers an important mechanism for understanding and coping with stress is the sense of coherence (SOC). It reflects a person's ability to perceive life as comprehensible, manageable, and meaningful. The SOC includes 3 dimensions: comprehensibility; manageability; and meaningfulness. Haugan and Dezutter argue that individuals who find meaning in life, even amidst illness, manage their conditions better and report higher levels of well-being.[11] This aligns with Aaron Antonovsky's salutogenic theory, where SOC plays a central role.[12,13] Research from the University of Crete, Greece, supports SOC as a strong determinant of health, linking it to improved clinical and laboratory outcomes.[14–17] Studies from Crete reveal that individuals with strong religious beliefs report lower depression levels, and higher SOC scores correlate with reduced symptoms on the Beck Depression Inventory (BDI).[16] Interventions to strengthen SOC – through behavior change or reframing life events – enhance stress resilience, particularly in high-pressure professions, such as family medicine.[18]

Simultaneously, compassionate care is emerging as a vital tool for supporting physicians' well-being. Defined as "feeling that arises in witnessing another's suffering and that motivates a subsequent desire to help," "a sensitivity to the suffering of others, combined with a desire to alleviate it,"[19] compassion deepens patient–physician relationships and acts as a protective factor against burnout. Research also links compassion – toward self and others – to greater psychological well-being.[20,21]

Paradoxically, suffering itself can enhance compassion. Personal loss, while painful, often increases one's capacity for empathy. As Megan Shen writes, "the most significant payoff to suffering is compassion, not just resilience."[22] In an era of growing disconnection, compassion is arguably one of the most powerful tools for healing and growth.

In today's global context, where social and environmental health determinants are increasingly relevant, family medicine must expand its research scope. Topics like loneliness are gaining prominence. Recent studies from the University of Crete contribute valuable insight into this growing field.[23,24] These findings underscore the need for primary care interventions that enable patients, particularly those with severe or terminal illnesses, to reflect on the meaning of their lives. Such approaches may help individuals better cope with the psychological stress of modern crises and should inform future research in family medicine. However, realizing this vision requires specific foundational steps. As noted in Herbert's editorial, 6 key recommendations are essential for advancing family medicine research – among them, a more substantial commitment to evidence-based practice, physician support in clinical settings, and increased research funding.[25]

Conclusions

Ultimately, alleviating suffering in primary care is not only a clinical goal but a profoundly human one. Physicians who find purpose and joy in their work can have a profound impact on both patient outcomes and their well-being. Research on suffering, meaning, hope, and compassion represents a promising and necessary new frontier in PHC. This field urgently needs the attention of academic institutions and funding bodies committed to strengthening primary care and family medicine.

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Transcranial direct current stimulation (tDCS): A new, (still) legal form of "neurodoping" in sports?

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Abstract

Transcranial direct current stimulation (tDCS) has emerged as a widely accessible, noninvasive technique capable of modulating cortical excitability. A rapidly expanding body of sports-science literature suggests that it can produce modest but measurable gains in endurance, strength, skill acquisition, and perceived exertion. This editorial reviews the physiological mechanisms underlying tDCS, evaluates the evidence for its ergogenic effects, and situates the technology within the broader framework of "neurodoping". Applying the 2021 World Anti-Doping Agency (WADA) Code, I argue that tDCS already satisfies 2 of the 3 criteria for prohibition – namely, potential performance enhancement and violation of the spirit of sport – while failing the 3rd criterion, as standard protocols pose minimal health risk. This editorial also considers practical and ethical counterarguments to a ban, including tDCS's low cost, relative safety, requirement for continued training effort, and the near-impossibility of detection or enforcement. Drawing parallels with accepted performance aids such as mindfulness, nutrition and altitude tents, this editorial concludes that outright prohibition could drive use underground and impede open scientific scrutiny. Instead, it advocates rigorous long-term safety monitoring, transparent research, and nuanced policy development that distinguishes therapeutic from performance applications. Ultimately, it frames tDCS as a "still-legal" yet ethically contested innovation at the frontier of sports technology, urging stakeholders to balance principles of fair play with scientific evidence as the debate over neurodoping continues to evolve.

Key words: sport, transcranial direct current stimulation, tDCS, neurodoping

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Highlights

- Transcranial direct current stimulation (tDCS) shows small, inconsistent performance gains with big individual differences.
- Short-term safety of tDCS is good; long-term and DIY misuse remain concerns.
- Use of tDCS is effectively undetectable, making enforcement difficult.
- tDCS likely meets World Anti-Doping Agency (WADA) "enhancement" and "spirit" criteria but not health risk; a ban is premature.
- Recommended: monitor, issue guidance, require disclosure, and fund targeted research.

Introduction

Athletic activity is fundamental to maintaining health and serves as a source of recreation and competition across hundereds of sports. In most disciplines, victory or defeat is determined by individual points or fractions of a second. Athletic performance is influenced by multiple factors, including athletes' physical and psychological characteristics, environmental conditions and potential distractions. Athletes, coaches and scientists must account for these influences to minimize negative effects and maximize potential benefits.

For decades, a great scientific race has been underway to identify methods with demonstrated efficacy in enhancing athletic performance. In this race, 3 paths can be distinguished in the search for performance enhancements. The 1st priority is the development of safe and effective methods that comply with the anti-doping rules established by the World Anti-Doping Agency (WADA). Such methods must uphold the principles of fair play and honest competition, ensure athlete safety and remain free of ethical or moral objections. Moreover, even when proven effective, they should be accessible to all.

The 2nd path involves developing methods that violate WADA rules but are not yet recognized by WADA as doping or cannot currently be detected. The 3rd and final path comprises methods that are or will be recognized as doping but for which reliable detection is problematic. This 3rd path carries a real risk of exposure and often leads to high-profile scandals, revocation of titles and exclusion of athletes from competition.

What is transcranial direct current stimulation?

Transcranial direct current stimulation (tDCS) is a non-invasive technique that delivers a weak, continuous electrical current to the brain through 2 or more saline-soaked sponge electrodes – typically 1 anode and 1 cathode – placed on the scalp.[1] The procedure is painless, generally well tolerated and considered safe, with few reported side effects.[2] A small, battery-powered stimulator generates a direct current, typically between 0.5 and 2 mA, a fraction of which penetrates the skull to reach cortical tissue. The polarity of the current determines its effect: Anodal stimulation generally depolarizes neurons and increases excitability, whereas cathodal stimulation hyperpolarizes neurons, reducing activity.[3,4] Current flows between the electrodes, making standard tDCS relatively diffuse; however, the use of smaller electrodes can enhance the focality of stimulation.[5] Transcranial direct current stimulation sessions typically last 15–30 min, with 20-min protocols being most common. The physiological aftereffects outlast the stimulation: as little as 3 min can induce measurable changes, while 10 or more minutes at 1–2 mA can stabilize these changes for at least 1 h.[4,6] A single 15-min session can modulate cortical excitability for approx. 90 min, and repeated sessions can further extend these effects.[7–9] Such aftereffects are thought to result from subthreshold membrane polarization and subsequent synaptic plasticity, rather than direct neuronal firing.[10] Multiple studies have demonstrated that tDCS can induce long-lasting, long-term potentation (LTP)-like or long-term depression (LTF)-like plasticity in the cortex, resembling classic Hebbian learning mechanisms.[11] This plasticity depends critically on NMDAR (N-methyl-D-aspartate receptor) signaling and modulatory factors such as brain-derived neurotrophic factor (BDNF). For example, pairing anodal polarization with low-frequency synaptic input can produce persistent LTP in rodent cortex, an effect abolished by NMDAR antagonists.[8] Importantly, these mechanistic effects are highly context-dependent: The impact of tDCS interacts with the preexisting state of the cortex and the ongoing activity of targeted networks. Stimulation during rest compared to task performance, or even differences in arousal state or time of day, can reverse or attenuate the expected excitability changes – a phenomenon known as metaplasticity.[12,13] Moreover, tDCS does not act in isolation on a single brain region but instead modulates distributed networks. Brain-network models emphasize that perturbing 1 node (e.g., M1) can propagate effects across connected circuits.[14] Empirical imaging studies confirm that tDCS alters large-scale functional connectivity, so behavioral outcomes depend on the broader network state.[15,16] In summary, the primary physiological signatures of tDCS – membrane polarization, shifts in excitation–inhibition

balance and plasticity – are well established, but their expression varies with ongoing brain dynamics.

A critical perspective also acknowledges that brain stimulation operates within a body–brain milieu. Neuromodulation does not override the influence of autonomic, endocrine or emotional states. For example, emotional arousal induces autonomic responses, such as heart rate acceleration or deceleration, which in turn affect brain function.[17] Fear and stress recruit prefrontal–limbic circuits that regulate both cortical excitability and learning.[18,19] Moreover, the same threatening stimulus can elicit different heart rate and brain responses depending on context – for instance, during anticipation or extinction training.[20,21] Hormonal factors such as cortisol also feedback on synaptic plasticity and memory networks.[22,23] In the sporting context, variables such as stress, fatigue, motivation, and metabolic state are likely to interact with any neuromodulatory intervention. These layers of brain–body–emotion interaction imply that even well-documented physiological effects of tDCS must be interpreted within a dynamic system. Simple cause–effect assumptions (e.g., tDCS → performance improvement) risk overlooking how internal states gate plasticity.

Finally, it is evident that individuals differ markedly in their neurophysiological responses to tDCS. Genetic polymorphisms, cortical anatomy, neurotransmitter levels, and prior experience can all influence sensitivity to stimulation.[24–26] For example, studies of conditioned fear have shown that genetic variation can substantially modify autonomic responses, such as heart rate, to identical stimuli.[27] By analogy, 2 athletes receiving the same tDCS protocol may experience very different neural effects. This baseline variability is often obscured in group-averaged data but contributes to failed replications and mixed outcomes. In practice, any ergogenic benefit of tDCS in sport will likely interact with an individual's unique neurochemical and neuroplastic profile.

There are 3 areas of scientific interest for tDCS. The 1st is reducing the symptoms of neurological and psychiatric conditions. Transcranial direct current stimulation has been shown to reduce symptoms of depression,[28] schizophrenia,[29] addiction,[30] anxiety disorders,[31] neurodegenerative diseases,[32] post-traumatic stress disorder (PTSD),[31] neurodevelopmental disorders,[33] epilepsy,[34] and many other conditions. The 2nd area of application is the use of tDCS to improve cognitive function in healthy individuals. Transcranial direct current stimulation has been shown to improve working memory,[35] associative memory,[36] episodic memory,[37] creativity,[38] decision-making,[39] inhibitory control,[40] numerical cognition,[41] foreign language learning,[42] and many other domains related to human cognition. The 3rd, and most interesting, area is improving performance in sports. Numerous studies have shown that tDCS improves the psychophysical performance of athletes by increasing limb strength and endurance and reducing perceived exertion and fatigue. We will continue to a detailed discussion of the effects of tDCS on performance in athletes in the next section.

What is "neurodoping"?

Neurodoping, also referred to as brain doping or cognitive enhancement in sports, denotes the use of interventions aimed at improving an athlete's mental or cognitive functions – such as focus, learning or decision-making – to enhance performance.[43] It includes both pharmacological approaches, such as nootropic agents or psychostimulants (e.g., modafinil, methylphenidate (Ritalin), or Alzheimer's medications like donepezil, memantine and rivastigmine), and non-pharmacological techniques involving direct brain stimulation (e.g., tDCS or transcranial magnetic stimulation (TMS)).[44,45] These interventions aim to enhance athletic performance by sharpening cognitive functions – such as concentration, alertness and strategic thinking – while also improving motor skills, endurance, motivation, and other psychological factors that contribute to success.[44,46] For example, stimulant "smart drugs" such as modafinil or methylphenidate (Ritalin) can increase alertness and reaction time, benefits thought to aid not only physical sports but also mind sports such as chess.[44,47] The rise of neurodoping, however, raises significant ethical concerns: Critics argue that artificially enhancing the brain confers an unfair advantage and undermines both fairness and the "spirit of sport" that underpins athletic competition.[46] Debates also extend to the question of an athlete's praiseworthiness when using such aids, raising doubts about whether achievements attained with cognitive enhancements are truly deserved – a discussion often framed in terms of moral desert, that is, whether neurodoping diminishes an athletes praiseworthiness or prizeworthiness.[44] From a regulatory perspective, neurodoping currently occupies a grey area: WADA has not yet explicitly banned most neurodoping methods. Under existing definitions of doping, which apply only to substances and methods on the Prohibited List, these brain-enhancing techniques remain legal in sport for now.[45,48] The absence of prohibition has sparked active debate among experts and policymakers. Some have urged WADA to ban neurostimulation techniques such as tDCS, arguing that they fulfill the criteria for doping and threaten both sporting integrity and athlete safety.[46] Others, however, advocate more permissive approaches, suggesting that safe and accessible neuroenhancement could even help level the playing field. Proponents note that, unlike steroids or other traditional forms of doping – which can boost performance without effort – techniques like tDCS still require athletes to train rigorously to obtain benefits, potentially making them a fairer form of enhancement.[49] Real-world instances of neurodoping have already been observed, which further fuels this debate. For example, some Olympic athletes and professional sports teams have reportedly experimented with tDCS during training in hopes of gaining a competitive edge.[47] Additionally, in competitive chess (a sport with anti-doping rules), studies have found that cognitive stimulants such as modafinil or Ritalin can significantly improve

a player's performance, showing that even mind sports are subject to neurodoping considerations.[47] Finally, there are clear risks and side effects associated with neurodoping: Athletes who use neural enhancement without medical supervision risk adverse effects such as dermatological lesions or burns from improper electrode use in brain stimulation (e.g., tDCS devices can cause scalp burns if used incorrectly), and the off-label use of psychoactive "brain booster" drugs (for example, rivastigmine patches) has been linked to skin rashes and other health issues.[45]

Transcranial direct current stimulation in sports: Hit or miss?

Much research effort has been devoted to demonstrating the effectiveness of tDCS in improving athletic performance. Numerous review studies have shown that tDCS improves running and cycling performance,[50] endurance and maximal force production,[51] upper-limb motor and endurance performance,[52,53] exercise performance,[54] maximal strength and lower limb explosive strength,[42–55] performance in basketball players,[56] physical and psychological performance in national- or international-level athletes,[57] and visuomotor performance,[58] as well as lowers ratings of perceived exertion.[59] Across sport tasks, recent meta-analyses suggest small average ergogenic effects, with important differences by outcome and protocol. A 2022 synthesis of 43 studies reported an overall standardized mean difference (SMD) of 0.25 (95% confidence interval (95% CI): 0.14–0.36), with strength (SMD = 0.31) and visuomotor tasks (SMD = 0.29) tending higher than endurance (SMD = 0.18). However, effects were inconsistent across studies, meta-regressions did not isolate robust stimulation parameters, and authors cautioned that factors like genetics, sex and training history likely moderate responsiveness.[58] Other review concluded that any positive impact on exercise outcomes is small and potentially inflated by low study quality and selective reporting.[60] The sport-performance evidence is mixed and methodologically heterogeneous. While multiple trials report improvements, synthesis across studies yields small average effects and substantial between-study variability, with suspected publication bias. Many experiments are small and underpowered, and responses vary widely between individuals, with only ~39–45% showing the expected excitability or behavioral change in classic motor-cortex paradigms. These features, together with blinding/sham limitations (standard sham can be biologically active and participants often guess condition above chance), argue for a cautious interpretation of apparent benefits.[26,61–63]

Even a cursory examination of the scientific literature on tDCS suggests 2 conclusions. First, tDCS is being intensively studied in the context of sports. Thousands of studies have already been published in this area. This demonstrates the scientific interest in tDCS, which is good, as our understanding of the brain mechanisms of tDCS is still limited. However, to date, more is known about the mechanisms of tDCS in alleviating the symptoms of various diseases. Meanwhile, the mechanisms of tDCS in improving athletic performance are enigmatic.[64] In other words, tDCS has shown performance benefits in some cases, but we still do not fully understand how or why these occur. The 2nd conclusion follows from the 1st: Since so many experiments have been conducted using tDCS in sports, can we speak of a new era of "neurodoping" in sports? If researchers are intensively studying the use of tDCS in sports, should we suspect that tDCS has already been implemented in training and athletic preparation and is being used as a "secret weapon"? Transcranial direct current stimulation use is virtually undetectable. The only telltale signs of tDCS use are a slight redness of the scalp where the electrode was attached, but this redness is not always present and disappears very quickly.

Evaluating tDCS under the 2021 WADA doping criteria

The 2021 WADA Code sets out 3 criteria for determining whether a substance or method should be considered doping. A substance or method may be added to the Prohibited List if it meets any 2 of these 3 criteria: 1) medical or scientific evidence that it has the potential to enhance, or actually enhances, sport performance; 2) evidence that its use presents an actual or potential health risk to the athlete; and 3) WADAs determination that its use violates the spirit of sport. In this section, we analyze tDCS against each criterion, considering both athletic use (i.e., performance enhancement in healthy athletes) and clinical or therapeutic use (i.e., medical treatment), with reference to established knowledge about tDCS and the 2021 WADA Code.

Criterion 1: Potential to enhance sport performance

WADA's first criterion considers whether there is scientific or medical evidence that tDCS "has the potential to enhance, or actually enhances, sport performance." Here, we evaluate the performance-enhancing potential of tDCS in the context of athletic competition, as distinct from its application in clinical therapy.

Athletic use of tDCS: Performance enhancement potential

Transcranial direct current stimulation is an experimental, noninvasive brain stimulation technique that modulates cortical excitability and has the potential to improve certain cognitive and motor functions. As a tool for neuroenhancement, it has been applied to healthy individuals to improve motor control and learning, including in athletic contexts. For instance, studies have demonstrated that

tDCS can enhance motor skill acquisition and cognitive performance in healthy volunteers, effects that may translate into gains in athletic performance. Emerging evidence suggests that stimulating specific brain regions with tDCS may acutely improve motor skills or delay fatigue, while repeated sessions can accelerate longer-term skill acquisition. Notably, reports indicate that some elite athletes have already experimented with tDCS during training in an effort to gain a competitive advantage.

It must be emphasized, however, that scientific findings on the ergogenic effects of tDCS are mixed. Although individual studies have reported improvements in endurance, reaction time and skill learning, recent meta-analyses highlight inconsistent results and considerable methodological variability.[65] The precise neurophysiological mechanisms by which tDCS might enhance performance remain unclear, and several well-controlled trials have found minimal or no benefit. Nonetheless, WADA's threshold of "potential to enhance" does not require conclusive or consistent evidence of enhancement. The mere existence of plausible performance gains, supported by some scientific evidence, is sufficient to satisfy this criterion. By WADA's standards – which require only the potential for enhancement, not consistent proof – tDCS meets Criterion 1. It artificially modulates brain circuits in ways not achievable through conventional training, thereby offering athletes a possible edge. Thus, although its effects may be modest and continued training remains necessary, the potential for direct performance enhancement through brain stimulation fulfills the performance-enhancement criterion.

Clinical/therapeutic use of tDCS: Performance effects

In clinical and therapeutic contexts, the primary aim of tDCS is to restore or normalize function rather than to enhance performance beyond natural baselines. It has shown promise in treating neurological and psychiatric conditions, including motor recovery after stroke, relief of depression or chronic pain, and mitigation of cognitive decline. In such cases, any observed "performance" improvements – such as better memory in Alzheimer's disease or improved mobility in Parkinson's disease – represent medical benefits rather than competitive sport enhancements. Current evidence highlights tDCS as a valuable therapeutic tool for difficult-to-treat disorders and symptoms. Its use to help patients regain normal function or quality of life is not intended to confer a supernormal athletic advantage, but rather to counteract illness. Accordingly, in the therapeutic context, the effects of tDCS would not be classified as "performance enhancement" in the sporting sense; they represent rehabilitation. If an athlete were to use tDCS under medical supervision to treat a clinical condition – e.g., to manage depression or pain – this would constitute legitimate medical use. WADA provides for Therapeutic Use Exemptions (TUEs), which permit necessary treatments even when they involve otherwise prohibited methods. In short, clinical applications of tDCS are not intended to enhance sport performance, and any improvements are incidental to treating a health condition. Accordingly, therapeutic use of tDCS does not satisfy Criterion 1, except insofar as it may incidentally restore an athlete to their baseline capabilities.

Criterion 2: Actual or potential health risk to the athlete

The 2nd criterion concerns whether tDCS poses an "actual or potential health risk" to the user. In this section, we evaluate the safety profile of tDCS and its associated risks, distinguishing between potential misuse in athletic contexts and controlled clinical applications.

Athletic use of tDCS: Health risks and safety

A key consideration for Criterion 2 is the excellent safety profile of tDCS when protocols are properly followed. According to broad consensus among researchers and clinicians, there is "no evidence for a serious adverse event being caused by tDCS" in human trials.[66–68] Typically, tDCS delivers a weak current of 1–2 mA to the scalp for a limited duration, and decades of cumulative research have not identified lasting injuries under these conditions. Reviews of clinical trials report no unexpected or serious adverse events across hundreds of participants, including vulnerable groups such as older adults. Importantly, even in individuals with epilepsy – where brain stimulation might raise particular concern – no seizures or exacerbation of epileptic activity have been causally linked to tDCS in clinical studies.

The side effects of tDCS are typically mild, transient and localized. Common minor reactions include scalp itching, tingling, a burning sensation, or mild headache during or shortly after stimulation. These symptoms generally resolve quickly and have not been linked to lasting harm. Even in trials involving repeated sessions – for example, daily stimulation over several weeks – participants have tolerated the procedure well, with no cumulative adverse effects reported. This favorable safety record reflects both the low intensity of currents used and the careful exclusion of individuals with contraindications in research settings.

However, one must consider the "potential" health risks, including those arising from misuse. While tDCS itself, as used in published studies, appears safe, improper use could introduce dangers. Experts caution that if tDCS is performed incorrectly or without proper medical guidance, serious injuries are possible. For example, using electrodes over broken skin or wounds on the scalp can cause burns or skin lesions. Likewise, individuals with metal implants in the head (aneurysm clips, deep brain stimulators, cochlear implants, etc.) could be at risk – applying current near metal can concentrate current and

potentially lead to tissue damage or even life-threatening outcomes. These scenarios underscore that unsupervised, amateur "do-it-yourself" (DIY) tDCS (which has emerged in some communities) carries non-negligible risks. If athletes were to self-administer excessive stimulation in pursuit of performance gains, they might exceed established safety limits, possibly causing unknown long-term neural changes or acute injuries. Additionally, there is a theoretical concern that enhancing one brain function might impair another (a trade-off), as 1 study noted tDCS improving numerical skills at the expense of another cognitive ability. Such off-target or long-term effects remain under investigation.

Overall, in the athletic context, tDCS does not inherently represent a significant health risk when used within recommended parameters, as no direct evidence of harm has emerged from controlled studies. The routine practice of testing tDCS on healthy volunteers (even for multi-week protocols) without incident attests to its general safety. However, the absence of documented harm so far does not guarantee that tDCS is without health risks. WADA's Criterion 2 concerns potential as well as actual harm. We do not yet know the long-term consequences of repeated brain stimulation: Chronic use of tDCS could lead to unforeseen neural changes or side effects. For example, 1 study found that tDCS improved one cognitive skill while impairing another, hinting at complex trade-offs in brain function. Moreover, unsupervised or improper use (the so-called "DIY" tDCS trend) can introduce real dangers: users might over-stimulate, place electrodes incorrectly or ignore contraindications, potentially causing burns, tissue damage or unpredictable neural effects. Because these long-term and misuse-related risks are not fully understood, tDCS could be seen as posing a potential health risk. In summary, while short-term use appears safe, the unknown long-term and misapplication hazards mean Criterion 2 cannot be dismissed; there is at least a potential health risk that meets WADA's wording for this criterion.

Clinical and therapeutic use of tDCS: Health risk considerations

From a clinical perspective, the safety of tDCS is a paramount concern, and the literature gives a reassuring picture. In therapeutic trials, patients (including those who are elderly or have neurological disease) have undergone tDCS with very few adverse events. For instance, a review of 19 studies involving over 500 older adult patients reported no serious adverse events and no major safety issues attributable to tDCS.[68] Minor side effects in patient populations mirror those in healthy subjects – transient scalp discomfort or fatigue, which do not require medical intervention. In conditions like depression, stroke and chronic pain, tDCS has been delivered in repeated sessions with a strong safety record. One favourable reason tDCS is being explored as an alternative to pharmaceuticals is because it lacks the systemic side effects that many drugs have.

Clinically, one must still be cautious. Medical teams employing tDCS adhere to strict protocols to avoid risks (screening for implants, monitoring skin condition, using proper electrode preparations, etc.). In the hands of clinicians or researchers, tDCS is handled as a medical device with appropriate oversight. Under these conditions, tDCS does not pose a significant health risk to patients. On the contrary, it is often sought for patients precisely because it has a benign side-effect profile compared to medications (e.g., treating depression with tDCS to avoid the side effects of antidepressant drugs). Therefore, in the therapeutic use case, tDCS clearly fails to meet the "health risk" doping criterion. It is considered a safe treatment modality.

It is worth noting that if WADA were to ban tDCS as a doping method, athletes who genuinely need it for medical reasons could apply for TUEs to continue treatment. Such an exemption process underscores the difference between risk-laden abuse of a method by healthy athletes and legitimate medical use by those in need. In summary, tDCS is characterized by an absence of significant health risks in both healthy and clinical populations (aside from manageable minor effects), especially when compared to many pharmacological doping agents. Thus, it does not satisfy Criterion 2 under clinical-based use conditions. This form of intervention could help athletes suffering from injuries or neurological and psychiatric conditions recover faster.

Criterion 3: Violation of the "spirit of sport"

The 3rd criterion is more qualitative: It asks whether the use of tDCS is "against the spirit of sport", as defined by WADA's ethical principles. The spirit of sport is outlined in the Code's introduction as "the ethical pursuit of human excellence through the dedicated perfection of each athlete's natural talents". It encompasses values like health, fair play, honesty, respect for rules, and fairness on a level playing field. Doping is deemed fundamentally contrary to the spirit of sport because it undermines these values – it allows success to be achieved through artificial aid rather than natural talent and hard work. We consider whether using tDCS for performance enhancement would violate these principles, and contrast that with its use in a therapeutic context.

Athletic use of tDCS: Ethical and fairness issues

Using tDCS as a performance enhancer in sport raises significant ethical and fairness concerns, directly engaging WADA's "spirit of sport" criterion. Sport has traditionally celebrated achievements derived from athletes' natural abilities, refined through training, practice and dedication.

If competitors turn to electrical brain stimulation to gain an advantage, that edge is exogenous to their innate talent and training regimen, resembling the use of drugs or other artificial aids. In this sense, ergogenic use of tDCS may be regarded as a form of "neurodoping" – an external technical shortcut to improved performance. This clearly conflicts with the ethos of fair play and equal conditions. Athletes who do not use such technology would be at a disadvantage compared to those who do, creating an imbalance based not on skill or effort but on access to enhancement devices. This scenario is analogous to other banned methods of enhancement; indeed, researchers have explicitly begun calling on WADA to address "neurodoping" techniques like tDCS to preserve fair competition. If one athlete's endurance or reaction time is artificially boosted by a brain stimulation device, the competition is no longer solely a measure of natural training and talent – it has introduced a technological arms race, which undermines the integrity of sport.

Moreover, the spirit of sport also encompasses athletes' health and the natural enjoyment of sport. One might argue that even if tDCS is relatively safe, encouraging athletes to use any intervention on their brain for a competitive benefit puts psychological pressure on them to "keep up" with others, eroding the purity of sport. Respect for the rules and self-respect are also listed values – covertly zapping one's brain to win, especially if it were prohibited, would violate these principles. In essence, using tDCS to enhance performance violates the spirit of sport for the same core reasons as using a chemical stimulant or blood doping: It is an artificial enhancer granting an unearned advantage and compromising the ideal of sport as a test of natural human ability and honest effort.

It is interesting to note that some ethicists have debated whether certain forms of enhancement might be acceptable in the future (for instance, caffeine was once monitored by WADA but is now permitted in moderation). In the Practical Guide book, the authors discuss that "the simple act of neuroenhancement itself is not unethical, as it represents a fundamental component of human life and development" – people regularly seek to improve themselves (through education, caffeine, etc.). They argue that tDCS use "in and of itself" is not inherently immoral. However, context is crucial: The same source immediately cautions that using tDCS without understanding its long-term consequences is ethically questionable. In sport, even if one viewed tDCS as akin to intense training or a nutritional supplement, the counterargument is that it directly alters brain function in a way not achieved by natural means, pushing the athlete beyond their innate limits. Until proven otherwise, this is closer to cheating than to innovation. The neuroethics literature broadly agrees that current neurostimulation devices, if effective as enhancers, would contravene the unwritten contract of sport – much like mechanical doping (e.g., hidden motors in bicycles) or gene doping. From an athletic standpoint, therefore, the use of tDCS for performance enhancement satisfies WADA's "spirit of sport" violation criterion, as it conflicts with the principle of fair and natural competition. That said, the analysis is not without nuance.

If tDCS were truly safe with only modest effects, some argue that banning it while permitting other advanced aids is inconsistent. Later I point out that current WADA practice tolerates many enhancement-like strategies (mindset training, optimized equipment, etc.). Ethicists have suggested treating tDCS more like these – as part of science-backed training – until it clearly violates fairness. Likewise, Møller and Christiansen caution that the "neurodoping" scare is largely speculative; given tDCS's mild effects and non-toxicity, a blanket ban might be premature.[48]

Other scholars emphasize that enduring physical effort is central to sport's value. Erler argued that bypassing "inner challenges" via neuroenhancement could erode the character-building aspect of competition. He concluded there is at least reason to prohibit tDCS in endurance sports to preserve the role of effort and struggle.[69] In his view, even a safe, accessible enhancer might still diminish what it means to achieve victory.

Ultimately, many conclude that tDCS's ethical status depends on context. Used medically (to restore health) it upholds sport's values; used for unfair gain it violates them. The debate shows tDCS is not simply "ethical" or "unethical" in all cases. Instead, factors like intent, outcome size and accessibility matter. This complicates a binary view: If tDCS modestly augments training and is equally available, some argue it could fit within a broad interpretation of the spirit of sport.[70] Conversely, if it provides a large, exclusive boost, most agree it offends the ideals of fair competition.[48]

In summary, neuroethicists urge a balanced perspective: tDCS sits on a spectrum, and its ethical profile shifts with the circumstances of use. These analyses suggest WADA's "spirit" criterion should be applied with nuance, recognizing that safe, equitable applications of tDCS might align with sport's health and training values, whereas more secretive or unequal use would violate its core principles.

Clinical/therapeutic use of tDCS: Spirit of sport perspective

When tDCS is used therapeutically by an athlete (under medical supervision for a diagnosed condition), the ethical interpretation shifts. In this scenario, the athlete's intent is not to "boost" performance above normal, but to recover health or function. This is akin to an athlete taking prescribed insulin for diabetes or using a rehabilitation technology after injury. Such use aligns with the spirit of sport insofar as it supports the athlete's health – one of the core values listed by WADA – and helps them compete on normal rather than enhanced terms. The WADA Code explicitly recognizes this distinction through the TUEs process,

which upholds the spirit of sport by allowing necessary medical treatments while still forbidding abuse of those treatments for performance gain. If an athlete with clinical depression uses tDCS under a doctor's care to alleviate their symptoms, this could be viewed as ethical and within the spirit of sport, provided it simply brings the athlete back to their natural baseline. The spirit of sport concept includes values like "health" and "respect for self" – obtaining proper treatment honors those values.

Thus, therapeutic tDCS use does not violate the spirit of sport, whereas non-medical performance use does. The key factor is intent and effect: using technology to level the playing field (by restoring health) vs to tilt the playing field (by gaining an advantage). WADA's stance is that doping is contrary to sport's spirit because it's an unfair shortcut. A medical intervention under a TUE is not considered a shortcut but rather a legitimate support for an athlete's wellbeing. It's analogous to the ethical difference between taking an anabolic steroid to build extra muscle (doping) compared to taking prescribed cortisone to treat severe inflammation (medicine).

Neuroethicists stress that therapy and enhancement lie on a continuum, not a strict binary. Shook et al. warned that drawing the line "where therapy ends" and enhancement begins is a mistake – enhancement effects can emerge even during ostensibly therapeutic use.[71] In practice, tDCS used to restore an athlete's lost function (e.g. treating depression or injury) is ethically akin to medicine – it "helps them compete on normal terms rather than enhanced terms". WADA embodies this distinction via TUE: Treating a medical condition with tDCS (thereby returning an athlete to baseline) is compatible with the spirit of sport, whereas using the same technology explicitly to boost performance beyond natural abilities is not. As I noted, using tDCS like an insulin shot or rehab is aligned with sports values of health and respect, whereas using technology to level the playing field (by restoring health) vs to tilt it (by gaining an advantage) is the key ethical difference. When tDCS is applied under medical supervision for a diagnosed condition, its effects are restorative. This aligns with WADA's values of health and fairness. The WADA Code explicitly allows necessary medical treatments (via TUE), recognizing that returning an athlete to normal capabilities does not gain an "unnatural" edge.

Some ethicists argue that certain enhancements could be permissible under fair conditions. A recent review emphasizes that any discussion of enhancement must tackle equitable access: If only the wealthy can use an ergogenic technology, it will worsen social disparities and violate justice.[70] Ploesser et al. concluded that ethical approval of brain-enhancing methods hinges on ensuring they are safe, effective and widely available.[70] In sports, however, perfect equity is elusive. Critics point out that athletes already benefit from high-tech gear and coaching to which poorer competitors have less access. As Kayser et al. note, even commonplace equipment (e.g. specialized bikes, prosthetics, swimsuits) is not equally available to all athletes, calling into question simplistic "everyone plays fair" arguments.[72]

Does tDCS meet WADA's doping criteria?

Bringing the analysis together, a clear contrast emerges between the role of tDCS as a performance aid and its role as a therapeutic intervention.

A. Potential to enhance sport performance: tDCS has been shown to have the potential to improve various aspects of performance (motor learning, endurance, cognitive function). Even though training and effort are still required, these improvements come from artificially modulating brain activity. In other words, tDCS can provide an unnatural advantage by boosting an athlete's own capacities beyond what natural training alone can achieve. Under WADA's 1st criterion, even this potential for enhanced performance (via external brain stimulation) is sufficient to satisfy the performance-enhancement requirement.

B. Health risk to athletes: No. The use of tDCS does not represent an appreciable health risk based on current evidence. It has an excellent safety record with only minor side effects and no serious adverse events reported. While any misuse or unknown long-term effects warrant caution, tDCS is far safer than virtually all traditional doping substances. It fails to meet WADA's risk criterion 4.3.1.2 in the sense that it does not pose a significant actual or potential harm to athletes under normal use.

C. Violation of spirit of sport: tDCS use for performance enhancement is at odds with the spirit of sport (in athletic use) – it compromises fairness, equality and the natural talent narrative of athletic competition. This satisfies WADA's criterion 4.3.1.3 for prohibition. However, genuine medical use of tDCS (with no intent to unfairly enhance performance) would not violate the spirit of sport and is ethically acceptable, analogous to other medical treatments.

According to the Code, meeting any 2 of the 3 criteria is enough for WADA to prohibit a method. In the case of tDCS, Criteria 1 and 3 are fulfilled in the scenario of athletic performance enhancement, whereas Criterion 2 is not. Two out of 3 criteria being met suggests that tDCS could be considered for inclusion on the Prohibited List if WADA chooses. Indeed, some scholars have argued that tDCS already meets the necessary criteria and have urged WADA to act. On the other hand, the fact that tDCS poses minimal health risk and has unproven efficacy might weigh in the balance of WADA's policy decision. WADA has thus far (as of the 2021 Code) not banned tDCS or other neurostimulation methods; this may reflect the still-emerging evidence and practical considerations about enforceability and medical use. In a recent neuroethics analysis, experts

concluded that while tDCS likely satisfies WADA's technical criteria for doping, it may be more prudent to monitor the technology rather than outright prohibit it at this stage.

Arguments against banning tDCS in sport

Unlike conventional training aids, tDCS works by directly altering brain function through electrical stimulation. Other legal methods (such as sports psychology, mindfulness, virtual reality (VR), neurofeedback, nutrition, or strength training) work indirectly – they rely on the body's natural physiological and psychological adaptation to practice or diet.[73–80] In contrast, tDCS applies an external current to the scalp, artificially modulating neural circuits. This fundamental difference means tDCS is not simply another version of meditation or altitude training. It actively pushes the athlete's brain beyond its innate limits, rather than harnessing normal adaptation. This artificial intervention makes tDCS distinct from accepted aids: It introduces a technological shortcut that those other methods do not. Even if tDCS were cheap and widely available, the issue of fairness is not resolved by price alone. The key point is that tDCS artificially alters the brain in a way that other methods do not. Simply making it accessible does not change the fact that it provides an external enhancement. Fair competition requires measuring athletes by their own unenhanced abilities and effort; any method that gives one athlete an artificial boost – even if it's affordable – undermines this principle. In short, tDCS's affordability or accessibility does not remove the ethical concern of its artificial advantage.

Third, we must revisit the "spirit of sport". As outlined under WADA's 3rd criterion, the use of tDCS for performance enhancement only weakly challenges this principle. Although neurostimulation may appear to offer an artificial shortcut, it in fact requires sustained training and produces, at best, modest gains – comparable to those achieved with other accepted high-tech training aids. In short, when used transparently, tDCS does not fundamentally undermine the values of dedication and fair play.

In light of tDCS's excellent safety profile, its moderate and inconsistent performance benefits, and the practical difficulty of policing its use, there is currently little justification for banning it outright. Unlike potent doping agents, tDCS does not dramatically alter competition or endanger athletes' health. Banning it prematurely could even be counterproductive – an unenforceable ban may drive covert use and create more unfairness. A more sensible approach is to continue monitoring and researching tDCS in sport rather than imposing a hasty prohibition despite the unsettled science.

Moreover, the performance benefits of tDCS, while demonstrated in some studies, are not overwhelmingly potent or reliable – calling into question whether it even provides an undue advantage. Scientific reviews and meta-analyses indicate that tDCS's ergogenic effects are modest and inconsistent overall. For example, a meta-analysis of 36 studies found only a small average improvement in outcomes like exercise endurance, an effect that may be inflated by publication bias and methodological factors. Other analyses have concluded that tDCS has little to no significant impact on muscular strength or perceived exertion in exercise, with some positive findings hinging on isolated studies. While ongoing research continues to refine how, where and for whom tDCS might be most effective, it is clear that tDCS is not a guaranteed performance booster – many individuals experience no measurable benefit, and responders might gain only a marginal edge (e.g., a few percent improvement in a specific task). Such gains are comparable to those attainable through perfectly legal means like optimized nutrition (consider the small but crucial effects of carbohydrate loading or creatine supplementation) or psychological techniques. Critically, there is also high inter-individual variability in response to tDCS. Factors like neuroanatomy and genetics appear to make some athletes "non-responders" who get no boost at all from the same stimulation that aids others. This variability further blunts any blanket advantage – at the population level, tDCS is far from a surefire or unfair enhancement. Sports already tolerate many performance differences arising from genetics or variable responses to training; the uneven efficacy of tDCS is no different. In short, the current evidence paints tDCS as a low-risk, moderate-reward intervention. It does not dramatically distort competition outcomes in the way that potent doping agents can, which is likely why WADA has so far held off on prohibition pending more conclusive data. Given this evidence, a preeemptive ban would be scientifically premature, especially when less effective or comparably marginal aids (with far greater uncertainty of benefit) are not banned. A more sensible approach is continuing to study and monitor tDCS use in sport, rather than outlawing it despite the unsettled science.

Even for those who remain ethically uneasy about neurostimulation in sport, an outright ban on tDCS would pose severe practical challenges. Unlike pharmacological agents, tDCS leaves no trace in blood or urine, making enforcement of any prohibition extremely difficult. Anti-doping authorities would have no reliable test to catch an athlete who used tDCS during training or prior to competition. Policing a tDCS ban could only be done by witness reports or catching an athlete with a device in hand, an approach that is neither systematic nor fair. This raises the specter of uneven enforcement and the potential for covert use: If a method is easily hidden and undetectable, a ban may simply drive its use underground, favoring those willing to break the rules. That outcome – clandestine neurostimulation by some athletes while rule-abiding ones abstain – would undermine the very fairness and integrity that doping rules intend to protect. The history of altitude

simulation illustrates this point. WADA considered banning hypoxic altitude tents on "spirit of sport" grounds, as they mimic high-altitude training artificially, but ultimately chose not to ban them in part due to enforceability and fairness concerns. It was recognized that prohibiting altitude tents would not only be hard to police but would also reward athletes who live or train at high elevations while penalizing those who tried to legally simulate those conditions. A similar logic applies to tDCS: Banning it could generate new inequities, favoring athletes who either use it covertly or who possess equivalent innate advantages. Moreover, effective regulation would be nearly impossible without intrusive surveillance. In contrast, allowing tDCS keeps the playing field transparent and subject to open scientific oversight. If it remains legal, athletes and coaches can discuss and publish their experiences with tDCS, and researchers can openly collaborate with teams to optimize safe use – all of which contributes to a better understanding of its true effects. This transparency is lost under a ban. It is telling that WADA has thus far opted not to list tDCS or other noninvasive brain stimulation methods as prohibited, despite tDCS seemingly fulfilling 2 of the 3 formal doping criteria. The likely reasons are the ones outlined above: Minimal health risk, still-emerging evidence of efficacy and serious doubts about enforceability. In a recent neuroethics analysis, experts concluded that while tDCS technically meets the criteria, the prudent course is to monitor the technology rather than ban it at this stage. This approach mirrors how WADA handles certain borderline substances (like caffeine, now on the monitoring program but not banned) and innovative training tools – proceeding with caution, gathering data, but stopping short of prohibition unless a clear threat materializes.

On balance, the ethical, scientific and practical arguments weigh strongly against banning tDCS in sport. It can be likened to other permitted performance aids that support training or mental preparation without undermining the core values of competition. Transcranial direct current stimulation is safe, with no appreciable risk to athlete health. Its effects are moderate and variable, offering no guarantee of victory and leaving the essential nature of competition intact. It is also accessible and relatively affordable, preserving fairness as long as it is equally available to all. Finally, it continues to require hard work from athletes, aligning with the spirit of sport's emphasis on effort and dedication. Where concerns do exist (e.g., about fairness or future misuse), they are better addressed through regulation, education and ongoing research rather than an outright ban. Parallels can be drawn to advancements like nutritional science or sports psychology, which were once novel but are now integral to athlete preparation. Embracing tDCS as a legitimate tool encourages transparency and further study into its benefits and limits, ultimately helping sports authorities make evidence-based decisions. In contrast, a ban would be premature and unenforceable, potentially stifling research and driving use into the shadows. In summary, while tDCS does satisfy 2 of WADA's doping criteria (enhancement potential and a surface-level departure from "natural" performance), those concerns are mitigated by its safety, equity of access and requirement for genuine athletic effort. Just as other enhancement technologies have been allowed to coexist with the ethos of sport, tDCS can be accommodated within athletics without undermining integrity or fairness.

Risk of abuse and injury

The human body has natural limits to its physiological capabilities. We experience fatigue, which signals that we should stop exercising and rest. We feel muscle pain, which signals that we should stop activity and investigate the cause, as damage may have occurred. These signals warn us that we have exceeded a critical point in our capabilities. By ignoring them, we expose ourselves to the risk of injury or even life-threatening situations. In the previous sections, I outlined evidence that tDCS can enhance physical performance in various domains (usually to a small degree). An important question that cannot be ignored is whether raising the threshold of our natural limits beyond safe levels is completely safe. If our bodies have limits: Can pushing them beyond physiological limits result in injury or even life-threatening situations? Unfortunately, there is no research on the long-term effects of tDCS on improving athletic performance. It is unknown whether athletes who regularly use tDCS and achieve better results are exposed to these new risks. There is therefore an urgent need to monitor the long-term effects of tDCS used to enhance athletic performance, and above all, its safety. Such action is necessary, of course, to refute these suspicions, but also to avoid providing arguments to anti-doping organizations that tDCS is a dangerous technique and should be banned.

Across thousands of sessions in clinical and healthy cohorts, conventional-parameter tDCS (typically ≤2–4 mA for ≤20–30 min) shows a strong safety record: Serious adverse events are not established and the most common effects are mild, transient sensations (itching, tingling, erythema) that also occur with sham; importantly, systematic reviews of repeated-session studies likewise do not show risk escalation within tested ranges.[81] Nevertheless, these data come largely from short-to-medium-term protocols under supervision, not from chronic, performance-oriented use by healthy athletes, so caution is warranted when generalizing. The gap between laboratory protocols and season-long training cycles justifies explicit monitoring.

Several specific unknowns merit attention. First, domain-specific cognitive trade-offs have been documented: Stimulation that facilitates one aspect of learning can impair automaticity or slow responses in other domains or subgroups,[82,83] implying that a gain in a targeted skill (e.g., vigilance under fatigue) could be counterbalanced

by decrements in decision speed, response inhibition or multitasking – capacities central to sport performance. These findings argue for assessing off-target cognition during long-term use.

Second, dose–response non-linearities and metaplasticity raise the possibility of subtle maladaptive plasticity with repeated tDCS sessions. Motor cortex studies demonstrate that increasing current intensity can invert expected polarity effects – e.g., cathodal stimulation at 2 mA may enhance rather than suppress excitability – and that session spacing can reverse or prolong aftereffects via homeostatic mechanisms.[84–86] Over longer timescales, athlete-style protocols that cluster stimulation around training may therefore bias neural networks in unanticipated directions unless carefully monitored and adjusted.

Third, although rare and primarily reported in clinical samples, affective switches (hypo/mania) highlight that mood/arousal networks are susceptible to neuromodulation.[87] Athletes operate under fluctuating sleep, travel stress, stimulants, and supplements – all probable modulators of state-dependent responses – so mood and sleep should be part of surveillance even in healthy users.

Fourth, dermatologic injury remains a practical concern in cumulative or unsupervised use. Case reports describe electrode-site burns resulting from poor contact, dried sponges, high impedance, or excessively prolonged sessions, while systematic reviews identify skin reactions as the most common – though typically transient – adverse effects.[88–90] Device quality, electrode contact area and impedance are therefore critical factors, particularly outside controlled laboratory settings.

Fifth, brain-state dependence – fatigue, sleep loss, circadian phase, stress – can gate both the magnitude and direction of tDCS effects.[91] Sleep deprivation, for example, upscales cortical excitability and alters plasticity; acute stress can potentiate stimulation effects on emotional working memory.[92,93] In sport, where load, travel and arousal vary, unmeasured state shifts could mask benefits or foster maladaptation over time.

Finally, the context of use is critical. Expert groups have cautioned against DIY enhancement, noting that protocols often drift, device quality varies and side-effect reporting is inconsistent. By contrast, remotely supervised home-use (RS-tDCS) programs employing certified hardware, impedance monitoring and telehealth oversight have demonstrated good feasibility and tolerability, suggesting a safer operational model should athletes use tDCS outside clinical settings.[94,95]

Conclusions

The tDCS has moved from curiosity to a seriously discussed tool in high-performance sport. Across studies, its ergogenic effects are real but modest on average, heterogeneous across individuals and protocols, and confounded by methodological issues – features that warrant caution in interpreting headline gains. Mechanistic understanding in athletic contexts also lags behind clinical insight, and tDCS use is practically undetectable outside of self-report, which complicates governance.

Assessed against the 2021 WADA Code, the technology clearly satisfies the "potential to enhance performance" criterion and, when used explicitly for competitive advantage, raises a credible "spirit of sport" concern. By contrast, within established parameters tDCS shows a strong safety profile; the principal unknowns relate to long-term, performance-oriented use and to misuse outside supervision. Therapeutic applications under medical oversight remain ethically and regulatorily distinct.

Policy therefore sits on a knife-edge between letters and limits of the Code and what is practicable and proportionate. On the one hand, meeting 2 of 3 criteria would technically justify prohibition. On the other, enforceability is poor, the mean performance effect is small and variable, and a ban risks driving clandestine use and eroding transparency – considerations that have previously informed a "monitor rather than prohibit" stance for borderline methods.

A measured course of action follows from these findings: 1) continue open monitoring rather than immediate prohibition; 2) publish sport-specific guidance on safe, supervised use (including screening, dosing, skin care, and device quality), with clear separation between therapeutic use and performance enhancement; 3) require disclosure in elite training environments to preserve transparency; and 4) prioritize research on long-term safety, state-dependence, individual variability, and off-target cognitive trade-offs in athlete-style protocols. Should future evidence demonstrate robust, reliable and material competitive advantages – or reveal substantive health risks – revisiting inclusion on the Prohibited List would be justified. For now, the balance of scientific, ethical and practical considerations supports cautious oversight and ongoing evidence-gathering over an outright ban.

Use of AI and AI-assisted technologies

Not applicable.

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A systematic review and network meta-analysis of RCTs: The effect of systemic immunotherapies on treatment outcomes and quality of life in patients with metastatic colorectal cancer

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Abstract

Background. The impact of different systemic treatments on the health-related quality of life (HRQoL) in patients with metastatic colorectal cancer (mCRC) is still unclear.

Objectives. To compare and evaluate the effects of various systemic interventions on the HRQoL in patients with mCRC.

Material and methods. A thorough search was conducted using four electronic databases (PubMed, Embase, Scopus, and Cochrane Library) to locate relevant literature published in peer-reviewed journals. The risk ratio (RR) and 95% confidence intervals (95% CIs) were calculated. The heterogeneity was examined using p-value, Cochrane Q and I^2 statistics. The analysis was performed with RevMan 5.4. At least 2 treatment regimens were tested in phase II or III trials. The primary objectives were short- and long-term mean changes in EORTC QLQ-C30 GHS/QoL (European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire – Core 30, Global Health Status/Quality of Life) and EQ-5D health utility scores (EuroQol 5 Dimension). Multivariate meta-regression was used to combine direct and indirect comparison data into a network meta-analysis with a random-effects consistency model. The surface under the cumulative ranking (SUCRA) probability curve was used to compare different systemic therapy combinations.

Results. This meta-analysis involved 15 relevant randomized clinical trials (RCTs) with 7,699 patients with mCRC. The study had a low risk of bias (RoB) ($p > 0.05$ for Egger's regression test) and moderate heterogeneity ($I^2 < 60\%$). Results indicated that systemic therapies were substantially more effective than other agents in improving the overall survival (OS) of patients (RR: 0.85 (95% CI: 0.79–0.90); $p < 0.001$, $I^2 < 60\%$], ensuring progression-free survival (PFS) (RR 0.80 (95% CI: 0.75–0.85); $p < 0.001$; $I^2 < 60\%$), suggesting that there was moderate heterogeneity. Long-term findings demonstrated that cetuximab was the most effective treatment and was linked to a significant improvement in GHS/QoL.(coefficient [95% CI] = 0.23 [–0.68 to 0.96], $p = 0.747$). In terms of the long- and short-term results of change in QLQ-C30 GHS/HUS QoL score, cetuximab performed the best (SUCRA 95.12%) among all therapies. It also showed a substantial advantage in comparison to chemotherapy (mean deviation (MD) 0.06, 95% CI: 0.01 to 0.09).

Conclusions. This network meta-analysis found that cetuximab monotherapy improves HRQoL and prolongs OS and PFS in patients with mCRC.

Key words: colorectal cancer, metastasis, health-related quality of life, immunotherapies, patient-reported outcome

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Introduction

Colorectal cancer (CRC) is a malignancy that specifically targets the colon, the large intestine or the rectum. It has the potential to inflict significant damage and result in fatalities. The risk of CRC increases with age.[1] Colorectal cancer is the 3rd most prevalent cancer worldwide and is responsible for the 3rd greatest number of cancer-related deaths.[2] Screening can identify initial instances of CRC, which manifest as benign polyps without any symptoms. Colorectal cancer symptoms may vary depending on the size and location of the tumor. However, most people notice changes in their bowel habits, changes in their stool consistency, the presence of blood in their stool, and abdominal discomfort.[3,4] Metastatic colorectal cancer (mCRC) occurs in around 25% of all patients, and around 50% of individuals without metastases will eventually develop metastasis.[5]

Factors such as the tumor's size and location and extent of metastasis determine the CRC treatment. Typical treatment methods consist of surgical removal of the malignancy, systemic therapy (such as chemotherapy, immunotherapy, hormone therapy, or targeted therapy) and radiation therapy.[6–8] Systemic therapies are medications that target and eradicate cancer cells across the entire body, regardless of their location. The most commonly used systemic treatments for CRC are irinotecan, cetuximab, bevacizumab, and panitumumab.[9–11] The longevity of patients with mCRC has recently increased as a result of breakthroughs in targeted therapy. The 3-year survival rate for patients with mCRC has surged from 25% to 30% over the course of a decade.[12] Several randomized clinical trials (RCTs) have investigated different systemic interventions for the mCRC management.[13–15] Nevertheless, the efficacy results of those RCTs have been inconsistent, which makes it difficult to draw a definitive conclusion regarding the preferred strategy.

With the improvement in survivability rates and treatment choices, it has become critical to prioritize a satisfactory HRQoL alongside extending life expectancy. Therefore, researchers evaluated HRQoL as a measure of patients' self-reported outcomes to assess the impact of different systemic interventions on patients' health. There are 5 areas of quality of life (QoL) that HRQoL looks at: physical, role, cognitive, emotional, and social functioning. Health-related quality of life uses 2 different scales: the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire – Core 30, Global Health Status/Quality of Life (EORTC QLQ-C30 GHS/QoL) general health status and QoL questionnaire and the EuroQoL-five-dimension index questionnaire (EQ-5D).[16]

The EORTC QLQ-C30 questionnaire includes a global health status and quality of life (GHS/QoL) scale based on a 7-point Likert scale from "very poor" to "excellent," and the EQ-5D questionnaire, which includes the visual analogue scale (VAS) and health utility scores (HUS).[17] Nevertheless, there is a lack of research comparing the HRQoL of patients with mCRC across various systemic interventions. Therefore, we conducted a network meta-analysis (NMA) of 15 RCTs,[18–32] which were selected in accordance with pre-specified inclusion and exclusion criteria, to comprehensively compare the impacts of various systemic interventions on HRQoL in patients with mCRC and to compile relevant references for patients, clinicians and cancer management guidelines.

Objectives

The rationale of this systematic review and network meta-analysis was to compare and evaluate the effects of various systemic interventions on the HRQoL in patients with mCRC.

Materials and methods

The study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) extensions for NMA.[33]

Eligibility criteria

The following eligibility criteria were implemented using the PICOS (Population, Intervention, Comparison, Outcomes, and Study) framework: The Population of this study included adult patients who diagnosed with advanced or metastatic unresectable CRC, based on either histological or cytological confirmation. There were no restrictions placed on individual-level characteristics. All systemic approaches, such as pharmacological, surgical, radiological, and combination therapies, were included in the analyzed interventions and comparisons. Included RCTs should have reported at least 1 of the following outcomes as the primary clinical outcome: EORTC QLQ-C30 GHS/QoL score, EQ-5D VAS and HUS. We selected the EORTC QLQ-C30 and EQ-5D as assessments of HRQoL frequently included in RCTs encompassing mCRC. The GHS/QoL subscale from the EORTC QLQ-C30 questionnaire has a standard range of values from 0 to 100. A high score on the GHS/QoL scale indicates excellent global health status and a higher QoL, and vice versa. A change of 5 to 10 points on the GHS/QoL scale is accepted as the minimum clinically important difference (MCID) for GHS/QoL.

Information sources

To identify pertinent RCTs and published studies, we implemented an exhaustive search of PubMed, Scopus, Embase, and Cochrane Library by May 31, 2024. The key words that we used were "colorectal cancer," "systemic treatment," "metastatic colorectal cancer," "advanced colorectal cancer,"

Table 1. Database search strategy

Database	Search strategy
Scopus	#1 "Colorectal cancer," OR "systemic treatment," OR "metastatic colorectal cancer," OR "advanced colorectal cancer," OR "Health related quality of life," OR "Quality of life," OR "QoL," OR "patient reported outcome," OR "Colorectal carcinoma" OR "treatment section". #2 "Network meta-analysis," OR "systematic review," OR "systematic review and meta-analysis," OR "", OR "Meta-analysis," OR "RCT," OR "randomized controlled trial" #3 #1 AND #2
PubMed	#1 "Colorectal cancer" OR "systemic treatment" OR "metastatic colorectal cancer" [MeSH Terms] #OR "advanced colorectal cancer" OR "Health related quality of life" OR "QoL" OR "patient reported outcome" [All Fields]" OR "Colorectal carcinoma" OR "treatment section" OR "" [All Fields] #2 "Network meta-analysis," OR "systematic review,"" [MeSH Terms] OR "systematic review and meta-analysis," OR "Meta-analysis," OR "RCT" [All Fields] OR "randomized controlled trial" #3 #1 AND #2
Embase	#1 "Colorectal cancer"/exp$ OR "systemic treatment"/expOR "metastatic colorectal cancer"/exp OR "advanced colorectal cancer"/exp OR "Health related quality of life"/exp OR "Quality of life"/OR "QoL"/exp OR "patient reported outcome"/exp OR "Colorectal carcinoma"/exp OR "treatment section"/exp #2 "Network meta-analysis"/exp OR "systematic review"/exp OR "systematic review and meta-analysis"/exp OR "meta-analysis"/exp OR "RCT"/exp OR "randomized controlled trial"/exp #3 #1 AND #2
Cochrane Library	#1 (Colorectal cancer): ti, ab, kw@ OR(systemic treatment): ti, ab, kw OR(metastatic colorectal cancer): ti, ab, kw OR (advanced colorectal cancer): ti, ab, kw OR (Health related quality of life): ti, ab, kw OR (Quality of life) OR (QoL): ti, ab, kw OR (patient reported outcome): ti, ab, kw OR (Colorectal carcinoma): ti, ab, kw OR (treatment section): ti, ab, kw (Word variations have been searched) #2 (Network meta-analysis): ti, ab, kw OR (systematic review):ti, ab, kw OR (systematic review and meta-analysis): ti, ab, kw or (meta-analysis):ti, ab, kw or (RCT): ti, ab, kw or (randomized controlled trial):ti, ab, kw (Word variations have been searched) #3 #1 AND #2

MeSH terms – Medical Subject Headings; $ exp – explosion in Emtree (searching of selected subject terms and related subjects); @ ti, ab, kw – either title or abstract or key word fields.

"health-related quality of life," "quality of life," "QoL," "patient reported outcome," "colorectal carcinoma," "treatment section," "network meta-analysis," "systematic review," "systematic review and meta-analysis," "meta-analysis," "RCT," and "randomized controlled trial." Table 1 outlines the search strategy.

Search strategy

During the Scopus search, the specified key words were entered into the Title (ti)-Abstract (abs)-Keyword (key) field. The Cochrane search terms encompassed "metastatic colorectal cancer" and "systemic interventions." We employed the PICO framework[34] to establish precise selection criteria. In this context, "P" refers to patients diagnosed with colorectal cancer, "I" represents the inclusion of multiple systemic interventions, "C" represents a control group, and "O" encompasses the enhancement of clinical outcomes, specifically the improvement in HRQoL. During the course of this investigation, the research strategy was restricted to the utilization of RCTs. The inclusion criteria required that only papers written in English were considered. This was done to guarantee the accuracy of the data interpretation and analysis, taking into account the expertise of the research team. Furthermore, there were no publication date restrictions.

Selection process

The titles and abstracts of the included articles were initially reviewed by 2 researchers. This study was primarily designed using the phase II or III clinical trials, which examined multiple distinct treatments. For the purpose of eliminating redundancy, we exclusively included studies that yielded the most up-to-date and enlightening results and excluded studies that were not relevant to any comparisons or investigated different doses with the same combinations.

Data extraction

Two researchers independently extracted the pertinent data. Discrepancies were resolved in collaboration with other investigators. The retrieved information included following specific characteristics of trials that met the eligibility criteria: study ID and year, journal of publication, country of the study, total number of participants, number of participants in the intervention arm, number of participants in the control arm, intervention medications, control medications, age of participants, sex (M/F), line of treatment, and primary outcomes. Clinical outcomes analyzed were mean change from baseline in EORTC QLQ-C30 GHS/QoL and EQ-5D HUS scores.

Study risk of bias assessment

The Cochrane Collaboration's risk of bias (RoB) tool[35] was used to evaluate the quality of the included studies. The qualifying studies were classified into 3 categories: high, low or uncertain risk. The existence of publication bias was evaluated with a funnel plot[36] and the application of the Egger's regression test.[37] Any result with a p-value of less than 0.05 was considered evidence of bias.[38]

Synthesis methods

The impact of the intervention on EORTC QLQ-C30 GHS/QoL scores in the short and long term and the mean change in EQ-5D HUS from baseline to endpoint were the primary outcomes. The long-term period was defined as the time from baseline to the endpoint, while the short-term period was defined as a period of 8–12 weeks from baseline. The data from the included trials were used to calculate the risk ratio (RR) and 95% confidence interval (95% CI)[39] for overall survival (OS) and progression-free survival (PFS) of patients receiving the intervention treatment compared to the control group using the DerSimonian and Laird random-effects model.[40] A random-effects consistency model[41] was implemented to conduct a network meta-analysis. This involved employing a multivariate meta-regression technique[42] to combine data from both direct and indirect comparisons. The network meta-analysis maintained the transitivity assumption, which ensured that interventions were comparable. The coherence between direct and indirect evidence was evaluated using node-splitting methods, and any inconsistencies were carefully examined for methodological or clinical reasons. The I^2 statistic was used to assess the heterogeneity of the included studies. A value greater than 50% indicated a moderate degree of heterogeneity.[43] A two-tailed test with a p-value less than 0.05 was considered statistically significant.[44] In addition, treatment solutions were evaluated and graded based on the surface under the cumulative ranking (SUCRA) probability.[45] A greater efficacy was associated with higher SUCRA scores. The SUCRA values were used to determine the relative ranking of treatments. The scores, which ranged from 0 to 100%, represented the likelihood that each treatment was the most effective option within the network. Higher values indicated superior relative performance.

Results

Literature search results

A comprehensive electronic search of several databases was carried out and a total of 203 articles were identified that met the inclusion and exclusion criteria as indicated in the PICOS. A total of 48 entries were excluded due to their duplication, and 155 records were identified. Subsequently, 104 records underwent review, and 51 were excluded due to the presence of invalid titles and abstracts. Following the completion of complementary screening, a total of 51 records were assessed to determine their eligibility. However, after applying the established criteria for including and excluding studies, 36 studies were found to be ineligible and were therefore excluded. The studies were excluded mainly because they did not meet the criteria for inclusion, did not provide sufficient data to create

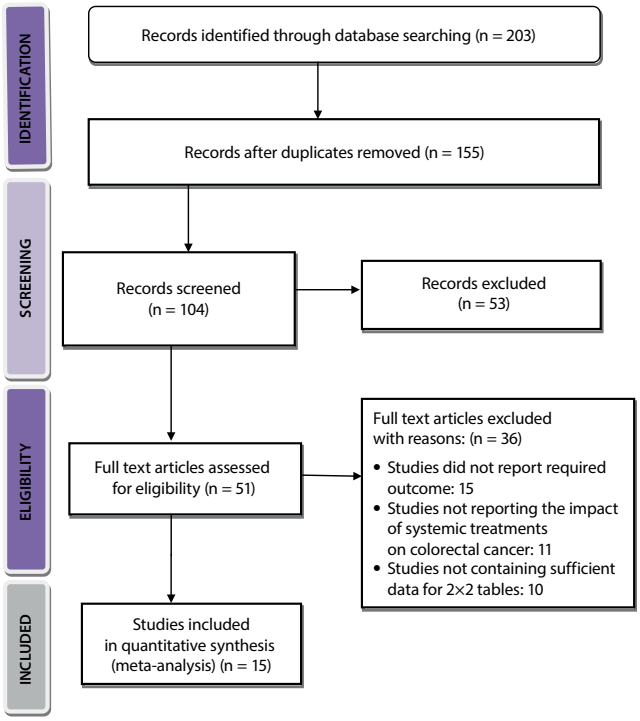

Fig. 1. Study flow diagram of Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA)

2×2 tables and did not have relevant outcome measures. Finally, 15 RCT studies that met the specified inclusion criteria and were published between 2000 and 2024 were included in this review and meta-analysis, as illustrated in Fig. 1.

This meta-analysis is a comprehensive examination of 7,699 individuals diagnosed with CRC, spanning a variety of age groups. The efficacy of various therapeutic agents, including bevacizumab, panitumumab, cetuximab, FOLFOX, CAPOX, irinotecan, fluorouracil, leucovorin, and folinic acid, was evaluated in the included RCTs. The main characteristics of the studies included in this meta-analysis are shown in Table 2. The meta-analysis was subsequently conducted using the aforementioned event data.

Risk of bias assessment

To ascertain the study's overall quality rating, we implemented an evaluation of the potential RoB. The results of the RoB assessment for each of the 15 included RCTs are presented in Table 3, which was conducted using the pre-established questionnaire. According to the traffic light plot in Fig. 2 and the summary plot for bias assessment in Fig. 3, the current meta-analysis presents a low RoB. We discovered that 11 of the 15 RCTs had a low RoB. However, 2 RCTs conducted by Peeters et al.[24] and Tol et al.[31] demonstrated a moderate degree of bias resulting from deviations from the intended intervention. Lang et al.[21] and Thomsen et al.[30] conducted 2 additional RCTs that had a substantial RoB in outcome measurement.

Table 2. Characteristics of the included studies

Study ID and year	Journal of publication	Country of study	Total participants	Participants in intervention arm	Participants in control arm	Intervention	Control	Age of participants	Sex (M/F)	Line of treatment	Primary outcomes
Avallone et al. 2021[18]	*JAMA Network Open*	Italy	230	115	115	modified FOLFOX-6/modified CAPOX + bevacizumab administered 4 days before chemotherapy	modified FOLFOX-6/modified CAPOX + bevacizumab administered on the same day as chemotherapy	61 ±8	119/111	F	LT & ST GHS
Ballhausen et al. 2023[19]	*European Journal of Cancer*	Germany	248	125	123	panitumumab + FU/FA	FU/FA	66 ±14	133/115	F	LT & ST GHS
Bennett et al. 2011[20]	*British Journal of Cancer*	USA	576	284	292	panitumumab + FOLFOX4	FOLFOX4	60.1 ±11.3	131/445	F	LT & ST HUS
Lang et al. 2013[21]	*European Journal of Cancer*	Hungary	627	300	327	cetuximab + FOLFIRI	FOLFIRI	60 ±14	125/502	F	GHS (ST and LT)
Liu et al. 2020[22]	*Frontiers in Pharmacology*	China	320	160	160	traditional Chinese medicine combined with chemotherapy and cetuximab or bevacizumab	placebo combined with chemotherapy and cetuximab or bevacizumab	63.89 ±10.11	123/97	F + S	LT GHS
Odom et al. 2010[23]	*International Journal of Colorectal Diseases*	USA	208	112	96	panitumumab + BSC	BSC	62 ±10	136/72	S	LT & ST HUS
Peeters et al. 2014[24]	*Annals of Oncology*	Belgium	597	303	294	panitumumab + FOLFIRI	panitumumab	60 ±18	127/470	S	LT & ST HUS
Price et al. 2014[25]	*Lancet Oncology*	Australia	872	499	500	panitumumab	cetuximab	61 ±8	127/	S	LT & ST HUS
Raimondi et al. 2020[26]	*European Journal of Cancer*	Italy	210	107	103	panitumumab + 5-FU/LV	panitumumab	64 ±10	131/79	F	LT & ST GHS
Saltz et al. 2000[27]	*New England Journal of Medicine*	USA	457	231	226	irinotecan + FU + LV	FU + LV	63 ±17	119/338	F	LT & ST GHS
Seymour et al. 2013[28]	*Lancet Oncology*	UK	460	230	230	panitumumab + irinotecan	irinotecan	63 ±9	139/321	S	LT GHS
Sobrero et al. 2008[29]	*Journal of Clinical Oncology*	Italy	1298	648	650	cetuximab + irinotecan	irinotecan	65 ±10	126/1172	S	LT & ST GHS
Thomsen et al. 2017[30]	*European Journal of Cancer*	Norway	509	343	169	FLOX/I2: cetuximab + FLOX/C	cetuximab and intermittent FLOX	61 ±13	178/165	F	LT & ST GHS
Tol et al. 2010[31]	*New England Journal of Medicine*	the Netherlands	736	368	368	chemotherapy + bevacizumab	chemotherapy + bevacizumab + cetuximab	62 ±18	119/617	F	LT GHS
Yamaguchi et al. 2017[32]	*Clinical Colorectal Cancer*	Japan	351	170	181	cetuximab + FOLFIRI	FOLFIRI	62 ±15	110/241	F	LT & ST GHS

F – first-line treatment; S – second-line treatment; LT – long-term; ST – short-term; GHS – quality of life questionnaire QLQ – C30 global health status; BSC – best supportive care; FOLFOX – levo-folinic acid, fluorouracil, and oxaliplatin; modified CAPOX – capecitabine and oxaliplatin; HUS – EQ-5D-5L Euro-Quality of Life 5-dimension level health utility scores; FU – fluorouracil; LV – leucovorin; FA – folinic acid.

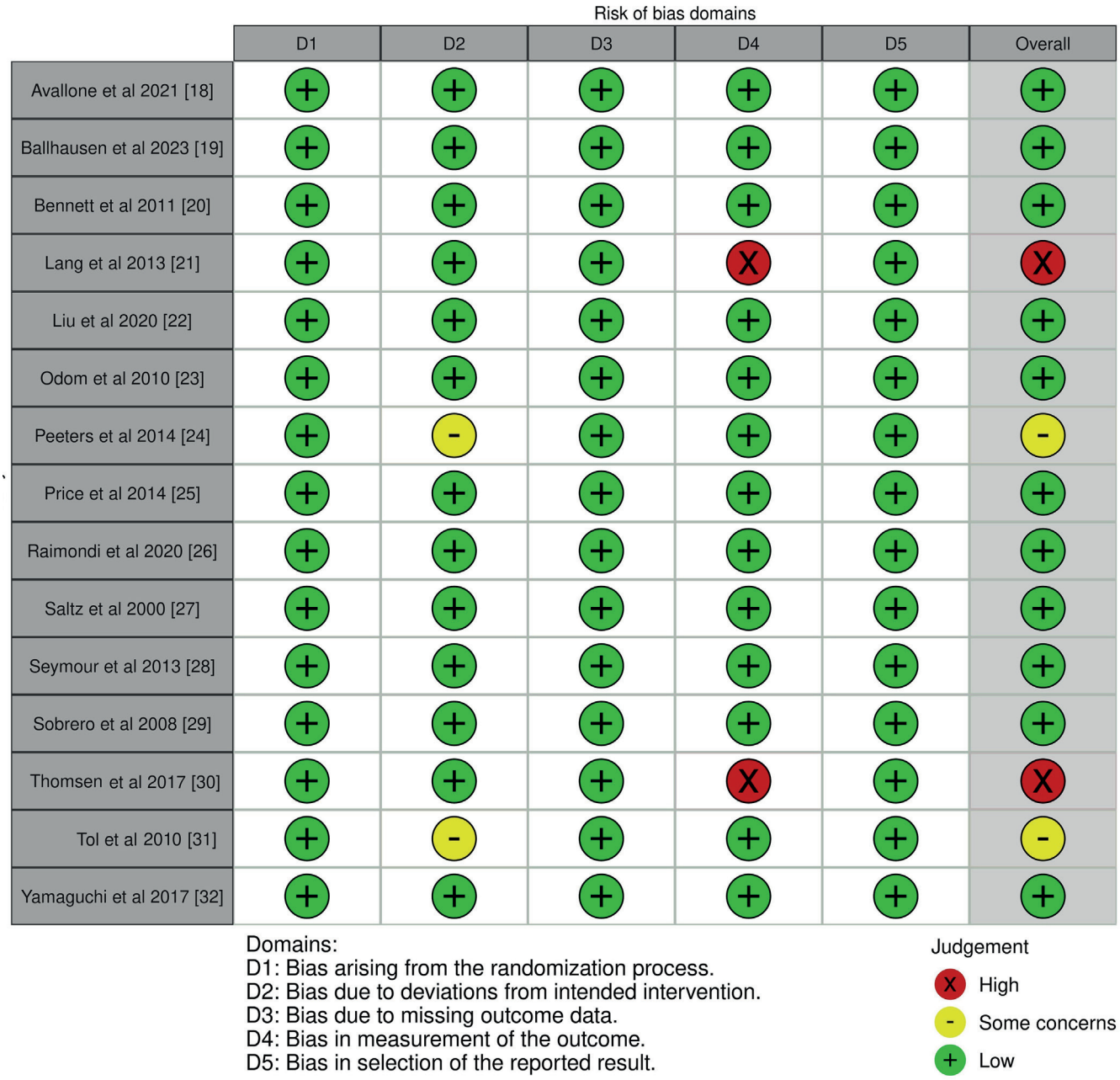

Fig. 2. Traffic light plot to analyse the risk of bias

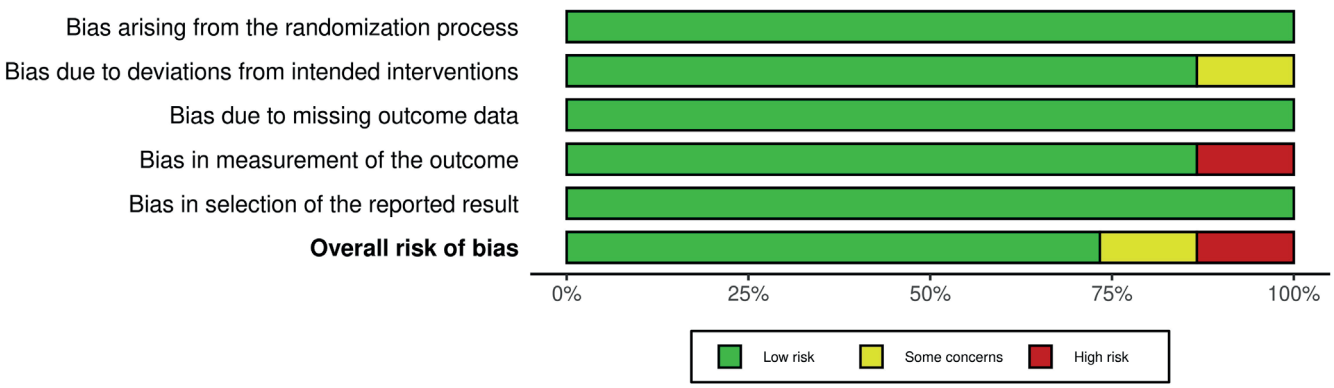

Fig. 3. Summary plot depicting the risk of bias

Table 3. Risk assessment for included studies

Study ID and year	Avallone et al. 2021[18]	Ballhausen et al. 2023[19]	Bennett et al. 2011[20]	Lang et al. 2013[21]	Liu et al. 2020[22]	Odom et al. 2010[23]	Peeters et al. 2014[24]	Price et al. 2014[25]	Raimondi et al. 2020[26]	Saltz et al. 2000[27]	Seymour et al. 2013[28]	Sobrero et al. 2008[29]	Thomsen et al. 2017[30]	Tol et al. 2010[31]	Yamaguchi et al. 2017[32]
Was a consecutive or random sample of patients enrolled?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Did the study avoid inappropriate exclusions	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Did all patients receive the same reference standard	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Were all patients included in the analysis	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Was the sample frame appropriate to address the target population?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Were study participants sampled in an appropriate way?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Were the study subjects and the setting described in detail?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Were valid methods used for the identification of the condition?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Was the condition measured in a standard, reliable way for all participants?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y – yes; N – no.

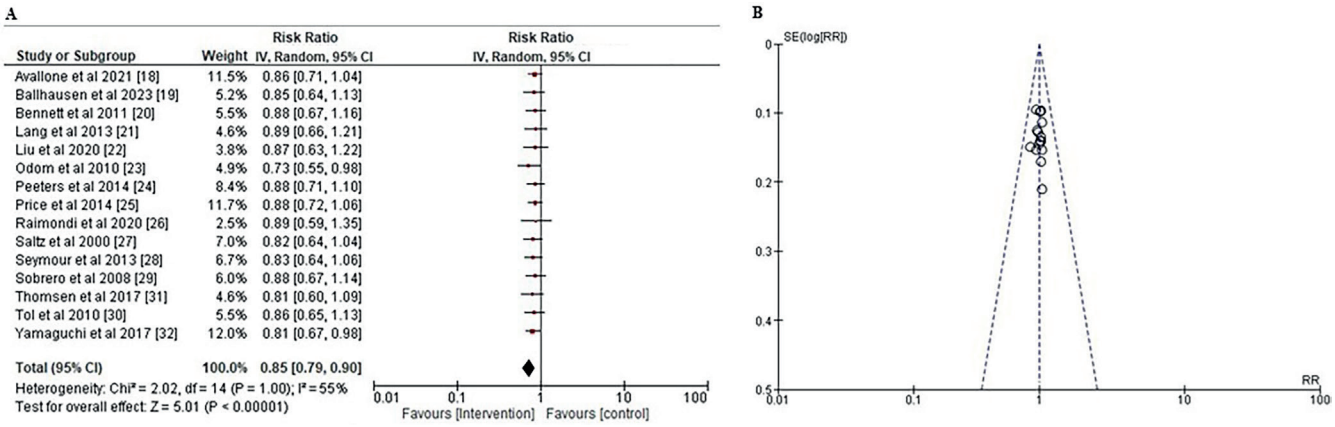

Fig. 4. A. Forest plot illustrating the overall survival (OS) of patients receiving the intervention treatment compared to the control group; B. Funnel plot

95% CI – 95% confidence interval; RR – risk ratio; SE – standard error.

Findings of the statistical analysis

The current meta-analysis included 7,699 patients with CRC from 15 selected RCTs to evaluate the impact of different systemic interventions on HRQoL in patients with mCRC. The following conclusions were obtained from the statistical analysis of the primary study outcome:

The impact of various systemic interventions on patients' overall survival rate

Measuring the OS of patients in a clinical trial is a reliable method to assess the effectiveness of a newly developed medicine. Overall survival in the context of cancer treatments refers to the duration of time from diagnosis or the initiation of treatment for cancer until death.[46,47] To assess the effect of various systemic interventions on the OS of patients with CRC, the RR, along with the 95% CI based on the event data extracted from the included studies, was calculated as shown in Fig. 4A. Results indicated that systemic therapies were substantially more effective than other agents in improving the OS of patients with a risk ratio less than 1 (RR: 0.85 (95% CI: 0.79–0.90); $\chi^2 = 2.02$, degrees of freedom (df) = 14, Z = 5.01, and $p < 0.001$). The I^2 value of 55% indicated moderate heterogeneity. In addition, the symmetrical funnel diagram shown in Fig. 4B and

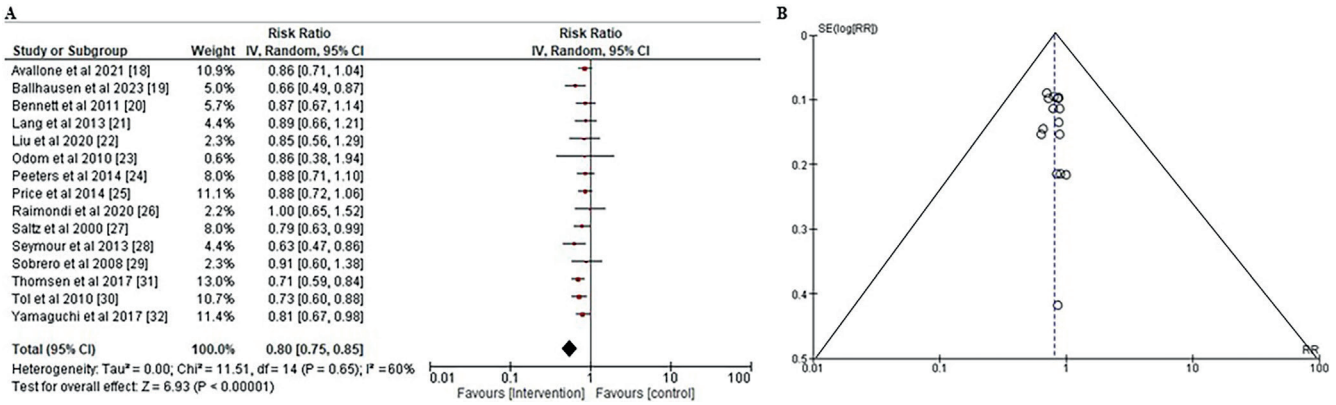

Fig. 5. A. Forest plot illustrating progression-free survival (PFS) of patients receiving intervention therapy compared to control; B. Funnel plot

95% CI – 95% confidence interval; RR – risk ratio; SE – standard error.

the statistically insignificant p statistic of Egger's test (p = 0.262), which is higher than the predetermined significance threshold of 0.05, indicate a low probability of publication bias.

The impact of various systemic interventions on progression-free survival of patients

Progression-free survival refers to the time after surgical removal of the tumor and the first signs of tumor recurrence or disease progression.[48] To evaluate the impact of a variety of systemic interventions on the PFS of CRC patients, the RR and the 95% CI were calculated using the event data extracted from the included studies, as illustrated in Fig. 5A. The findings showed that systemic interventions were significantly more successful than other treatments in ensuring PFS with a RR of less than 1 (RR: 0.80 (95% CI: 0.75–0.85); $\chi^2 = 11.51$, df = 14, Z = 6.93, and p < 0.001). The I^2 value of 60% indicated of moderate heterogeneity. Moreover, the symmetrical funnel plot illustrated in Fig. 5B, in conjunction with the statistically insignificant p-value of Egger's test (p = 0.327), above the preset significance threshold of 0.05, suggested a minimal likelihood of publication bias.

Results of network meta-analysis

A NMA was conducted to evaluate the efficacy of various systemic interventions for CRC. This analysis entailed the synthesis of data from the included RCTs, which included both direct and indirect evidence. Figure 6 illustrates a network depiction that illustrates the relationships and interactions between various medications, as well as a collection of network estimates that illustrate the effects of various systemic interventions for all fundamental comparisons. The network graph is represented by nodes or vertices, which represent the total number of interventions. Edges or lines are used to represent the total number of direct comparisons between the nodes of the network. The number of studies

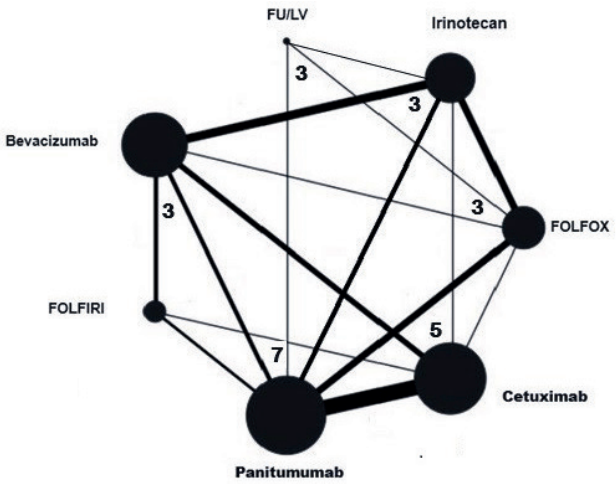

Fig. 6. Network plot for overall analysis of quality-of-life questionnaire (QLQ-C30) for all patients

represents the total number of studies included in the network. The degree of a node is the number of edges incident to the node, with loops counted twice.[49]

We observed a consistent correlation between the direct and indirect evidence while conducting the pairwise meta-analyses and analysing the data. No significant divergences between direct and indirect estimates were observed during the node splitting analysis, as all p-values in the inconsistency test were greater than 0.05. The consistency and inconsistency models did not exhibit any discernible distinctions. There was no significant difference in the consistency and inconsistency models for the improvement in OS and PFS using systemic interventions such as FOLFOX, bevacizumab, panitumumab, cetuximab, FOLFIRI, irinotecan, fluorouracil, leucovorin, and folinic acid ($\chi^2 = 0.53$, p = 0.854).

Furthermore, our results did not differ significantly between direct and indirect evidence: 1) for panitumumab vs control (coefficient [95% CI] = −0.06 [−0.47 to 0.72], p = 0.631), 2) for cetuximab vs control (coefficient [95% CI] = 0.23 [−0.68 to 0.96], p = 0.747), 3) bevacizumab vs control (coefficient [95% CI] = −0.42 [−1.87 to 1.46], p = 0.681), or 4) irinotecan vs control (coefficient

[95% CI] = −0.34 [−1.54 to 1.31], p = 0.549). Long-term results showed that cetuximab performed best and was associated with significantly improved GHS/QoL.

Cumulative ranking of different systemic treatments for colorectal cancer

The SUCRA curve was developed to create a hierarchy of systemic therapies that considers the variability of their respective treatment effects. The SUCRA value quantifies the average rank and posterior probability of each medicine being among the top n alternatives. A higher SUCRA rating indicates a superior ranking for the therapy. The SUCRA curve in Fig. 7 compares the effectiveness of various systemic interventions, including FOLFOX, bevacizumab, panitumumab, cetuximab, FOLFIRI, irinotecan, fluorouracil, leucovorin, folinic acid, and their combinations. The results indicate that cetuximab had the highest performance (SUCRA 95.12%) in improving both short-term and long-term QLQ-C30 GHS/HUS QoL scores. Furthermore, cetuximab showed a significant advantage over chemotherapy (MD 0.06, 95% CI: 0.01–0.09).

Discussion

Our findings indicate that there is a significant advantage in continuing the full induction regimen until disease progression, as opposed to periods of observation or maintenance therapy, in terms of both PFS and OS. Furthermore, our findings indicate that cetuximab offers a substantial benefit compared to alternative control treatments in terms of prolonging the time before disease progression and improving OS rates. An epidermal growth factor receptor inhibitor medication, cetuximab, is used to treat mCRC. It is marketed under the brand name Erbitux. Cetuximab is administered intravenously as a chimeric monoclonal antibody.[50] In general, the preferred maintenance treatment appears to be cetuximab monotherapy or a combination of cetuximab and bevacizumab. Both single-agent panitumumab and bevacizumab seem to be viable choices. Monotherapy has demonstrated significant improvements in patient-reported HRQoL compared to combination therapy and immunotherapy and targeted therapy. Immunotherapy and targeted therapy showed the best results in terms of HRQoL.[51–53] The addition of targeted therapy to chemotherapy did not improve patient performance in terms of HRQoL. The results demonstrated that cetuximab was highly effective in improving HRQoL in the management of mCRC, particularly when the baseline gene expression was not specified. This was particularly the case in patients who had previously undergone systematic treatment. A review of the overall impact on patients' QoL, effectiveness and safety indicated that cetuximab is a beneficial treatment for patients who have wild-type Kirsten rat sarcoma virus (KRAS) mutations. The sensitivity analysis confirmed the robustness of the main results. After differentiating

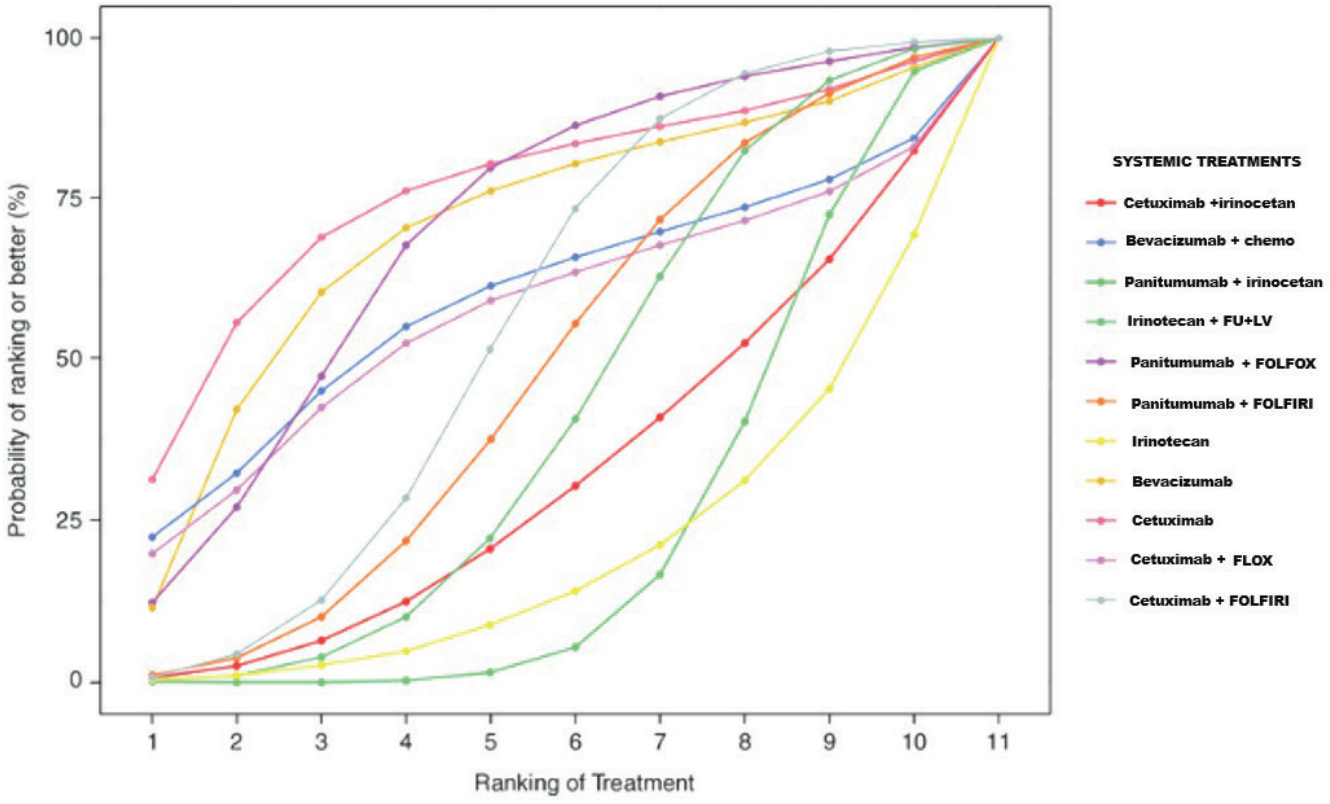

Fig. 7. Surface under the cumulative rank (SUCRA) curve for different systemic treatments of colorectal cancer

between chemotherapy medicines and removing RCTs with inconsistent baseline characteristics, the overall conclusions remained unaltered. Studies have been conducted to determine the most effective length of first-line induction chemotherapy in mCRC following an initial response or stable illness. These studies showed that it is possible to take a "management break" and then resume the same treatment when the disease progresses. Our study confirms that maintenance therapy with systemic treatment is a viable alternative to continuing the full course of treatment.

One significant benefit of reducing treatment intensity after attaining the highest level of response (usually within 3–4 months) with initial therapy is the reduction of harmful side effects, enhancement of overall wellbeing and potential cost improvement. The included RCTs clearly showed that patients who received maintenance treatment had lower rates of adverse effects than those who received the continuous induction regimen. These non-cytotoxic biologic medicines enable the preservation of benefits while further minimizing toxic effects. The use of cetuximab, panitumumab and bevacizumab has led to improved outcomes when incorporated into initial cytotoxic induction regimens for patients with mCRC. Although cost-effectiveness analysis has its limitations, it can provide valuable information to support shared decision-making with the patient in choosing the most effective maintenance approach. However, the pairwise comparison in the meta-analysis showed that this improvement was statistically significant for each of them compared with observation.

Earlier meta-analyses investigated the effectiveness of continuous vs intermittent chemotherapy approaches in patients with mCRC by analyzing data from RCTs.[54–56] The results of this study suggest that intermittent methods do not lead to a clinically meaningful reduction in OS. Conversely, our study employed a network meta-analysis approach, exclusively encompassing RCTs that employed combination induction chemotherapy. The findings suggest that systemic interventions represent an established treatment protocol for patients with mCRC. Nevertheless, biomarkers are an indispensable tool in future research, facilitating the implementation of precision medicine, identification of distinct patient subgroups, optimization of treatment strategies, prediction of disease progression, and early detection of responses to therapy. Biomarkers play an essential role in risk assessment, enabling clinicians to personalize interventions and enhance patient outcomes. Therefore, integration of biomarkers in research paradigms will revolutionize patient care, yielding personalized and targeted treatments that maximize efficacy while minimizing adverse effects.[57] Likewise, to move personalized medicine forward, make early intervention easier and improve therapeutic efficacy, more research needs to be done on figuring out predictive biomarkers and reducing side effects. By identifying and characterizing biomarkers, medical practitioners can implement precision-based treatment plans that minimize the risk of harm to patients and enhance patient safety and outcomes, thereby advancing the frontiers of precision medicine.[58] The integration of biomarkers in clinical practice for systemic immunotherapies in CRC will enable personalized treatment planning, enhanced patient selection and real-time monitoring. This will optimize treatment outcomes, reduce toxicity and streamline clinical trials, ultimately revolutionizing CRC management with more effective and adaptive treatment strategies.[59,60]

Limitations

Our study has limitations when it comes to network analysis and individual trials. Our initial analysis was conducted using data at the study level, rather than individual patient data, which restricted the effectiveness of our research. In addition, the lack of blinding of outcome assessors and the need for cautious interpretation of PFS in clinical trials pose a potential risk of detection bias. Furthermore, the majority of included RCTs did not differentiate between mutation types and gene expression levels when reporting HRQoL, so it was not possible to make indirect comparisons based on mutation target or patient gene expression levels. Moreover, the HRQoL-related data in this investigation primarily came from populations where the baseline expression levels of patients were not specified. Hence, it is necessary to get further clinical evidence to confirm the efficacy of various therapy methods in populations with precise levels of target expression. The lack of data on physical functioning, social functioning and tiredness ratings limited the ability to examine more complex components of HRQoL.

Conclusions

Immunotherapies and targeted medicines provide the best results in terms of improving patient HRQoL, but complicated therapy regimens are not as beneficial. Monotherapy, particularly with cetuximab, substantially improves HRQoL and provides a longer duration of OS and PFS compared to other control treatments. Patients with mCRC who have achieved stable disease after a certain length of induction treatment (3–4 months) are advised to undergo a maintenance schedule using cetuximab. However, the selection of the most efficient maintenance solutions should be based on considerations such as patient preference, the cost of treatment and the possibility for harmful consequences.

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Sex-related differences in the association of obesity described by emergency medical teams on outcomes in out-of-hospital cardiac arrest patients

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Abstract

Background. Out-of-hospital cardiac arrests (OHCA) are a major global health concern, occurring frequently worldwide. Obesity may impact outcomes in OHCA patients.

Objectives. This study aimed to assess the impact of obesity on the return of spontaneous circulation (ROSC) in OHCA patients, considering sex differences.

Material and methods. A retrospective cohort study was conducted, analyzing medical records of patients assisted by the Emergency Medical System (EMS) in Poland from January 2021 to June 2022. The study included 33,636 patients with OHCA. Obesity status was determined using ICD-10 codes (E66) and descriptive diagnoses recorded by EMS teams.

Results. Univariate analysis indicated that obesity decreased the odds of ROSC by 25.47% (odds ratio (OR) = 0.75, 95% confidence interval (95% CI): 0.61–0.92) in women and by 19.76% (OR = 0.80, 95% CI: 0.66–0.97) in men. However, multivariate analysis, adjusting for confounding variables, did not confirm a statistically significant impact of obesity on ROSC outcomes. The likelihood of ROSC was significantly higher in individuals with an initial ventricular fibrillation (VF) or pulseless ventricular tachycardia (pVT) rhythm compared to Asystole/pulseless electrical activity (PEA), being 4.204 times higher in women (95% CI: 3.525–5.014) and 3.655 times in men (95% CI: 3.320–4.023). Out-of-hospital cardiac arrest in a public place increased the odds of ROSC more than twofold for both sexes (women: OR = 2.20, 95% CI: 2.00–2.43; men: OR = 2.13, 95% CI: 1.98–2.29). Among women without obesity, hypertension decreased the odds of ROSC by 11.11% (OR = 0.89, 95% CI: 0.81–0.99).

Conclusions. Our study demonstrated that obesity was not an independent predictor of ROSC in OHCA patients. Different predictors of ROSC were identified for men and women. Initial VF/pVT rhythm, location of OHCA and age were the significant factors influencing ROSC.

Key words: obesity, cardiopulmonary resuscitation, OHCA, ROSC

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Background

Out-of-hospital cardiac arrests (OHCA) are a significant global health issue, with an average incidence of 55 cases per 100,000 people annually.[1,2] It is estimated that there are between 300,000 and 700,000 cases each year in the European Union, with a survival rate of 8–10%.[2–4] These situations, which occur suddenly and unexpectedly, are associated with a high risk of mortality and severe neurological failure.[5,6] In Spain, there are approx. 30,000 cases of OHCA annually, resulting in around 20,000 deaths. In Poland, there are approx. 64,000 cases of OHCA annually, with a survival rate close to 20%.[7–9] Although the return of spontaneous circulation (ROSC) occurs in 10–50% of patients with OHCA after starting advanced life support (ALS), mortality rates remain very high. According to the European Resuscitation Council (ERC), OHCA results in approx. 275,000 deaths annually in Europe.[9] Other factors influence the survival of these patients, such as the place of occurrence of the event, the presence or absence of obesity, or their sex.[3,10,11]

We are faced with another global public health problem, obesity. This disease affects up to 53% of the European population and 42% of Americans.[3,12–14] An abnormal body mass index (BMI), which is more prevalent among women than in men,[15,16] is a factor that worsens the prognosis of patients with OHCA. This is due to the pathophysiology of the disease itself and the increased risk of cardiovascular events such as hypertension (HT), coronary events or heart failure (HF), as well as the difficulty when performing chest compressions.[3,5,12] Although acute coronary syndrome (ACS) is the leading cause of OHCA and obesity is a recognized risk factor for ACS, studies such as those by Tanaka et al. and Berdowski et al. confirm these associations but do not account for sex differences.[1,17]

The scientific literature agrees that there are differences in the diagnosis and treatment of cardiovascular events according to sex, with women who suffer OHCA having more unfavorable prognostic factors[3,17,18] and fewer probability of overall survival and survival with a good neurological condition.[19,20] In general, we observed an underdiagnosis of cardiovascular risk in women,[20,21] with this sex being the most affected by cardiovascular diseases (CVD).[18,20,22] On the one hand, studies show that women have a greater probability of experiencing OHCA in the absence of witnesses because women tend to spend more time alone or at home since they have a greater life expectancy than men and are more likely to be widowed.[19,20,22–24] These factors cause a delay in cardiopulmonary resuscitation (CPR) of patients, which gives them lower survival rates. On the other hand, there is evidence that the factors associated with CVD (diabetes, obesity, psychological stress) are more prevalent in the female sex, which gives them a greater cardiovascular risk.[19–21] Additionally, fewer women compared to men have been observed in CVD research studies, and specific guidelines for women are often based on studies of men, resulting in lower diagnostic ability and delayed treatment and detection of the disease.[18,25–27]

While earlier studies suggested an "obesity paradox" where higher BMI was associated with better outcomes in OHCA patients,[28,29] more recent studies have debunked this theory, indicating that obesity is not a protective factor.[30,31] Recent findings highlight that this paradox might be more accurately described as a "BMI paradox", as greater adiposity, when measured using alternative anthropometric indices, is associated with worse outcomes.[32,33] It is of great importance to determine which patient conditions can aggravate or improve the chance of ROSC, some of them being modifiable factors (such as obesity, comorbidities and the time between OHCA and CPR initiation) or non-modifiable factors (such as age, sex or initial heart rhythm).[8,30]

Other factors can also intervene in the prognosis of patients with OHCA, such as the initial rhythm of ventricular fibrillation (VF) or pulseless ventricular tachycardia (pVT) or their age and sex.[3,5,28] It is of great importance to determine which patient conditions can aggravate or improve the chance of ROSC, some of them being modifiable factors (obesity or comorbidities) or not (age, sex or initial heart rhythm).

Patients experiencing OHCA with a shockable rhythm have been shown to have a higher rate of survival and recovery. This is attributed to the higher ROSC rate achieved through the application of an external defibrillator, which significantly increases the likelihood of ROSC.[6,17,34] Differences in the prevalence and diagnosis of cardiovascular events and obesity in women indicate the need to adopt new approaches to address the sex difference in the treatment of patients with OHCA. In the general population, younger women have a lower prevalence of CVD compared to men. However, women who experience OHCA are typically post-menopausal, with higher rates of obesity and consequently a higher prevalence of CVD compared to men. These 2 factors significantly influence both the cause and subsequent survival of patients.[13,15,20,22] No scientific literature has been found that addresses these differences, so we believe it is important to study them to perform an individualized evaluation of patients with OHCA. In our previous study (in which we did not disaggregate patients by gender), obesity results were an independent factor that decreased the odds of ROSC by more than 8%.[5] Given the lack of literature and the results of our previous research, we decided to investigate sex differences in this area.

Objectives

This study aimed to assess the association of obesity on the ROSC among patients with OHCA in relation to sex differences.

Methods

Study design and setting

A retrospective cohort study was conducted, involving an analysis of medical records for patients assisted by the Emergency Medical System (EMS) in Poland from January 2021 to June 2022. For this study, data were obtained from the Command Support System of the National Emergency Medical Service. The dataset included all documented cases of OHCA with International Classification of Diseases (ICD-10) code I46, where CPR was initiated. In Poland, there are 2 types of emergency medical teams (EMTs). Specialized EMTs (S-EMTs) comprise at least 3 persons, including a physician and an emergency nurse or paramedic. Basic EMTs (B-EMTs) comprise at least 2 persons qualified to perform medical emergency activities, including an emergency nurse or paramedic. As of the 1st half of 2023, there were 1,600 EMTs in Poland, including 1,284 B-EMTs and 316 S-EMTs.

Study population

A total of 4,925,214 EMS records were analyzed. Patients were included if they had an ICD-10 diagnosis code of I46 (cardiac arrest) and were treated with CPR at the scene. Exclusions were made for cases involving crime, suicide, trauma, or patients who died before EMS arrival or lacked recorded OHCA rhythm. Ultimately, data from 33,636 patients with OHCA were included in the analysis. Obesity status was determined using ICD-10 codes (E66) and descriptive diagnoses recorded by EMS personnel. Data from both the ICD-10 section and the descriptive diagnosis section of the medical records were used to categorize patients by sex and obesity status. The flowchart of patient is presented in Fig. 1.

Data collection

Data were collected retrospectively from EMS records. The following variables were analyzed: sex, age, location of OHCA (non-public place vs public place, e.g., parks, streets, squares, sidewalks, libraries, schools, government buildings, public transportation facilities), initial rhythm, and presence of comorbidities such as HT, diabetes mellitus (DM), HF, history of cerebral stroke (CS), and ACS.

Variables

The primary outcome was the ROSC. Key exposures included obesity status, initial rhythm of OHCA (VF/pVT vs Asystole/PEA), location of OHCA (public place vs private place) and patient age. Comorbidities such as HT, DM, HF, CS, and ACS were also considered.

Bias

Efforts to minimize bias included using standardized ICD-10 codes for obesity and consistent methods of data collection across all EMS records. Potential sources of bias included the subjective assessment of obesity by EMS teams and the retrospective nature of the study.

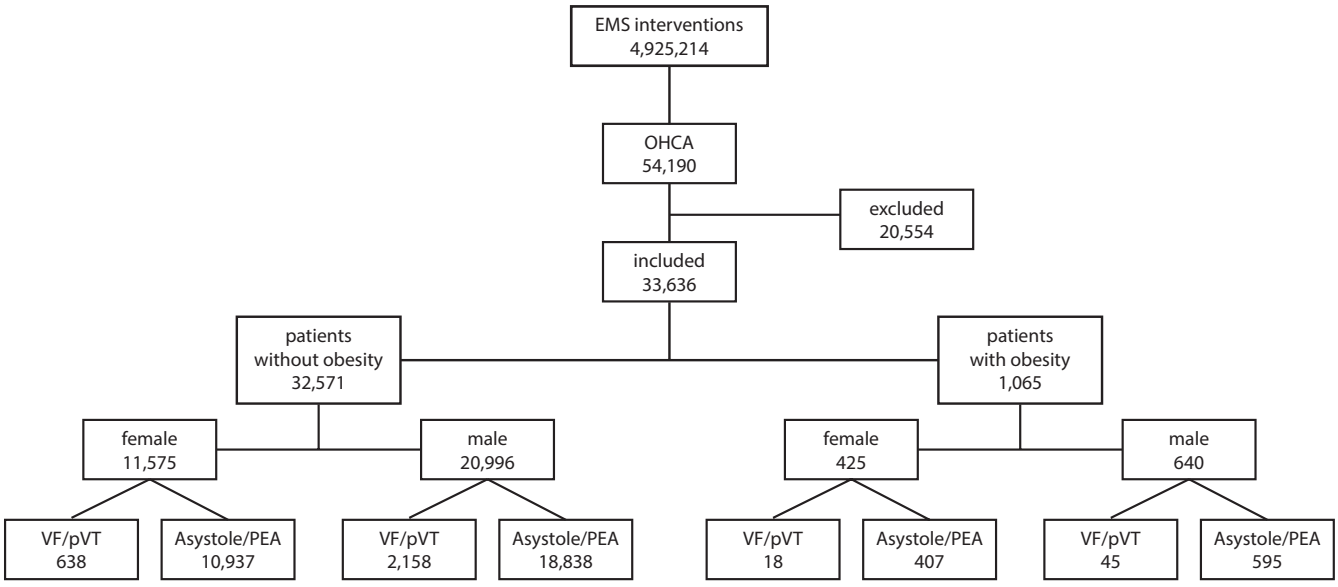

Fig. 1. Study flowchart

EMS – Emergency Medical System; PEA – pulseless electrical activity; OHCA – out-of-hospital cardiac arrests; VF/pVT – ventricular fibrillation/pulseless ventricular tachycardia.

Study size

The study included all available EMS records from the specified period, resulting in a final sample size of 33,636 OHCA patients.

Ethical considerations

The study was conducted according to the principles of the Declaration of Helsinki and was approved by the independent Bioethics Committee of Wroclaw Medical University, Poland (approval No. KB-895/2022). It also followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Statistical analyses

Data preprocessing and visualization were performed using Python 3.10.6 (https://www.python.org/downloads/release/python-3106/; packages: numpy 1.23.5, pandas 2.0.1, matplotlib 3.7.1, seaborn 0.12.2, and forestplot 0.3.1) in Visual Studio Code 1.80.0 (https://code.visualstudio.com/updates/v1_80). Statistical modeling was conducted with Statistica v. 13.3 (StatSoft Inc., Tulsa, USA) under a license provided by Wroclaw Medical University. The α value used for statistical inference was 0.05. Classical comparisons were performed with the independent samples t-test (by groups) or the χ^2 test, depending on the compared data (continuous or categorical). Due to the large number of observations, the assumption of normal distribution was based on the central limit theorem (CLT), assuming that the variable (i.e., age) in both groups had a distribution that differed statistically insignificantly from the normal distribution. Homoscedasticity assumption was checked with the use of Levene's test (Supplementary Table 1). If this assumption was not met, Cochran–Cox correction was applied to the t-test. In case of referring to variables of normal distribution, inherent moments of this distribution (mean and standard deviation (SD)) were chosen as descriptive statistics. In case of referring to variables of multinomial distribution, observed counts and frequencies were reported. In context of employing the χ^2 test, the assumption that the estimated counts were not <5 in ≥20% of the cells of each contingency table was met.

Logistic regression (binomial distribution of the predicted variable, link function: logit, dummy coding) was used for statistical modelling to analyze the odds of ROSC. Since it was assumed that the variable in both groups had a distribution that was statistically insignificantly different from the normal distribution, the assumption of no outliers for logistic regression was not checked and it was considered to be met. Linearity assumption for the relationship between the continuous explanatory variables and the logit of the response variable was plotted and checked with use of Box–Tidwell test. Despite the violation of this assumption by the test results ($p < 0.05$), age was left untransformed in the models because the visible deviation from linearity was attributed to individuals of extreme age (i.e., >96 years).

Results

Sex and obesity-related differences in age, comorbidities and the incidence of ROSC

There was no difference in age, regardless of whether the group was subsequently stratified by sex only (Table 1) or by both sex and obesity status (Table 2). Obesity, DM, CS, HT, and HF were significantly more frequent among women. Conversely, ACS occurred more frequently among men (Table 1). Markedly, a nearly twofold higher incidence of the VF/pVT initial rhythm was observed among

Table 1. Sex-related differences in age, comorbidities and the incidence of ROSC

Parameter	Descriptive statistic/category	Female (n = 12,000)	Male (n = 21,636)	Total (n = 33,636)	Statistic	df	p-value
Age [years]	mean (SD)	67.66 (15.51)	67.85 (15.15)	67.79 (15.28)	t = –1.093	24090.89	0.274
Obesity	yes	425 (3.54%)	640 (2.96%)	1,065 (3.17%)	$\chi^2 = 8.576$	1	**0.003**
DM	yes	2,246 (18.72%)	3,101 (14.33%)	5,347 (15.90%)	$\chi^2 = 110.97$	1	**<0.001**
CS	yes	703 (5.86%)	1,110 (5.13%)	1,813 (5.39%)	$\chi^2 = 8.022$	1	**0.005**
HT	yes	3,210 (26.75%)	4,251 (19.65%)	7,461 (22.18%)	$\chi^2 = 225.56$	1	**<0.001**
HF	yes	1,149 (9.57%)	1,622 (7.50%)	2,771 (8.24%)	$\chi^2 = 44.100$	1	**<0.001**
ACS	yes	378 (3.15%)	964 (4.46%)	1,342 (3.99%)	$\chi^2 = 34.345$	1	**<0.001**
Initial rhythm	VF/pVT	656 (5.47%)	2,203 (10.18%)	2,859 (8.50%)	$\chi^2 = 220.680$	1	**<0.001**
	Asystole/PEA	11,344 (94.53%)	19,433 (89.82%)	30,777 (91.50%)			
Location	a public place	2,232 (18.60%)	4,117 (19.03%)	6,349 (18.88%)	$\chi^2 = 0.926$	1	0.336
ROSC	yes	4,221 (35.17%)	7,818 (36.13%)	12,039 (35.79%)	$\chi^2 = 3.090$	1	0.079

n – number of patients; df – degrees of freedom; ROSC – return of spontaneous circulation; PEA – pulseless electrical activity; DM – diabetes mellitus; CS – cerebral stroke; HT – atrial hypertension; HF – heart failure; ACS – acute coronary syndrome; VF/pVT – ventricular fibrillation/pulseless ventricular tachycardia. Values in bold are statistically significant.

Table 2. Obesity-related differences in age, comorbidities and the incidence of ROSC: In the context of sex variation

Parameter	Descriptive statistic/ category	Female (n = 12,000)					Male (n = 21,636)				
		non-obese (n = 11,575)	obese (n = 425)	statistic	df	p-value	non-obese (n = 20,996)	obese (n = 640)	statistic	df	p-value
Age [years]	mean (SD)	67.68 (15.51)	67.28 (15.57)	t = 0.517	11,901	0.605	67.84 (15.15)	68.28 (15.24)	t = –0.714	21,450	0.475
DM	yes	2,038 (17.61%)	208 (48.94%)	χ^2 = 264.568	1	**<0.001**	2,844 (13.55%)	257 (40.16%)	χ^2 = 358.191	1	**<0.001**
CS	yes	671 (5.80%)	32 (7.53%)	χ^2 = 2.231	1	0.135	1,073 (5.11%)	37 (5.78%)	χ^2 = 0.574	1	0.449
HT	yes	2,970 (25.66%)	240 (56.47%)	χ^2 = 198.624	1	**<0.001**	3,949 (18.81%)	302 (47.19%)	χ^2 = 316.830	1	**<0.001**
HF	yes	1,047 (9.05%)	102 (24.00%)	χ^2 = 105.889	1	**<0.001**	1499 (7.14%)	123 (19.22%)	χ^2 = 130.675	1	**<0.001**
ACS	yes	361 (3.12%)	17 (4.00%)	χ^2 = 1.043	1	0.307	921 (4.39%)	43 (6.72%)	χ^2 = 7.935	1	**0.005**
Initial rhythm	VF/pVT	638 (5.51%)	18 (4.24%)	χ^2 = 1.293	1	0.256	2,158 (10.28%)	45 (7.03%)	χ^2 = 7.159	1	**0.007**
	Asystole/PEA	10,937 (94.49%)	407 (95.76%)				18,838 (89.72%)	595 (92.97%)			
Location	a public place	2,156 (18.63%)	76 (17.88%)	χ^2 = 0.151	1	0.698	4,007 (19.09%)	110 (17.19%)	χ^2 = 1.453	1	0.228
ROSC	yes	4,092 (35.35%)	129 (30.35%)	χ^2 = 4.493	1	**0.034**	7,612 (36.25%)	206 (32.19%)	χ^2 = 4.451	1	**0.035**

n – number of patients; df – degrees of freedom; ROSC – return of spontaneous circulation; PEA – pulseless electrical activity; DM – diabetes mellitus; CS – cerebral stroke; HT – atrial hypertension; HF – heart failure; ACS – acute coronary syndrome; VF/pVT – ventricular fibrillation/pulseless ventricular tachycardia. Values in bold are statistically significant.

men (10.18% vs 5.47%, p < 0.001, Table 1). The difference in ROSC incidence between the 2 sexes was insignificant (p = 0.079, Table 1). Likewise, the frequencies of OHCA occurring in a public place were similar (p = 0.336, Table 1).

Upon subsequent stratification of the 2 sexes by obesity status, a markedly higher incidence of DM, HT and HF was observed, regardless of sex (Table 2). Although the obesity-related change in frequency of ACS and the VF/pVT initial rhythm was insignificant among women (p = 0.307 and p = 0.256, respectively), men showed higher incidence of ACS (p = 0.005) and lower incidence of the VF/pVT initial rhythm (p = 0.007) among the obese individuals, compared to non-obese (Table 2). There were no changes in the obesity-associated distribution of the location among the 2 sexes. Regardless of sex, ROSC occurred significantly less frequently among obese individuals (30.35% vs 35.35% among women and 32.19% vs 36.25% among men).

Exploring the modulation of the odds of ROSC by obesity – insights from univariate and multi-factor models

According to the univariate model (Table 3), obesity decreased the odds of ROSC by 25.47% among women (p = 0.034) and 19.76% (p = 0.035) among men. Upon adding other features to the model, obesity became insignificant (Table 3). However, it was on the brink of statistical significance (p = 0.051) among women, according to the model adjusted by 3 key factors modulating the odds of ROSC: initial rhythm, location, and age. According to models with all factors (Table 3), the individuals with the VF/pVT initial rhythm were 4.204-fold more likely (vs Asystole/PEA) to develop ROSC among women, but only 3.655-fold more likely among men. If OHCA happened in a public place, both sexes showed similar, over twofold increase in odds of ROSC (2.204 among women, 2.130 among men). The association of age on these odds was similar among the 2 sexes, decreasing the odds for ROSC (by 1.21% and 1.52% in women and men, respectively, per 1-year increase in age). Moreover, unlike men (p = 0.832), women showed a 10.01% decrease in the odds for ROSC upon the presence of hypertension (p = 0.044).

After stratification by sex and obesity (Table 4), several factors appeared to influence the odds for ROSC differently. Although the public place location significantly increased the odds for ROSC among both sexes, women showed to have significantly lower location-associated OR if they were obese (2.126 vs 4.264 in woman with obesity and without, respectively). Moreover, exclusively among non-obese women, hypertension decreased the odds of ROSC by 11.11% (p = 0.032).

Discussion

The study aimed to assess the association of obesity with the incidence of ROSC among OHCA patients, specifically in context of between-sex differences. Regardless

Table 3. The odds of ROSC in the context of obesity and other selected factors: After stratification by sex

Sex	Model	Feature	Category	OR	OR –95% CI	OR 95% CI	p-value
Female	obesity only (AIC = 15,435.15, BIC = 15,449.92, D = 15,431.15)	obesity	yes	**0.797**	0.646	0.983	**0.034**
	adjusted by initial rhythm, location and age (AIC = 14,696.43, BIC = 14,733.35, D = 14,686.43)	obesity	yes	0.805	0.648	1.001	0.051
		initial rhythm	VF/pVT	**4.183**	3.513	4.981	**<0.001**
		location	a public place	**2.202**	1.998	2.427	**<0.001**
		age	–	**0.988**	0.986	0.991	**<0.001**
	adjusted by all featured factors (AIC = 14,698.39, BIC = 14,772.23, D = 14,678.39)	obesity	yes	0.835	0.669	1.042	0.110
		initial rhythm	VF/pVT	**4.204**	3.525	5.014	**<0.001**
		location	a public place	**2.204**	2.000	2.429	**<0.001**
		age	–	**0.988**	0.986	0.991	**<0.001**
		DM	yes	0.961	0.864	1.069	0.462
		CS	yes	0.947	0.800	1.121	0.529
		HT	yes	**0.909**	0.828	0.998	**0.044**
		HF	yes	1.053	0.920	1.205	0.455
		ACS	yes	0.885	0.703	1.116	0.302
Male	obesity only (AIC = 28,050.88, BIC = 28,066.83, D = 28,046.88)	obesity	yes	**0.835**	0.705	0.987	**0.035**
	adjusted by initial rhythm, location and age (AIC = 26,306.74, BIC = 26,346.61, D = 26,296.74)	obesity	yes	0.886	0.744	1.056	0.176
		initial rhythm	VF/pVT	**3.655**	3.320	4.023	**<0.001**
		location	a public place	**2.131**	1.981	2.291	**<0.001**
		age	–	**0.985**	0.983	0.987	**<0.001**
	adjusted by all featured factors (AIC = 26,311.52, BIC = 26,391.25, D = 26,291.52)	obesity	yes	0.874	0.732	1.044	0.138
		initial rhythm	VF/pVT	3.641	3.305	4.011	<0.001
		location	a public place	2.130	1.981	2.291	<0.001
		age	–	0.985	0.983	0.987	<0.001
		DM	yes	1.041	0.954	1.137	0.366
		CS	yes	1.112	0.975	1.268	0.113
		HT	yes	1.008	0.933	1.090	0.832
		HF	yes	0.978	0.874	1.095	0.700
		ACS	yes	1.079	0.936	1.244	0.297

n – number of patients; AIC – Akaike information criterion; BIC – Bayesian information criterion; 95% CI – 95% confidence interval; D – deviance; OR – odds ratio; ROSC – return of spontaneous circulation; DM – diabetes mellitus; CS – cerebral stroke; HT – atrial hypertension; HF – heart failure; ACS – acute coronary syndrome; VF/pVT – ventricular fibrillation/pulseless ventricular tachycardia. Values in bold are statistically significant.

of sex, ROSC occurred significantly less frequently among obese individuals, as observed in several studies.[4,12,30] Despite corroborating evidence found in scientific literature, the most solid studies indicate that obesity is a factor which increases the risk of mortality in patients with OHCA since it decreases the probability of ROSC, increasing the deterioration of the vascular system or making CPR difficult.[3,4,12,30,35,36] In this study, the term "obesity" refers to patients described as obese by EMS teams based on available information, which may include visual assessment or medical records. This approach, while necessary in the urgent context of OHCA, may introduce a degree of subjectivity that could affect the accuracy of obesity categorization. It is important to note that measuring weight and height in these urgent and vulnerable situations is not always feasible, and these parameters are often determined upon taking a glance at the patient. This limitation should be considered when interpreting the results of our study. Our univariate analysis showed a significant association between obesity and decreased odds of ROSC, but the multivariate analysis indicated that this association was not statistically significant. It is important to note that this loss of significance in multivariate settings does not necessarily mean that obesity does not modulate the odds of ROSC in real-life settings. The more rational conclusion would be that other factors, such as initial rhythm and

Table 4. The odds of ROSC in the context of selected factors: Upon stratification by sex and obesity status

Sex	Non-obese							Obese						
	model	feature	category	OR	OR –95% CI	OR 95% CI	p-value	Model	Feature	Category	OR	OR –95% CI	OR 95% CI	p-value
Female	adjusted by initial rhythm, location and age (AIC = 14,201.60, BIC = 14,230.99, D = 14,193.60)	initial rhythm	VF/pVT	**4.241**	3.551	5.065	**<0.001**	adjusted by initial rhythm, location and age (AIC = 496.18, BIC = 512.35, D = 488.18)	initial rhythm	VF/pVT	2.576	0.969	6.847	0.058
		location	a public place	**2.207**	1.999	2.437	**<0.001**		location	a public place	**2.096**	1.240	3.543	**0.006**
		age	–	**0.989**	0.986	0.991	**<0.001**		age	–	**0.975**	0.961	0.988	**<0.001**
	adjusted by all featured factors (AIC = 14,201.48, BIC = 14,267.61, D = 14,183.48)	initial rhythm	VF/pVT	**4.264**	3.564	5.101	**<0.001**	adjusted by all featured factors (AIC = 500.70, BIC = 537.08, D = 482.70)	initial rhythm	VF/pVT	**2.759**	1.033	7.374	**0.043**
		location	a public place	**2.211**	2.002	2.441	**<0.001**		location	a public place	**2.126**	1.250	3.615	**0.005**
		age	–	**0.989**	0.986	0.991	**<0.001**		age	–	**0.975**	0.961	0.989	**<0.001**
		DM	yes	0.959	0.859	1.070	0.450		DM	yes	0.990	0.636	1.539	0.964
		CS	yes	0.909	0.764	1.081	0.281		CS	yes	1.994	0.930	4.274	0.076
		HT	yes	0.900	0.818	0.991	0.032		HT	yes	1.074	0.687	1.678	0.754
		HF	yes	1.087	0.945	1.249	0.244		HF	yes	0.708	0.416	1.204	0.203
		ACS	yes	0.890	0.703	1.127	0.333		ACS	yes	0.668	0.202	2.205	0.508
Male	adjusted by initial rhythm, location and age (AIC = 25,537.18, BIC = 25,568.96, D = 25,529.18)	initial rhythm	VF/pVT	**3.701**	3.358	4.079	**<0.001**	adjusted by initial rhythm, location and age (AIC = 772.63, BIC = 790.43, D = 764.63)	initial rhythm	VF/pVT	**2.103**	1.113	3.975	**0.022**
		location	a public place	**2.131**	1.979	2.294	**<0.001**		location	a public place	**2.138**	1.387	3.295	**0.001**
		age	–	**0.985**	0.983	0.987	**<0.001**		age	–	**0.985**	0.974	0.996	**0.007**
	adjusted by all featured factors (AIC = 25,542.23, BIC = 25,613.72, D = 25,524.23)	initial rhythm	VF/pVT	**3.690**	3.346	4.070	**<0.001**	adjusted by all featured factors (AIC = 774.05, BIC = 814.10, D = 756.05)	initial rhythm	VF/pVT	**1.990**	1.042	3.801	**0.037**
		location	a public place	**2.130**	1.979	2.293	**<0.001**		location	a public place	**2.168**	1.402	3.351	**0.001**
		age	–	**0.985**	0.983	0.987	**<0.001**		age	–	**0.985**	0.974	0.996	**0.009**
		DM	yes	1.052	0.961	1.151	0.270		DM	yes	0.842	0.583	1.217	0.360
		CS	yes	1.089	0.953	1.246	0.210		CS	yes	1.995	0.998	3.990	0.051
		HT	yes	1.014	0.936	1.097	0.740		HT	yes	0.966	0.677	1.379	0.850
		HF	yes	0.948	0.843	1.066	0.374		HF	yes	1.496	0.970	2.307	0.068
		ACS	yes	1.071	0.926	1.239	0.356		ACS	yes	1.251	0.631	2.480	0.521

n – number of patients; AIC – Akaike information criterion; BIC – Bayesian information criterion; 95% CI – 95% confidence interval; D – deviance; OR – odds ratio; ROSC – return of spontaneous circulation; DM – diabetes mellitus; CS – cerebral stroke; HT – atrial hypertension; HF – heart failure; ACS – acute coronary syndrome; VF/pVT – ventricular fibrillation/pulseless ventricular tachycardia. Values in bold are statistically significant.

location of OHCA, may play a more critical role in influencing ROSC outcomes,[37,38] compared to obesity status.

This study, along with research by Fehler et al.,[5] Czapla et al.,[3,4] Tanaka et al.,[17] and Haskins et al.,[39] not only analyzed but also observed that patients presenting with VF/pVT had a higher likelihood of ROSC. When we consider the differences between sex, we have seen an almost twofold higher incidence of the initial VF/pVT rhythm among men, which has been reported in other studies.[18,23,24] Although other investigators have reported that in their population samples, women were generally older than men, there was no difference in age between both sexes in our data. The low survival rate among women is driven by higher prevalence of chronic diseases (DM, HT, CS, CVD) and a greater occurrence of OHCA at home or without witnesses.[18,22] Moreover, the presence of obesity among women statistically significantly decreased the effectiveness of CPR among women with obesity experiencing OHCA in a public place. Not only are women less likely to experience OHCA in a public place, but they are also negatively affected by obesity in terms of being subject to effective CPR in public places.[21,23–25] The most significant predictors of ROSC in our study were the initial VF/pVT rhythm and the occurrence of OHCA in a public place. Women with an initial VF/pVT rhythm had a 4.204 times greater chance of achieving ROSC, compared to a 3.655-fold difference found among men. Additionally, OHCA in a public place increased the odds of ROSC more than twofold for both sexes. These findings highlight the paramount importance of initial rhythm and the context of the arrest location over the isolated effect of obesity.

In our results, we have seen how an increase of 1 year in the age of the patients similarly decreases the probabilities of ROSC between both sexes, just as we observed in our previous study[5] and the rest of the studies of the scientific literature,[2,4,17,40] with similar values among all the results, but with the lack of taking gender differences into account in case of studies performed by other investigators. Other studies have reported that although the association of age affects both sexes equally, in the phase after cardiac arrest, older women showed a higher mortality rate compared to men.[40] However, when women of premenopausal age were compared with men of the same age, the former had higher levels of survival.[27,40–42] Awad et al. only described this difference in the group of women with initial non-shockable heart rhythm.[40] We can observe the cumulative effect of chronic diseases in our multivariate analysis since women with hypertension showed a 10% decrease in the odds of ROSC, which increases the number of deaths in women, in contrast to the data obtained in our previous study, where no difference was made between sexes, and hypertension only showed a decrease of 3.4%.[5] Stratification by age and sex also showed an 11% decrease in the odds of ROSC among non-obese women, with a smaller reduction seen in all non-obese patients in our previous study.[5] Considering solely the obesity factor, we once again observed that the female sex is the most affected since this pathology decreased the probabilities of ROSC by 25.47% vs the reduction of 19.76% among men. However, in the multivariate analysis taking all factors into account, women with initial VF/pVT rhythm were more likely to develop ROSC than men with the same type of initial rhythm, which opposes the previous results.[4,18,25,39] The mentioned studies did not consider the association of obesity on the ROSC, highlighting a need for further research in this aspect. Our results indicate that while obesity has a significant impact in univariate analysis, its effect is mitigated when other critical factors are considered in a multivariate context. This aligns with recent studies that question the so-called "obesity paradox" in cardiac arrest outcomes. Therefore, our findings contribute to a growing body of evidence suggesting that the role of obesity in ROSC is complex and influenced by multiple interacting factors.

The reported results, including those found in the scientific literature,[18,22–25,43] show differences in the prevalence of OHCA between women and men, as well as the associated pathologies or the diagnosis and treatment they receive. Most studies indicate that women are more likely to experience OHCA with associated pathologies.[15,18,22,25] Additionally, they show lower survival rates or worse associated outcomes.[18,22,23,25] Our findings confirm the differences observed between both sexes. Since obesity, in particular, makes CPR more difficult in the case of women, new approaches must be adopted in the treatment of OHCA. The fact of finding some contradictory results, such as the higher frequency of initial VF/pVT rhythm among women, highlights the need to investigate sex-associated variability and adopt measures that improve survival rates among the female sex; when women receive the correct measures, they show a better recovery than men.[23,27]

Implications for practice

Given these findings, it is essential for clinical practice to prioritize factors which have a more direct and substantial impact on ROSC outcomes, such as ensuring rapid defibrillation and effective CPR, especially in public settings. While addressing obesity remains important for overall cardiovascular health, it may not be the most critical factor in the acute setting of OHCA. Our results are in line with the available scientific literature that describes a higher prevalence of CVD among women and worse survival outcomes related to OHCA, derived from a greater odds of occurring.[22,25–27,44] These events occur when the women are alone at home without the presence of witnesses or have a lower frequency of shockable rhythms.[4,17,27] Additionally, it has been observed that women receive fewer invasive treatments once they are admitted to the hospital after experiencing OHCA, which leads to worse health outcomes. Other studies have indicated that health illiteracy, older

age of women or low socioeconomic status of women are correlated with an underestimation of CVD risk.[27] These facts, together with the greater prevalence of chronic diseases in women, such as obesity, negatively influence treatment and success rates for ROSC among women with OHCA. It is necessary to include a sex-related approach in the care of women with OHCA, and in general with any disease, since we see worse health outcomes associated with the female sex. Furthermore, improving the accuracy of obesity recognition and its documentation in medical records is crucial. This could help in better understanding the impact of obesity on OHCA outcomes and in developing targeted interventions to improve survival rates. In this context, prospective studies should be conducted to assess the impact of obesity on OHCA outcomes. This approach would provide a more comprehensive understanding of the role of obesity in OHCA and help in formulating effective strategies to enhance patient outcomes.

Limitations

This study has several important limitations. The assessment of obesity by EMS personnel, based on available information such as visual evaluation, medical records, ICD-10 codes (E66), and descriptive diagnoses recorded, may introduce some inaccuracies. We do not have detailed information on how the diagnosis of obesity (E66) was determined, whether through direct measurements or visual assessment. It is likely that only patients who appeared overtly obese were categorized as such. This limitation should be considered when interpreting the findings, as it may impact the accuracy of the obesity categorization. This method of assessment may lead to an underestimation of obesity prevalence compared to studies using precise BMI measurements. Additionally, due to the retrospective nature of this study and the emergency context in which EMS personnel operate, it was not possible to obtain precise measurements of BMI, body composition or other detailed anthropometric data. The retrospective nature of this study and the characteristics of the database used introduce additional limitations. One concern is the uncertainty surrounding the data associated with comorbidities, as the EMS staff might not have interacted with family witnesses or had access to the patient's medical history. The emergency medical records lacked specific information on whether bystanders-initiated CPR before the arrival of the medical team, as well as the duration of such CPR, making it impossible to incorporate these factors into the analysis. Additionally, our dataset did not include detailed time-related data such as EMS response times, activation of the emergency system and bystander use of automated external defibrillators (AEDs). Our dataset also lacked specific clinical parameters like end-tidal carbon dioxide ($ETCO_2$) values, which are increasingly recognized as important prognostic markers in cardiac arrest. Moreover, there was limited information on airway management, including successful intubation rates and the use of alternative airway devices in cases of failed intubation attempts, particularly among obese patients. The anonymity of medical records precluded the examination of long-term survival. However, it is essential to note that a notable strength of the study lies in its extensive sample size, encompassing the entire Polish population.

Conclusions

Our study demonstrated that obesity was not an independent predictor of ROSC in OHCA patients. Different predictors of ROSC were identified for men and women. Major findings from this study indicate that initial VF/pVT rhythm and occurrence of OHCA in a public place significantly increase the likelihood of achieving ROSC. Women with an initial VF/pVT rhythm had a 4.204 times greater chance of ROSC compared to 3.655 times increase in men. Among both sexes, increasing age was associated with decreased odds of ROSC. These factors had a more significant impact on ROSC outcomes than obesity.

Further research is necessary to better understand the underlying mechanisms driving these differences and to develop targeted interventions that can enhance the effectiveness of resuscitation efforts for all OHCA patients.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.13754402. The package includes the following files:

Supplementary Table 1. Detailed report on models related to location and initial rhythm, including ORs and 95% CIs for cardiac arrest outcomes.

Supplementary Table 2. Further analysis of initial rhythm and location, adjusted by sex, age and other factors affecting cardiac arrest outcomes.

Supplementary Table 3. Assessment of multicollinearity between variables used in logistic regression models, based on the variance inflation factor (VIF).

Supplementary Table 4. Results of a 3-way logistic regression model showing the combined effects of sex, location and initial rhythm on the odds of ROSC.

Supplementary Table 5. A logistic regression model demonstrating how location and age modulate ROSC odds, with and without interaction with initial rhythm.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Identification of IGFBP3 and LGALS1 as potential secreted biomarkers for clear cell renal cell carcinoma based on bioinformatics analysis and machine learning

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Abstract

Background. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma (RCC). Due to the lack of symptoms until advanced stages, early diagnosis of ccRCC is challenging. Therefore, the identification of novel secreted biomarkers for the early detection of ccRCC is urgently needed.

Objectives. This study aimed to identify novel secreted biomarkers for diagnosing ccRCC using bioinformatics and machine learning techniques based on transcriptomics data.

Material and methods. Differentially expressed genes (DEGs) in ccRCC compared to normal kidney tissues were identified using 3 transcriptomics datasets (GSE53757, GSE40435 and GSE11151) from the Gene Expression Omnibus (GEO). Potential secreted biomarkers were examined within these common DEGs using a list of human secretome proteins from The Human Protein Atlas. The recursive feature elimination (RFE) technique was used to determine the optimal number of features for building classification machine learning models. The expression levels and clinical associations of candidate biomarkers identified with RFE were validated using transcriptomics data from The Cancer Genome Atlas (TCGA). Classification models were then developed based on the expression levels of these candidate biomarkers. The performance of the models was evaluated based on accuracy, evaluation metrics, confusion matrices, and ROC-AUC (receiver operating characteristic-area under the ROC curve) curves.

Results. We identified 44 DEGs that encode potential secreted proteins from 274 common DEGs found across all datasets. Among these, insulin-like growth factor binding protein 3 (IGFBP3) and lectin, galactoside-binding, soluble, 1 (LGALS1) were selected for further analysis using the RFE technique. Both IGFBP3 and LGALS1 showed significant upregulation in ccRCC tissues compared to normal tissues in the GEO and TCGA datasets. The results of the survival analysis indicated that patients with higher expression levels of these genes exhibited shorter overall and disease-free survival times (OS and DFS). Decision tree and random forest models based on IGFBP3 and LGALS1 levels achieved an accuracy of 98.04% and an AUC of 0.98.

Conclusions. This study identified IGFBP3 and LGALS1 as promising novel secreted biomarkers for ccRCC diagnosis.

Key words: TCGA, GEO, machine learning, clear cell renal cell carcinoma, bioinformatics

Background

Renal cell carcinoma (RCC) represents the most common type of kidney cancer, accounting for approx. 90% of all cases.[1] Globally, RCC is the 14th most commonly diagnosed malignancy, with over 400,000 new cases reported annually.[2] Smoking, alcohol consumption, obesity, and high blood pressure are associated risk factors for RCC.[3] Renal cell carcinoma is often asymptomatic in its early stages, with 60% of cases being discovered incidentally during imaging studies for unrelated conditions. When symptomatic, patients may present with a triad of flank pain, hematuria and an abdominal mass, although this classic presentation is relatively uncommon. Systemic symptoms, including fever, weight loss and paraneoplastic syndromes, may result from advanced disease.[4,5] The treatment of RCC has undergone significant evolution over the past few decades. Surgical resection is the standard of care for patients with localized RCC, while targeted therapies and immunotherapy have been promising treatment options for advanced and metastatic RCC.[6,7]

There are 3 common pathological RCC subtypes, including clear cell RCC (ccRCC), which makes up 70–80% of cases; papillary RCC, which comprises 10–15%; and chromophobe RCC, which accounts for 5%.[8] Clear cell RCC is the primary cause of death in kidney cancer patients due to its asymptomatic nature in the early stages and resistance to chemotherapy and radiotherapy.[9] Early detection of ccRCC is challenging, relying on a combination of imaging techniques and histological examination. Therefore, identifying novel secreted biomarkers is crucial for its effective diagnosis.

Transcriptomics data, encompassing the complete set of all RNAs transcribed by specific tissues or cells, are widely used to identify novel biomarkers and promising drug targets in many diseases, including cancers.[10] Public transcriptomics databases such as The Cancer Genome Atlas (TCGA)[11,12] and the Gene Expression Omnibus (GEO)[13,14] have become invaluable resources for researchers in this field. A combination of bioinformatics and machine learning approaches to analyze public transcriptomics data has emerged as a pivotal approach to cancer research, offering unprecedented opportunities to identify novel biomarkers and potential drug targets for various cancers,[15,16] such as colorectal cancer,[17] pancreatic cancer[18] and breast cancer.[19] However, our latest review found no reports identifying potential secreted biomarkers for ccRCC using bioinformatics and machine learning approaches on public transcriptomics datasets.

In this study, bioinformatics and machine learning analysis were used to identify novel secreted biomarkers for ccRCC diagnosis using transcriptomics datasets from the GEO and TCGA databases. Differentially expressed genes (DEGs) were identified by comparing ccRCC tissues with normal kidney tissues, and potentially secreted proteins among the common DEGs were further analyzed. The optimal number of features for building machine learning models was determined using the recursive feature elimination (RFE) technique. Subsequently, classification models were developed based on the expression levels of candidate-secreted biomarkers. Finally, the expression levels and clinical associations of these candidate biomarkers were validated using additional transcriptomic data from the TCGA database.

Objectives

This research aimed to discover novel secreted biomarkers for diagnosing ccRCC by integrating bioinformatics and machine learning techniques with public transcriptomics data.

Materials and methods

Transcriptomics datasets

Three microarray datasets of ccRCC and normal kidney tissues, including GSE11151, GSE40435 and GSE53757, were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo). The datasets GSE11151 and GSE53757 were generated using the Affymetrix Human Genome U133 Plus 2.0 Array platform (Thermo Fisher Scientific, Waltham, USA), which was utilized for transcriptional profiling, while GSE40435 was based on the Illumina HumanHT-12 V4.0 expression BeadChip platform (Illumina Inc., San Diego, USA).

Differentially expressed gene analysis

The DEGs were identified by comparing ccRCC and normal kidney tissues using the GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r) with an adjusted $p < 0.05$ and absolute log fold-changes ≥1.0 as criteria. GEO2R is a web-based tool provided by the Gene Expression Omnibus (GEO) for analyzing gene expression data. It allows researchers to compare 2 or more groups of samples to identify differentially expressed genes. Data visualization was performed using a volcano plot in RStudio (https://rstudio.com). Additionally, Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn) were generated to display common DEGs across the 3 transcriptomics datasets.

Gene expression analysis

Gene expression analysis was conducted on the GSE40435 dataset retrieved from the GEO database using the GEOquery package in R (R Foundation for Statistical Computing, Vienna, Austria).[20] Expression data were transformed using a base-2 logarithmic scale to normalize the distribution.

Identification of potential secreted biomarkers in common DEGs

Potential secreted biomarkers in ccRCC were identified based on overlapping genes between common DEGs and a list of 1,665 secreted proteins from The Human Protein Atlas (https://www.proteinatlas.org).[21,22]

Feature selection

The recursive feature elimination, based on the random forest classifier, was employed to select the minimal set of genes needed to create classification models. The feature-selection process was conducted using the scikit-learn library (https://scikit-learn.org/stable/modules/generated/sklearn.feature_selection.RFE.html). Feature subsets of sizes 44, 20, 10, 5, 3, 2, and 1 were used for training and evaluation. The performance of the random forest classifier for each gene subset was assessed using several evaluation metrics, including accuracy, precision, recall, and F1-score.

Machine learning for classification

Seven supervised machine learning algorithms, including decision trees, random forests, logistic regression, K-nearest neighbors, Gaussian naive Bayes (GNB), support vector machines, and multilayer perceptrons (MLPs), were used to develop classification models based on the selected potential secreted DEGs. The Python scikit-learn library (https://scikit-learn.org) was used to implement these algorithms. The transcriptomics data were split into training and test sets. The training set was used to develop models with 7 machine learning algorithms, and their performance was evaluated on the test set. GridSearchCV (https://scikit-learn.org/1.5/modules/generated/sklearn.model_selection.GridSearchCV.html) was employed to optimize hyperparameters for each model. The classification performance of each model was assessed using accuracy, precision, recall, F1-score, confusion matrix, and receiver operating characteristic (ROC) curves.

Validation of biomarkers gene expression and clinical association

The expression levels of potential biomarkers in ccRCC and normal kidney tissue were validated using the TCGA dataset, which includes 523 ccRCC samples and 100 normal kidney samples, employing Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn).[23,24] Additionally, the correlation between the expression levels of potential biomarkers and the survival of ccRCC patients was analyzed using GEPIA.

Statistical analyses

In the GEPIA, the significant difference between the 2 groups was compared using Student's t-test. The correlation between gene expression and both overall survival (OS) and disease-free survival (DFS) in ccRCC patients was evaluated using Kaplan–Meier analysis, accompanied by a log-rank test and hazard ratio (HR) calculation. Statistical significance was considered to be $p < 0.05$. In the box plots, the central line represents the median value of the data. The boxes extended from the 1st quartile (Q1) to the 3rd quartile (Q3), representing the interquartile range (IQR). The whiskers extended to the most extreme data points within 1.5 times the IQR from Q1 and Q3. Data points beyond this range were considered outliers.

Results

Identification of common DEGs in ccRCC

Differentially expressed genes were identified by comparing ccRCC and normal kidney tissues from 3 GEO datasets: GSE53757, GSE40435 and GSE11151. The selection criteria were an adjusted $p < 0.05$ and an absolute log fold change ≥1.0. Based on these criteria, 2,917 DEGs were identified in GSE11151, 1,521 in GSE40435 and 6,665 in GSE53757. Specifically, GSE11151 had 1,180 upregulated and 1,737 downregulated genes; GSE40435 had 680 upregulated and 841 downregulated genes; and GSE53757 had 3,124 upregulated and 3,541 downregulated genes (Fig. 1, Table 1).

Table 1. Number of differentially expressed genes (DEGs) in 3 ccRCC datasets

GEO accession	DEGs	Upregulated genes	Downregulated genes
GSE11151	2,917	1,180	1,737
GSE40435	1,521	680	841
GSE53757	6,665	3,124	3,541

ccRCC – clear cell renal cell carcinoma.

We further identified the common DEGs using a Venn diagram (Fig. 2). There were 274 common DEGs across all 3 datasets (GSE11151, GSE40435 and GSE53757). This identification of common DEGs could potentially help to identifying potential candidate biomarkers for ccRCC.

Identification of potential secreted DEGs in ccRCC

The list of human secretome proteins from The Human Protein Atlas, which includes 1,665 human-secreted proteins, was used to identify which DEGs potentially encode secreted proteins. A Venn diagram was used to determine the secreted proteins among the 274 common DEGs (Fig. 3). We identified 44 DEGs that potentially encode secreted proteins, including *ADM*, *ANGPT2*, *ANGPTL4*, *ANXA1*, *ANXA2*, *APOC1*, *C1QA*,

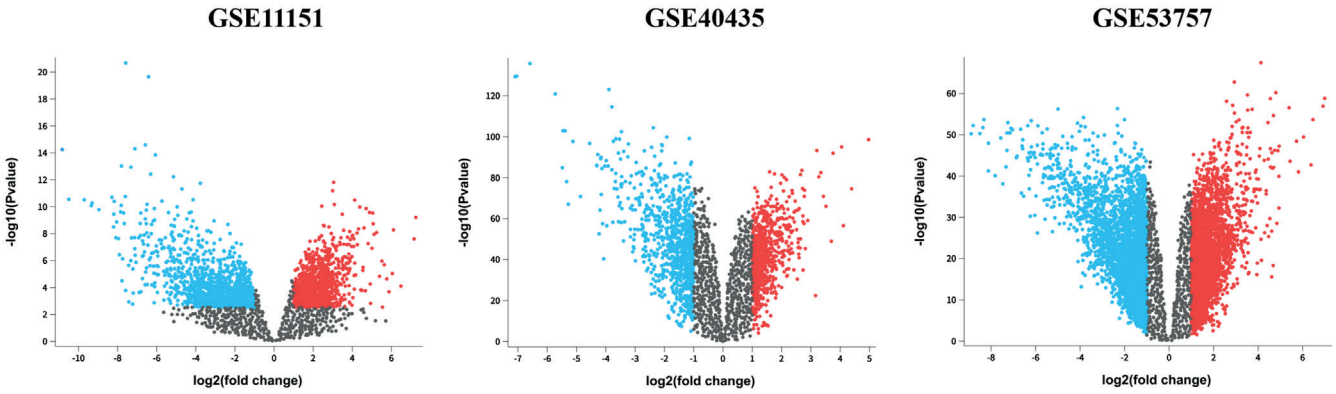

Fig. 1. Volcano plots demonstrating differentially expressed genes in 3 Gene Expression Omnibus (GEO) datasets (GSE11151, GSE40435 and GSE53757). The X-axis represents the log2 fold change, and the Y-axis represents the negative logarithm (base 10) of the p-value. Significantly upregulated genes are indicated in red, downregulated genes in blue, and nonsignificant genes in grey.

GSE – GEO accession number. A p-value < 0.05 was considered statistical significance.

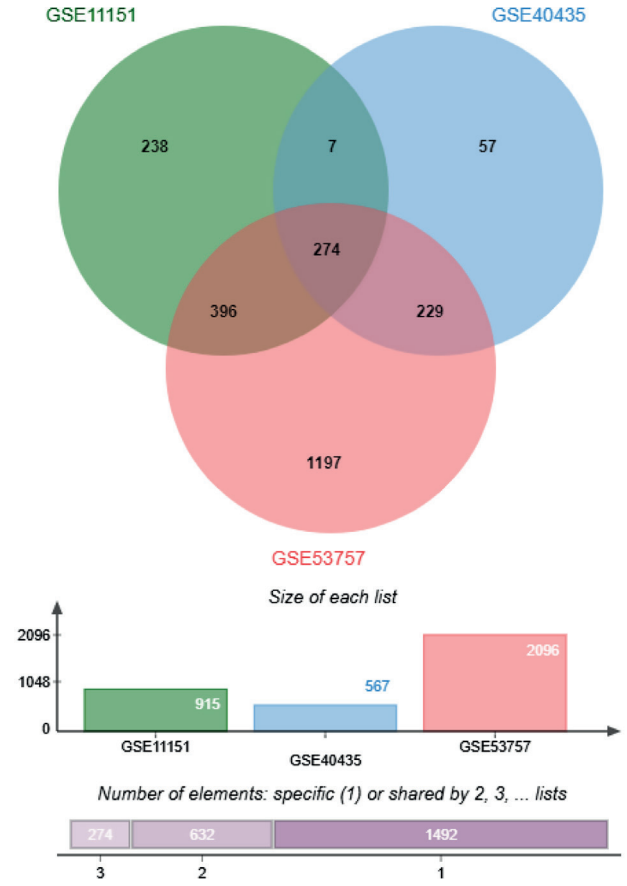

Fig. 2. Common DEGs in ccRCC across 3 Gene Expression Omnibus (GEO) datasets. The Venn diagram displays the overlap of DEGs among the 3 datasets. Numbers within the sections indicate the count of DEGs specific to 1 dataset or shared among multiple datasets. DEGs in GSE11151, GSE40435 and GSE53757 are represented in green, blue and red, respectively. The bar chart shows the total number of DEGs in each dataset

DEGs – differentially expressed genes; ccRCC – clear cell renal cell carcinoma; GSE – GEO accession number.

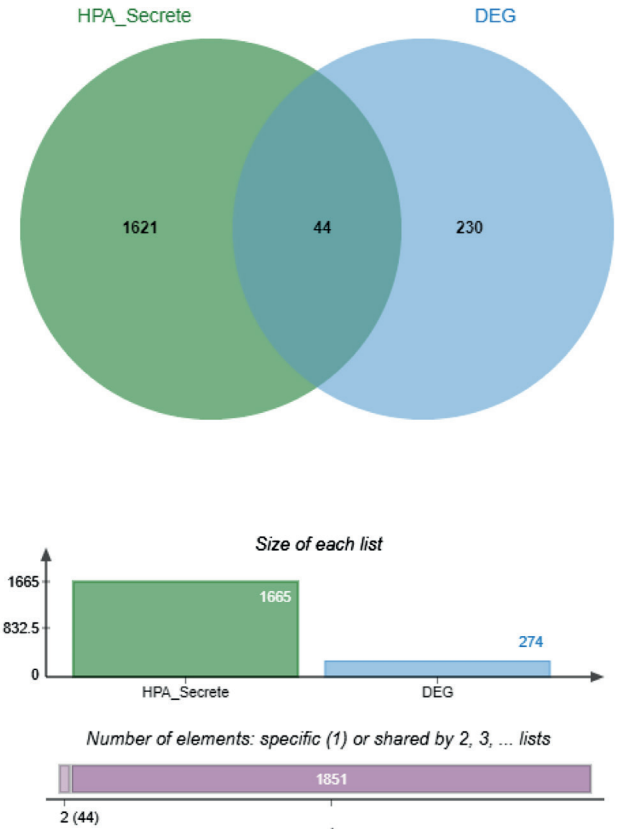

Fig. 3. Potentially secreted DEGs in ccRCC. The Venn diagram illustrates the overlap between the secreted gene list from The Human Protein Atlas (HPA_Secrete) and the common DEGs in ccRCC. The green circle represents the HPA_Secrete, with 1,665 genes. The blue circle represents the common DEGs in ccRCC, with 274 genes. The intersection of the 2 circles shows 44 potential secreted DEGs in ccRCC

DEGs – differentially expressed genes; ccRCC – clear cell renal cell carcinoma.

C1QC, *C3*, *CCL20*, *CD14*, *CHSY1*, *COL4A1*, *CTHRC1*, *CXCL10*, *CXCL9*, *EMILIN2*, *GNLY*, *GZMA*, *GZMH*, *IGFBP3*, *INHBB*, *ISG15*, *LAMA4*, *LAMC1*, *LGALS1*, *LOX*, *LY86*, *LY96*, *LYZ*, *NPTX2*, *OLFML2B*, *PLA2G7*, *PTHLH*, *RNASE6*, *RNASET2*, *SPARC*, *SRGN*, *STC2*, *TIMP1*, *TNFAIP6*, *VASH1*, *VEGFA*, and *VWF*. The overlap of the DEGs with the secretome database indicates that these 44 genes are strong candidates for secreted biomarkers for ccRCC.

Feature selection

The 44 DEGs that potentially encode secreted proteins in ccRCC were subjected to feature selection using the RFE technique based on the RandomForestClassifier. Various numbers of gene sets (1, 2, 3, 5, 10, 20, and 44 genes) were selected during the feature selection process. The RFE results indicated that sets of 3 and 5 genes resulted in the greatest accuracy (97.5%) (Table 2). However, we were able to achieve an accuracy of 96.3% using only 2 genes, namely insulin-like growth factor binding protein 3 (*IGFBP3*) and lectin, galactoside-binding, soluble, 1 (*LGALS1*), which we used to construct classification models.

The expression of IGFBP3 and LGALS1 in ccRCC patients based on the GEO dataset

The expression levels of *IGFBP3* and *LGALS1* were measured in adjacent non-tumor renal tissues and ccRCC tissues using the GSE40435 dataset. The pair plot revealed distinct clustering by tissue type, indicating a potential correlation between the expression levels of these genes and tissue classification (Fig. 4A). Additionally, the box plot showed that both *IGFBP3* and *LGALS1* expression levels were significantly elevated in ccRCC tissues compared to adjacent non-tumor renal tissues (Fig. 4B). These findings suggested that *IGFBP3* and *LGALS1* may serve as potential biomarkers for distinguishing ccRCC tissues from adjacent non-tumor renal tissues.

The performance of the classification model based on the expression levels of IGFBP3 and LGALS1

We evaluated the perfo rmance of 7 supervised machine learning algorithms, including Decision Trees, Random Forests, Logistic Regression, K-nearest Neighbors, Gaussian Naive Bayes, Support Vector Machines, and Multilayer Perceptrons, using the expression levels of *IGFBP3*

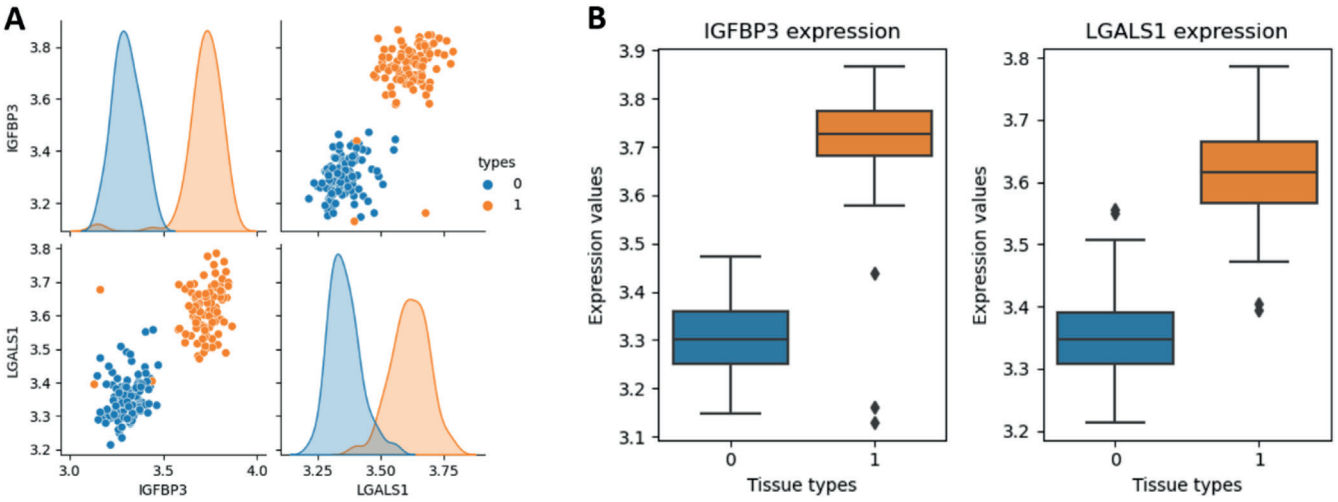

Fig. 4. Expression levels of IGFBP3 and LGALS1 in adjacent non-tumor renal tissues and ccRCC tissues. A. A pair plot displays the distribution and correlation of IGFBP3 and LGALS1 expression levels in adjacent non-tumor renal tissues (blue, Class 0) and ccRCC tissues (orange, Class 1); B. Box plots display the expression levels of IGFBP3 and LGALS1 between adjacent non-tumor renal tissues (Class 0) and ccRCC tissues (Class 1). In the box plots, the central line indicated the median, the box represented the interquartile range (IQR; Q1 to Q3) and the whiskers extended to 1.5 times the IQR from the quartiles. Outliers were plotted as individual points

IGFBP-3 – insulin-like growth factor binding protein 3; LGALS1 – lectin, galactoside-binding, soluble, 1; ccRCC – clear cell renal cell carcinoma.

Table 2. Performance of different feature sets in the feature selection process using RFE

Features	Accuracy [%]	Precision		Recall		F1-score	
		normal	ccRCC	normal	ccRCC	normal	ccRCC
44	96.3	0.96	0.97	0.98	0.94	0.97	0.96
20	96.3	0.96	0.97	0.98	0.94	0.97	0.96
10	96.3	0.96	0.97	0.98	0.94	0.97	0.96
5	97.5	0.96	1.00	1.00	0.94	0.98	0.97
3	97.5	0.96	1.00	1.00	0.94	0.98	0.97
2	96.3	0.96	0.97	0.98	0.94	0.97	0.96
1	91.4	0.93	0.89	0.91	0.91	0.92	0.90

RFE – recursive feature elimination; ccRCC – clear cell renal cell carcinoma.

Table 3. Classification performance of optimized decision tree and random forest models based on *IGFBP3* and *LGALS1*

Model	Accuracy [%]	AUC	Precision [%]		Recall [%]		F1-score [%]	
			normal	ccRCC	normal	ccRCC	normal	ccRCC
DT	98.04	0.98	0.96	1.00	1.00	0.96	0.98	0.98
RF	98.04	0.98	0.96	1.00	1.00	0.96	0.98	0.98

IGFBP3 – insulin-like growth factor binding protein 3; LGALS1 – lectin, galactoside-binding, soluble, 1; DT – decision tree; RF – random forest; AUC – area under the curve; ccRCC – clear cell renal cell carcinoma.

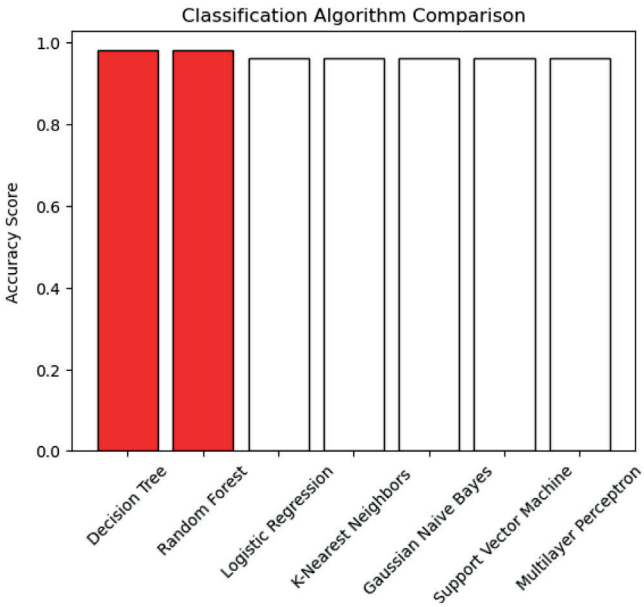

Fig. 5. Classification performance of 7 supervised machine learning algorithms. The bar chart demonstrates the accuracy scores of 7 supervised machine learning algorithms for classifying ccRCC. The decision tree and random forest algorithms achieved the highest accuracy scores, indicated by the red bars, while the other algorithms are represented by white bars

ccRCC – clear cell renal cell carcinoma.

and *LGALS1*. All algorithms demonstrated high accuracy, ranging from 96% to 98%. The Decision Tree and Random Forest models achieved the highest accuracy scores (Fig. 5). Consequently, these 2 models were selected for further optimization using GridSearchCV. After optimization, both models exhibited high performance, with an accuracy of 98.04% and an area under the ROC curve (AUC) of 0.98 (Table 3, Fig. 6).

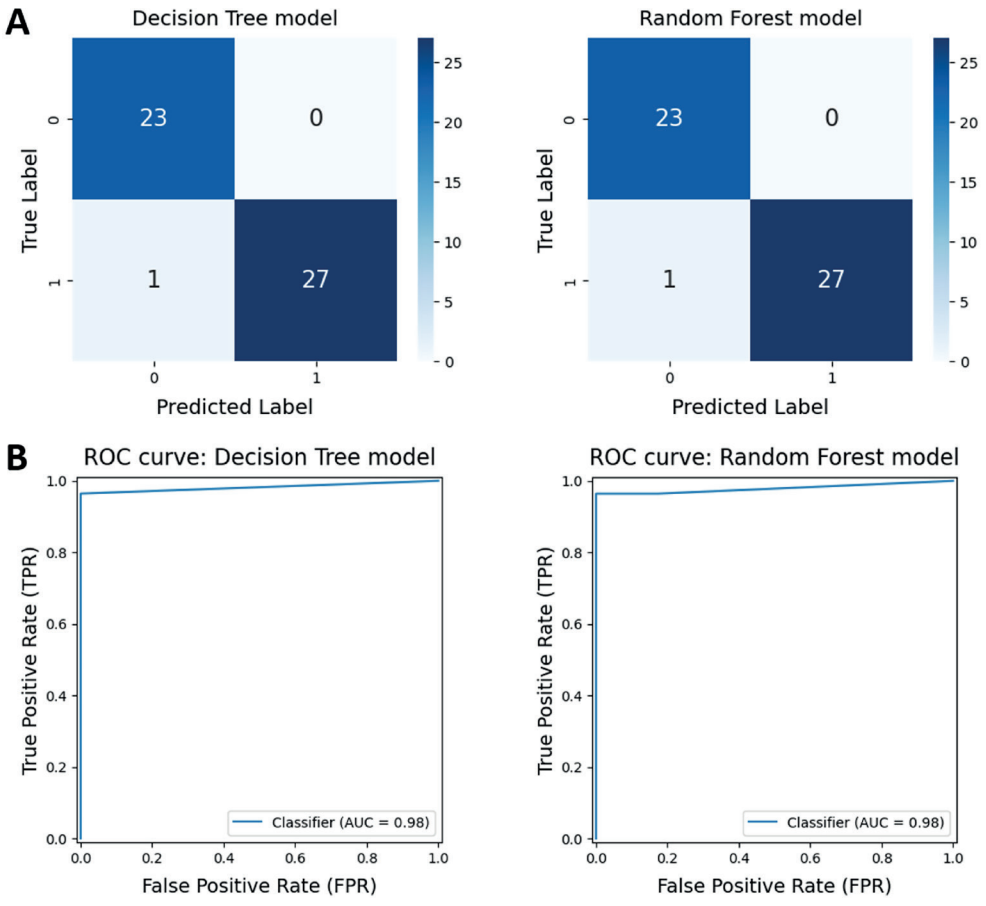

Fig. 6. Results from hyperparameter tuning of decision tree and random forest models. A. The confusion matrices show the performance of the decision tree and random forest models after hyperparameter tuning; B. The ROC curves for the decision tree and random forest models. Both models achieved a high AUC score of 0.98, indicating excellent performance in distinguishing between the adjacent non-tumor renal tissues (Class 0) and ccRCC tissues (Class 1)

ROC – receiver operating characteristic; AUC – area under the curve; ccRCC – clear cell renal cell carcinoma.

Validation of potential secreted biomarkers expression and clinical association based on the TCGA dataset

The expression levels of *IGFBP3* and *LGALS1* were confirmed using the TCGA dataset using the GEPIA online tool. Both *IGFBP3* and *LGALS1* were differentially expressed in kidney renal clear cell carcinoma (KIRC) samples, showing significantly higher expression compared to normal kidney tissue (Fig. 7).

The results of the survival analysis based on the TCGA dataset indicated that high IGFBP3 expression levels were associated with significantly reduced OS and DFS in ccRCC patients. Similarly, we found that high expression of *LGALS1* correlated with a trend of decreased OS and significantly affected DFS in ccRCC patients (Fig. 8A,B).

Discussion

Our bioinformatics analyses of 3 ccRCC datasets from the GEO identified 274 common DEGs. We then used the list of secreted proteins from the Human Protein Atlas to identify 44 potential secreted biomarkers for ccRCC.

The RFE technique, based on the RandomForestClassifier, highlighted a smaller subset of 2 genes that provided high classification accuracy, including *IGFBP3* and *LGALS1*. Decision Tree and Random Forest models based on the expression levels of *IGFBP3* and *LGALS1* demonstrated particularly high classification accuracy, underscoring their potential in diagnosing ccRCC patients.

Currently, several potential secreted biomarkers for diagnosing ccRCC have been identified. Carbonic anhydrase IX (CA9) is considered one of the promising biomarkers for ccRCC. Serum levels of CA9 were significantly higher in ccRCC patients than in those with non-CCRCC and benign tumours.[25] A similar finding was reported in 2018, in which plasma CA9 was evaluated in patients with ccRCC compared with patients with benign tumors and healthy controls.[26] However, the diagnostic performance of secreted CA9 in ccRCC remains unclear. Yang et al. identified 3 potential serum biomarkers for ccRCC using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. These biomarkers demonstrated a mean sensitivity of 88.38% and a mean specificity of 91.67%.[27] In 2017, Raf kinase inhibitor protein and phosphor Raf kinase inhibitor were also identified as potential urinary biomarkers for ccRCC using a proteomics technique with an AUC of 0.93.[28] In addition, Bao et al. identified hub genes associated with ccRCC from GEO dataset (GSE47352). They found that hub genes could distinguish ccRCC from paired normal tissue with an AUC ranging from 0.517 to 0.945.[29] Compared to the performance of currently established biomarkers for ccRCC diagnosis, the present study used a combination of bioinformatics and machine learning algorithms based on the expression levels of *IGFBP3* and *LGALS1* to achieve a notably higher diagnostic accuracy of 98.04% and an AUC of 0.98. Our results demonstrated the value of machine learning in achieving higher accuracy and consistency, which could lead to improved early detection and patient outcomes.

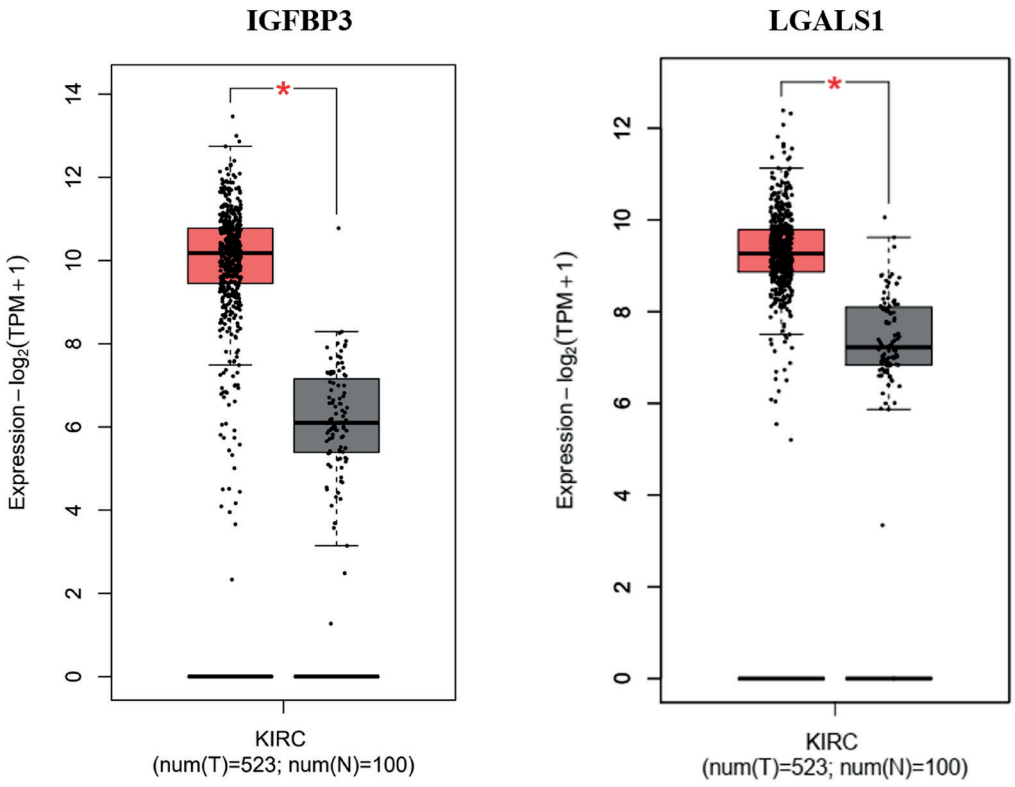

Fig. 7. Expression of IGFBP3 and LGALS1 in the The Cancer Genome Atlas (TCGA) dataset. Box plots display the expression levels of IGFBP3 and LGALS1 in kidney renal clear cell carcinoma (KIRC; red plot) tissues and non-tumor renal tissues (gray plot). In the box plots, the central line indicated the median, the box represented the interquartile range (IQR; Q1 to Q3) and the whiskers extended to 1.5 times the IQR from the quartiles. Outliers were shown as individual points

IGFBP-3 – insulin-like growth factor binding protein 3; LGALS1 – lectin, galactoside-binding, soluble, 1. *p < 0.05.

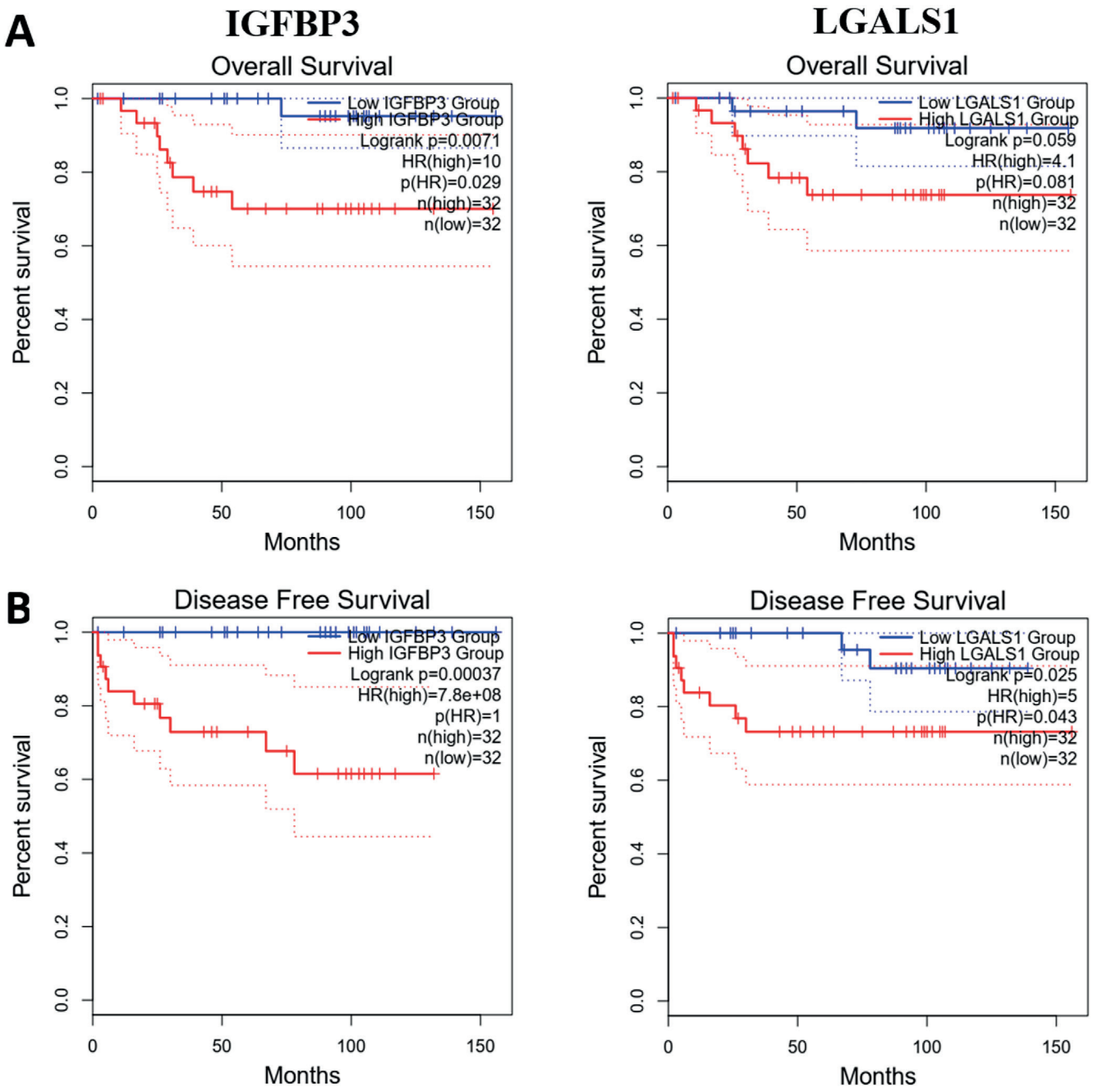

Fig. 8. Impact of IGFBP3 and LGALS1 expression on survival in ccRCC patients. The overall survival (A) and the disease-free survival (B) of ccRCC patients based on IGFBP3 and LGALS1 expression levels. Patients were divided into high- and low-expression groups

IGFBP-3 – insulin-like growth factor binding protein 3; LGALS1 – lectin, galactoside-binding, soluble, 1; ccRCC – clear cell renal cell carcinoma; HR – hazard ratio.

Transcriptomic data from the GEO and TCGA databases revealed high expression levels of *IGFBP3* and *LGALS1* in ccRCC tissue compared to normal kidney tissue. In addition, high expression of these genes was associated with shorter OS and DFS in ccRCC patients, underscoring their potential utility as diagnostic markers and prognostic indicators.

In cancer cells, *IGFBP3* regulates cell proliferation and apoptosis through both IGF-dependent and independent mechanisms. *IGFBP3* depletion suppresses glioma cell growth by inducing DNA damage and apoptosis. Furthermore, suppression of *IGFBP3* markedly increased the survival of brain-tumor-bearing mice.[30] Suppression of the IGFBP3-AKT/STAT3/MAPK-Snail signaling pathway by cyclovirobuxine resulted in a reduction of cell viability, proliferation, angiogenesis, migration, and invasion in ccRCC cells.[31] Overexpression of *IGFBP3* has been reported in several cancers, including breast cancer and nasopharyngeal carcinoma.[32,33] *IGFBP3* expression is associated with adverse outcomes such as metastasis, poor responses to chemoradiotherapy and decreased survival rates in cancer patients[33–35] Moreover, serum *IGFBP3*

is an independent prognostic risk factor in esophageal squamous cell carcinoma and esophagogastric junction adenocarcinoma.[36,37] Overexpression of *IGFBP3* has also been reported in ccRCC. A study by Braczkowski et al. demonstrated *IGFBP3* overexpression in ccRCC compared to adjacent non-cancerous kidney tissues using a quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay.[38] The distribution of *IGFBP3* genotypes was significantly associated with the histological grade and clinical stage of ccRCC patients.[39] This information suggests that *IGFBP3* could serve as a diagnostic and prognostic biomarker for ccRCC.

LGALS1, also known as galectin-1, is involved in various processes associated with cancer development and progression, including tumor transformation, cell cycle regulation, apoptosis, adhesion, migration, and inflammation.[40,41] Huang et al. reported that the suppression of *LGALS1* led to reduced cell invasion, clonogenic ability, epithelial–mesenchymal transition, and angiogenesis in renal cancer cell lines by upregulating C-X-C chemokine receptor type 4 through nuclear factor kappa B (NF-κB) activation.[42] Similarly, a report from 2014 highlighted that *LGALS1* plays a critical role in promoting the migration and invasion of ccRCC cells by activating the hypoxia-inducible factors/mammalian target of rapamycin signaling pathway.[43] Overexpression of *LGALS1* is correlated with tumor aggressiveness, including growth, cell migration, invasion, metastasis, and poor prognosis in several cancers such as hepatocellular carcinoma (HCC), upper urinary urothelial carcinoma, ovarian cancer, and squamous cervical cancer.[44–48] The potential of *LGALS1* as a serum biomarker has also been demonstrated in several cancers. Elevated plasma levels of galectin-1 have been found in pancreatic cancer,[49] classical Hodgkin lymphoma[50] and serous ovarian carcinoma. High serum levels of galectin-1 are associated with metastasis in epithelial ovarian cancer[51] and colorectal cancer.[52] In ccRCC, *LGALS1* expression was significantly associated with higher clinical grade and stage[53] and favorable outcomes from anti-PD1 treatment.[54] These results indicate the potential of using *LGALS1* as a prognostic marker and therapeutic target in ccRCC patients.

The results of our integrated bioinformatics and machine learning analysis indicate that *IGFBP3* and *LGALS1* are promising potential secreted biomarkers for the diagnosis of ccRCC.

Limitations

It is important to acknowledge the limitations of this study. The findings were derived from publicly available datasets from the GEO and the TCGA databases. The selection of these datasets may introduce potential biases, as they may not fully represent the broader patient population. Furthermore, the generalizability of our results may be constrained by variations in sample collection, processing methods and demographic factors across different studies. Accordingly, further research is planned to validate these findings in independent cohorts using serum or urine of ccRCC patients compared to healthy controls, with the objective of ensuring robustness and applicability to clinical settings.

Conclusions

The use of bioinformatics and machine learning enabled the identification of IGFBP3 and LGALS1 as potential secreted biomarkers for ccRCC. The classification models based on IGFBP3 and LGALS1 demonstrated the capacity to effectively differentiate ccRCC patients from healthy controls. Furthermore, the expression levels of IGFBP3 and LGALS1 were found to be useful not only for the diagnosis of ccRCC but also as prognostic biomarkers to predict patient outcomes.

Supplementary data

The Supplementary materials are available at https://doi.org/10.6084/m9.figshare.27154224.v1. The package includes the following files:

Supplementary File 1. DEG analysis from GEO database (GSE11151, GSE40435 and GSE53757).

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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RNA binding protein ELAVL1 is associated with severity and prognosis of hepatocellular carcinoma patients: A retrospective study

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Abstract

Background. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, which is characterized by a lack of sensitive and specific biomarkers.

Objectives. This study investigates the association between ELAV-like RNA binding protein 1 (ELAVL1) and HCC patient outcomes.

Materials and methods. This retrospective study encompassed 108 HCC patients who reported to Wuhan Fourth Hospital and Tongji Hospital, China, from January 2016 to August 2020. Clinical data collected included age, sex, body mass index (BMI), comorbidities, tumor-node-metastasis (TNM) stage, Barcelona Clinic Liver Cancer (BCLC) stage, and lymphatic metastasis. All patients received routine follow-up for survival and recurrence status ranged from 36 to 60 months. The serum levels of ELAVL1 were tested using enzyme-linked immuno-sorbent assay (ELISA). Levels of total bilirubin, alanine aminotransferase (ALT), aspartate transaminase (AST), HCC-related biomarkers of alpha fetoprotein (AFP), α-L-fucosidase (AFU), and carcinoembryonic antigen (CEA) were recorded.

Results. Our findings revealed a significantly higher expression of ELAVL1 in patients presenting with TNM stages III–IV, BCLC stages C–D, lymphatic metastasis, as well as deceased and recurrent patients. Receiver operating characteristic (ROC) curves showed that the areas under the curve (AUCs) for ELAVL1 in predicting mortality, recurrence and poor prognosis (defined as mortality or recurrence) in HCC patients were 0.818, 0.732 and 0.827, respectively. Patients with higher expression of ELAVL1 showed significantly higher frequencies of TNM III–IV stages, BCLC D stage, lymphatic metastasis, higher mortality, and recurrence ratio, as well as higher AFP and CEA levels. ELAVL1 was positively correlated with levels of AFP and CEA. Higher BCLC stage, lymphatic metastasis, age, AFP, and ELAVL1 were independent risk factors for poor prognosis of HCC patients.

Conclusions. Higher serum levels of ELAVL1 are associated with worse clinical outcomes and poorer prognosis in -HCC patients.

Key words: prognosis, hepatocellular carcinoma, retrospective study, clinical outcomes, ELAVL1

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Background

Annually, primary liver cancer accounts for over 900,000 new cases (4.7%) and more than 800,000 deaths globally.[1] The worldwide incidence rate of primary liver cancer stands at approx. 9.3 per 100,000 individuals, with an estimated mortality rate of 8.5 per 100,000. Notably, China has the highest burden of primary liver cancer, with over 380,000 new cases each year, representing nearly 60% of liver cancer patients across Asia.[2–4] In China, liver cancer is the 4th most common cancer and the 2nd leading cause of cancer-related deaths, with over 300,000 fatalities annually.[5,6] Recent research indicates that among all cancers, patients diagnosed with primary liver cancer and pancreatic cancer exhibit the poorest prognosis in China.[2]

Hepatocellular carcinoma (HCC), the most prevalent subtype of primary liver cancer, accounts for a significant majority, ranging from 75% to 85%, of all liver cancer cases.[7,8] Despite advances in treatment modalities, including targeted therapy and immunotherapy,[9] these approaches often fail to yield satisfactory outcomes for patients with advanced HCC, who continue to face a grim prognosis.[10,11] Recently, the combination of tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs) has shown higher efficacy and improved outcomes for HCC patients.[12] However, these combination treatments still necessitate further research and optimization to enhance their overall effectiveness and tolerability. In the realm of solid cancer diagnostics, histological analysis is regarded as the gold standard, with early detection primarily relying on imaging techniques and the analysis of serum/tissue biomarkers.[13] Compared with imaging methods, the detection of biomarkers can provide a more rapid, convenient and affordable method.[14] However, traditional biomarkers for HCC, such as alpha-fetoprotein (AFP), α-L-fucosidase (AFU) and carcinoembryonic antigen (CEA), lack sufficient sensitivity and specificity for effective HCC diagnosis.[15]

ELAV-like RNA binding protein 1 (ELAVL1), an RNA-binding protein, has been implicated in the progression of various cancer types, including prostate cancer,[16] gastric cancer[17] and breast cancer.[18] Nonetheless, the bulk of research to date has been confined to in vitro studies or animal models, with few investigations explicitly addressing the correlation between ELAVL1 and the clinical outcomes, prognosis or diagnostic relevance in HCC patients. Addressing the role of ELAVL1 in the clinical setting emerges as a critical and urgent need.

Objectives

In this study, we aimed to conduct a retrospective investigation to demonstrate the association of ELAVL1 with clinical outcomes and prognosis of HCC patients. This study might provide clinical evidence for novel biomarkers in diagnosis of HCC.

Materials and methods

Patients and study design

This retrospective study encompassed 108 HCC patients who presented to Wuhan Fourth Hospital and Tongji Hospital, China, from January 2016 to August 2020. This study analyzed recorded characteristics of patients and measured the expression of ELAVL1 using previously stored blood samples. The inclusion criteria were as follows: 1) confirmation of primary HCC diagnosis through both imaging techniques and histological analysis; 2) absence of prior treatment for HCC; 4) availability of complete clinical data. Exclusion criteria included incomplete clinical records, metastasis liver cancer or any treatment prior to diagnosis. The study received approval from the Ethical Committee of Wuhan Fourth Hospital and Tongji Hospital, China (approval No. KY2023-102-02).

Data collection of the patients

Clinical data collected included age, sex, body mass index (BMI), hepatitis B virus (HBV) status, hepatitis C virus (HCV) infection alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) and other comorbidities, as well as tumor-node-metastasis (TNM) stage, Barcelona Clinic Liver Cancer (BCLC) stage and lymphatic metastasis. All patients received the standard of care, which included routine follow-ups. The duration of survival and recurrence ranged from 36 to 60 months for all cancer patients treated at our hospital. Patients with less than 6 months of follow-up data were excluded unless deceased earlier. Follow-up concluded upon loss to follow-up or death. Overall survival and disease-free survival were calculated from diagnosis to the last follow-up or death.

Measurement of serum ELAVL1 and other serum indices

Briefly, 5 mL of fasting peripheral blood was collected from all patients at the time of diagnosis. These samples were originally collected for another planned study, which was ultimately not conducted. However, the informed consent obtained from the participants explicitly allowed for the potential use of their blood samples in future research endeavors. The samples were then stored at –80°C until analyzed for this research. The serum levels of ELAVL1 were tested using enzyme-linked immuno-sorbent assay (ELISA) using a human ELAVL1 kit (No. MBS7616762; MyBioSource, San Diego, USA) according to the manufacturer's instruction. Levels of total bilirubin, alanine aminotransferase (ALT), aspartate transaminase (AST), HCC-related biomarkers of AFP, AFU, and carcinoembryonic antigen (CEA) were tested with cobas® c602/c601 automatic biochemical analyzer (Roche Inc., Indianapolis, USA).

Statistical analyses

The Kolmogorov–Smirnov method was used to assess the distribution of the data (Supplementary Table 1). Non-normally distributed data were expressed as median (range, interquartile range (IQR)), and normally distributed data as mean ± standard deviation (±SD). The Mann–Whitney U test or unpaired Student's t-test was used for non-normally and normally distributed data, respectively. For the unpaired Student's t-test, Levene's test and Welch's correction were applied to account for heterogeneity of variances. Rates were compared using the χ^2 test of independence without rounding. Regression curve estimation and Spearman's analysis was used for analyzing the correlation. Spearman's correlation is appropriate for assessing monotonic relationships, whether they are linear or nonlinear. Additionally, regression curve estimation was employed to model the nonlinear relationships between predictors and the binary outcome, providing a more comprehensive understanding of the data. The receiver operating characteristic (ROC) curve evaluated prognostic value and the cutoff value was selected according to the Youden's index. Multivariable logistic regression identified mortality and recurrence risk factors using the enter method for predictor selection. The Box–Tidwell test and the variance inflation factor (VIF) values were used for measuring the linear relationship and multicollinearity. The Hosmer–Lemeshow goodness-of-fit test was used to assess the fit of the models, and casewise list (studentized residual) was used to identify outliers. Q–Q plots were used to assess the normality of residuals. A p-value <0.05 was considered statistically significant. Analyses were conducted using IBM SPSS v. 22.0 (IBM Corp., Armonk, USA) and GraphPad Prism v. 10.0 (GraphPad Software, Inc., San Diego, USA).

Table 1. Basic clinical characteristics of all patients

Variables		HCC patients (n = 108)
Age [years]		65.50 (47–80, 22)
Sex (male:female)		56:52
BMI [kg/m^2]		26.20 ±4.31
Comorbidities, n (%)	HBV infection	29 (26.85)
	HCV	19 (17.59)
	alcoholic liver disease	14 (12.96)
	NAFLD/NASH	25 (23.15)
	other	7 (6.48)
TNM stage, n (%)	I–II	57 (52.78)
	III–IV	51 (47.22)
BCLC stage, n (%)	0–A	57 (52.78)
	B	26 (24.07)
	C	14 (12.96)
	D	11 (10.19)
Lymphatic metastasis, n (%)		37 (34.26)
ALT [U/L]		174.86 (81.15–248.97, 79.97)
AST [U/L]		164.37 (81.47–248.00, 87.46)
AFP [ng/mL]		169.35 (100.85–297.59, 53.00)
AFU [U/L]		87.74 (40.14–149.20, 64.45)
CEA [U/L]		63.27 ±16.74
Median follow-up [months]		45 (5–60, 15)
Mortality, n (%)		31 (28.70)
Recurrence, n (%)		18 (16.67)

HCC – hepatocellular carcinoma; BMI – body mass index; HBV – hepatitis B virus; HCV – hepatitis C virus; NAFLD/NASH – non-alcoholic fatty liver disease/non-alcoholic steatohepatitis; TNM – tumor-node-metastasis classification; BCLC – Barcelona Clinic Liver Cancer; ALT – total bilirubin, alanine aminotransferase; AST – aspartate transaminase; AFP – alpha fetoprotein; AFU – α-L-fucosidase; CEA – carcinoembryonic antigen.

Results

ELAVL1 expression in serum samples of different HCC patients

The basic characteristics of all patients were shown in Table 1. Among all patients, 51 (47.22%) patients had TNM stage III–IV, 25 (23.15%) patients had BCLC stage C–D and 37 (34.26%) patients had lymphatic metastasis. A total of 31 (28.7%) patients died during the follow-up. The median follow-up duration was 45 (5–60, 15) months.

First, we detected and compared the serum levels of ELAVL1 in HCC patients (Fig. 1A–E). Our findings revealed a significantly higher expression of ELAVL1 in patients with TNM stages III–IV (162.19 ±61.20 pg/mL) and those with lymphatic metastasis (171.85 ±66.74 pg/mL), as well as those with BCLC stages C–D (181.31 ±53.26), in comparison to patients with TNM stages I–II (125.43 ±50.17 pg/mL) and without lymphatic metastasis (127.65 ±47.32 pg/mL), as well as those with BCLC stages 0–B (131.19 ±55.02), respectively. Besides, deceased (192.80 ±55.27 pg/mL) and recurrent patients (187.11 ±61.21 pg/mL) also showed remarkably higher ELAVL1 levels than survival patients (122.66 ±46.49 pg/mL) and non-recurrent (133.93 ±53.89 pg/mL) patients, respectively. All these results implied potential relationship between ELAVL1 level and disease severity, as well as prognosis of HCC patients.

Predictive value of ELAVL1 for poor prognosis of mortality and recurrence of HCC patients

Then, ROC curves were used to analyze the value of ELAVL1 for predicting mortality and recurrence of HCC patients. As shown in Fig. 2, the area under the curve (AUC) of ELAVL1 for predicting patients' mortality was 0.818, with sensitivity 80.65%, specificity 62.34% and cutoff value of >135.0 pg/mL. For predicting recurrence, the AUC of ELAVL1 was 0.732, with sensitivity 72.22%, specificity 55.56% and cutoff value >135.9 pg/mL. Next, we regarded

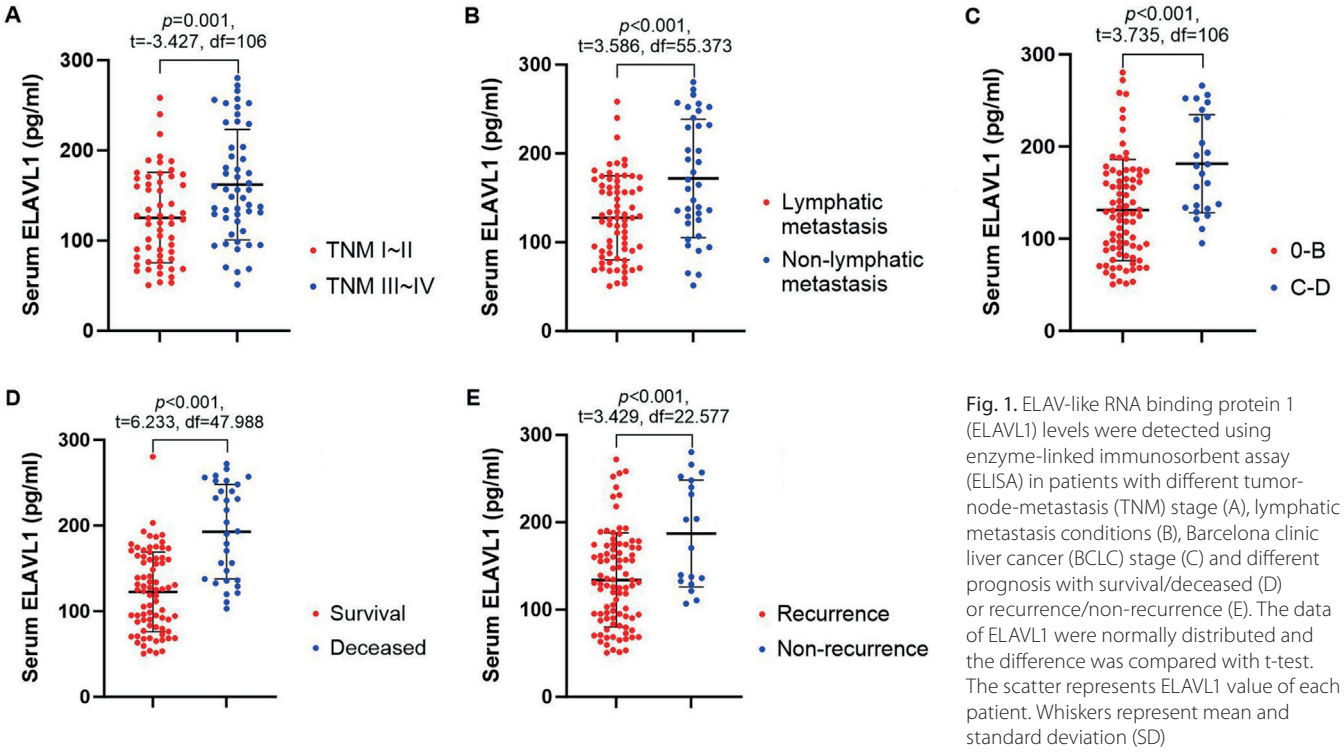

Fig. 1. ELAV-like RNA binding protein 1 (ELAVL1) levels were detected using enzyme-linked immunosorbent assay (ELISA) in patients with different tumor-node-metastasis (TNM) stage (A), lymphatic metastasis conditions (B), Barcelona clinic liver cancer (BCLC) stage (C) and different prognosis with survival/deceased (D) or recurrence/non-recurrence (E). The data of ELAVL1 were normally distributed and the difference was compared with t-test. The scatter represents ELAVL1 value of each patient. Whiskers represent mean and standard deviation (SD)

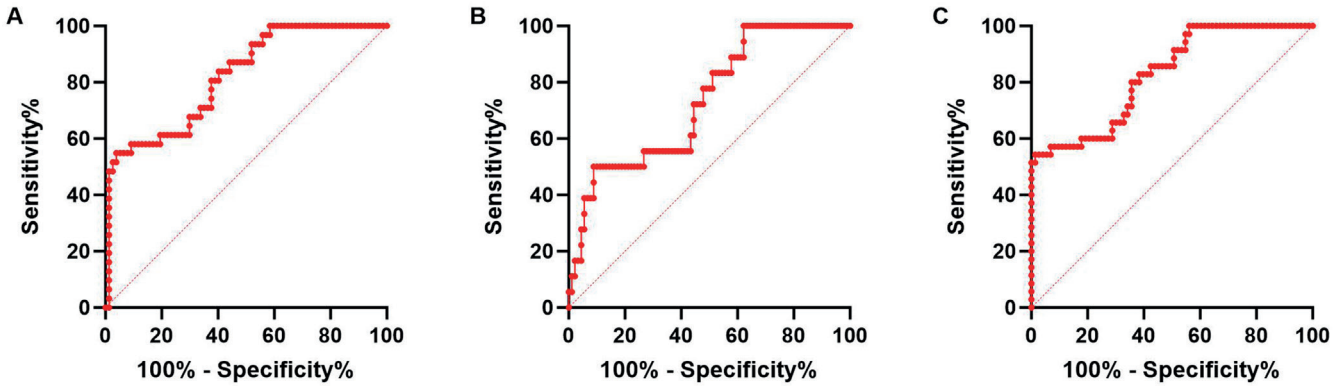

Fig. 2. Receive operating characteristic (ROC) curves of ELAV-like RNA binding protein 1 (ELAVL1) for predicting mortality (A), recurrence (B) and bad prognosis (C) of hepatocellular carcinoma (HCC) patients. The cutoff value was selected according to the Youden's index

both mortality and recurrence as poor prognosis of the patients, and ELAVL1 also demonstrated the predictive value of poor prognosis with AUC 0.827, sensitivity 80.00%, specificity 64.38%, and cutoff value >135.0 pg/mL. When choosing cut off value, we considered higher sensitivity first and used the Youden's index. All these results indicated the potential value for ELAVL1 to predict prognosis of HCC patients.

The relationship between ELAVL1 and clinical outcomes of HCC patients

To further investigate the role of ELAVL1 in HCC patients, we divided the patients into high and low ELAVL1 groups according to the above cutoff value for predicting poor prognosis of HCC (>135.0 pg/mL). As shown in Table 2, patients with higher expression of ELAVL1 showed significantly higher frequencies of TNM III–IV stages, BCLC stage D and lymphatic metastasis, as well as higher mortality and recurrence ratios. Meanwhile, the levels of AFP and CEA were markedly higher in patients with higher ELAVL1 compared with the low expression group. The results of Levene's tests for assumption of homogeneity of variances are shown in Supplementary Table 2. Further correlation analysis of Spearman's analysis (Table 3) and regression curve estimation (Fig. 3) showed that ELAVL1 was positively correlated with the levels of AFP and CEA. Regression curve estimation allows for fitting various types of curves (such as linear, logarithmic, reciprocal, quadratic, cubic, compound, power, S-curve, growth, exponential, and logistic models) to the data, providing a comprehensive analysis of the relationship

Table 2. Clinical characteristics in patients with high/low expressions of ELAVL1

Variables		Low ELAVL1 (n = 54)	High ELAVL1 (n = 54)	t, Z or χ^2	p-value
Age [years]*		67 (47–80, 20.25)	62.50 (47–79, 23)	–0.664	0.507
Sex (male:female)#		29:25	27:27	0.272	0.601
BMI [kg/m^2]&		26.32 ±4.38	26.08 ±4.28	0.296	0.768
Comorbidities, n (%)#	HBV infection	14 (25.93)	15 (27.78)	0.768	0.874
	HCV	9 (16.67)	10 (18.52)	0.731	0.854
	alcoholic liver disease	8 (14.81)	6 (11.11)	0.436	0.529
	NAFLD/NASH	12 (22.22)	13 (24.07)	0.756	0.867
	other	2 (3.70)	5 (9.26)	0.110	0.251
TNM stage, n (%)#	I–II	35 (64.81)	22 (40.74)	11.623	0.001
	III–IV	19 (35.19)	32 (59.26)		
BCLC stage, n (%)#	0–A	35 (64.81)	22 (40.74)	19.945	<0.001
	B	12 (22.22)	14 (25.93)		
	C	6 (11.11)	8 (14.81)		
	D	1 (1.85)	10 (18.52)		
Lymphatic metastasis, n (%)#		12 (22.22)	25 (46.30)	12.873	<0.001
ALT [U/L]*		182.69 (81.15–242.45, 77.96)	172.52 (84.05–248.97, 80.59)	–0.842	0.400
AST [U/L]*		154.95 (81.47–244.47, 87.04)	175.05 (82.22–248.00, 86.85)	–1.309	0.191
AFP [ng/mL]*		165.23 (102.03–286.05, 48.13)	181.29 (100.85–297.59, 63.16)	–2.851	0.004
AFU [U/L]*		85.55 (40.14–149.20, 65.77)	90.36 (40.32–145.57, 62.73)	–0.510	0.610
CEA [U/L]&		53.97 ±13.89	72.57 ±14.01	–6.926	<0.001
Median follow-up [months]*		46 (6–60, 12.25)	43 (5–60, 25.50)	–1.642	0.101
Mortality, n (%)#		6 (11.11)	25 (46.30)	30.255	<0.001
Recurrence, n (%)#		5 (9.26)	13 (24.07)	7.897	0.005

* Comparisons made with Mann–Whitney U test. # Comparisons made with χ^2 test of independence without rounding. & Comparisons made using unpaired student's t-test. ELAVL1 – ELAV-like RNA binding protein 1; BMI – body mass index; HBV – hepatitis B virus; HCV – hepatitis C virus; NAFLD/NASH – non-alcoholic fatty liver disease/non-alcoholic steatohepatitis; TNM – tumor-node-metastasis classification; BCLC – Barcelona Clinic Liver Cancer; ALT – total bilirubin, alanine aminotransferase; AST – aspartate transaminase; AFP – alpha fetoprotein; AFU – α-L-fucosidase; CEA – carcinoembryonic antigen. Comparison between 2 groups was performed with Mann–Whitney U test. χ^2 was used for comparing rates (without half adjust).

Table 3. Spearman's analysis for correlation among ELAVL1, AFP, AFU, and CEA

Variables	ELAVL1	
	Spearman's correlation	p-value
AFP	0.360	<0.001
AFU	0.054	0.581
CEA	0.484	<0.001

ALT – total bilirubin, alanine aminotransferase; AST – aspartate transaminase; AFP – alpha fetoprotein; AFU – α-L-fucosidase; CEA – carcinoembryonic antigen.

between variables by evaluating different models to identify the best-fitting curve.[19] In Fig. 3, positive correlations were observed in all analyses of the linear, logarithmic, reciprocal model, quadratic curve model, cubic curve model, compound model, power, S-curve, growth model, exponential model, and logistic models for both AFP and CEA with ELAVL1 (all p-values <0.001). These results further indicated that the high levels of ELAVL1 were associated with poorer clinical outcomes and prognosis of HCC patients.

Risk factors for poor prognosis of mortality and recurrence of HCC patients

Multivariable logistic regression was then used to analyze the independent risk factors for poor prognosis (mortality and recurrence) in HCC patients using the enter method for predictor selection. We used 2 separate models in the regression analysis. Model 1 included all counting data of sex, comorbidities, TNM stage, BCLC stage, and lymphatic metastasis, with Nagelkerke R^2 = 0.609. Model 2 included all continuous data of age, BMI, ALT, AST, AFP, AFU, CEA, and ELAVL1, with Nagelkerke R^2 = 0.750. The results of the Box–Tidwell test and the VIF values (Supplementary Tables 3–5) showed that the data met the criteria for a linear relationship and had no multicollinearity. Residual analysis identified 6 outliers for Model 1 and 4 outliers for Model 2, with the Hosmer–Lemeshow test yielding p-values of 0.908 and 0.344, respectively (Supplementary Tables 6–11). The outliers were not excluded as they did not affect the goodness-of-fit of the models. Additionally, The Q–Q plot showed that

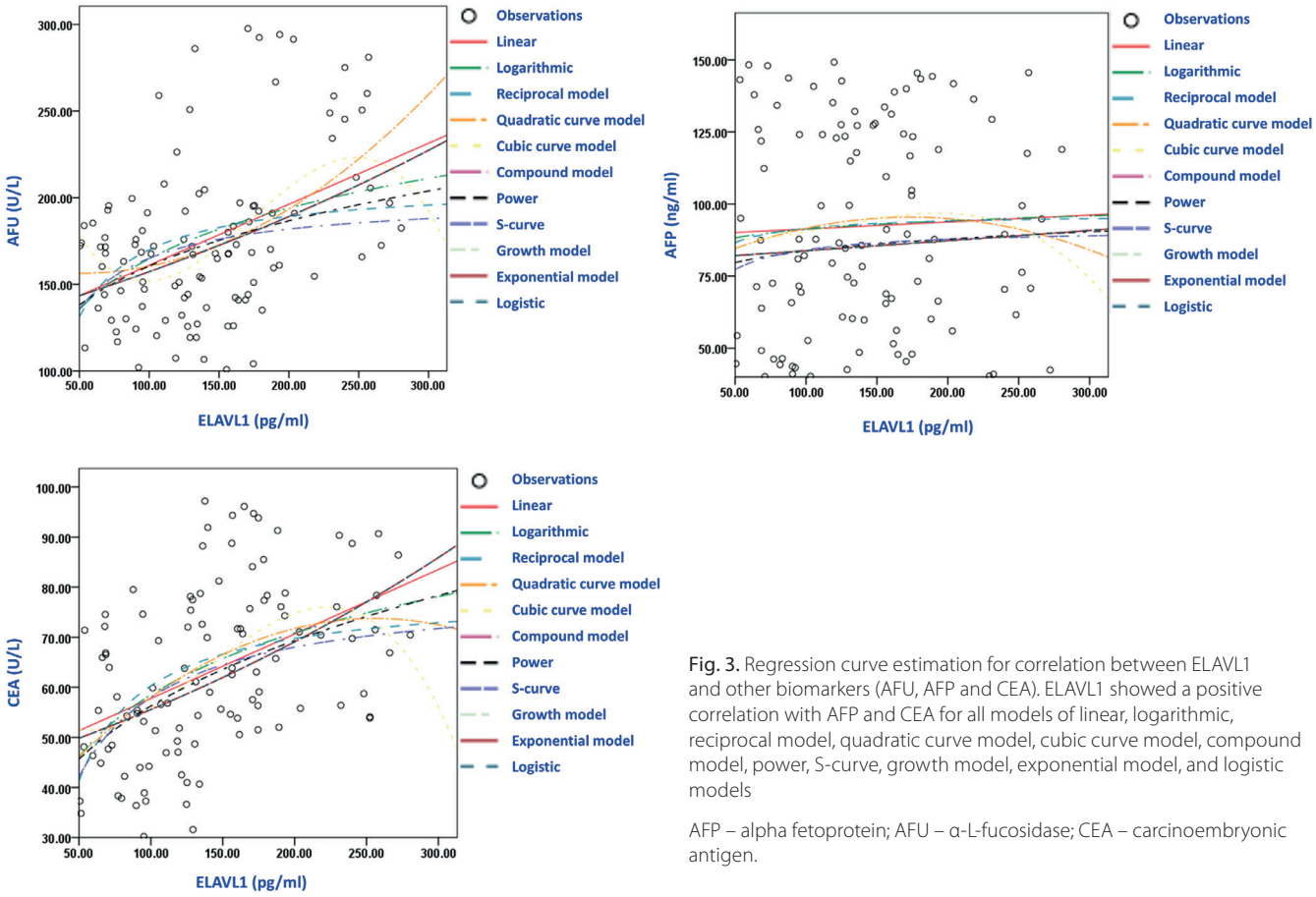

Fig. 3. Regression curve estimation for correlation between ELAVL1 and other biomarkers (AFU, AFP and CEA). ELAVL1 showed a positive correlation with AFP and CEA for all models of linear, logarithmic, reciprocal model, quadratic curve model, cubic curve model, compound model, power, S-curve, growth model, exponential model, and logistic models

AFP – alpha fetoprotein; AFU – α-L-fucosidase; CEA – carcinoembryonic antigen.

Table 4. Multivariable logistic regression for poor prognosis of HCC patients

Variables	OR	95% CI	p-value
Model 1			
Sex	1.480	0.438–4.997	0.528
Comorbidities	1.039	0.691–1.564	0.853
TNM stage	0.220	0.021–2.315	0.207
BCLC stage	4.934	1.544–15.764	0.007
Lymphatic metastasis	18.778	4.599–76.672	<0.001
Model 2			
Age	1.135	1.030–1.250	0.010
BMI	0.940	0.801–1.103	0.446
ALT	1.016	0.997–1.036	0.103
AST	0.999	0.985–1.013	0.863
AFP	1.074	1.035–1.115	<0.001
AFU	0.998	0.977–1.019	0.824
CEA	1.016	0.972–1.063	0.475
ELAVL1	1.031	1.012–1.050	0.001

HCC – hepatocellular carcinoma; OR – odds ratio; 95% CI – 95% confidence interval; TNM – tumor-node-metastasis; BCLC – Barcelona clinic liver cancer; BMI – body mass index; ALT – alanine aminotransferase; AST – aspartate transaminase; AFP – alpha fetoprotein; AFU – α-L-fucosidase; CEA – carcinoembryonic antigen; ELAVL1 – ELAV-like RNA binding protein 1.

the residuals were approximately normally distributed (Supplementary Fig. 1,2). As shown in Table 4, BCLC stage, lymphatic metastasis, age, AFP, and ELAVL1 were independent risk factors for poor prognosis in HCC patients.

Discussion

Early diagnosis is the key point for treatment efficacy of HCC patients, while current HCC biomarkers such as AFU and AFP have insufficient sensitivity and specificity for diagnosis of HCC. In the present study, we demonstrated that ELAVL1 was elevated in serum samples of HCC patients, associated with patients' TNM stage and lymphatic metastasis, and could be used as a potential biomarker for the poor prognosis.

ELAVL1 has been documented to play a facilitative role in the development of various cancers. In prostate cancer, ELAVL1 enhances cellular proliferation through the regulation of m6A modification, representing an epigenetic mechanism for RNA.[16] Additionally, ELAVL1 contributes to the progression of gastric cancer by promoting cytoplasmic translocation and inhibiting ferroptosis, a form of programmed cell death driven by iron accumulation.[17] In breast cancer, elevated ELAVL1 expression is associated with increased cell proliferation and metastasis, alongside chemoresistance,

by modulating the Rab10/TLR4 signaling pathway.[18] Furthermore, ELAVL1 has been shown to accelerate tumorigenesis in colorectal,[20] lung[21] and pancreatic cancers.[22] However, despite the abundance of cellular and animal studies, there is a scarcity of research on the clinical relevance of ELAVL1 in cancer patients, including those with HCC.

Recent studies have also highlighted the role of ELAVL1 in promoting cancer in HCC. It has been observed that ELAVL1 is more commonly expressed in HCC tissues compared to normal tissues and is linked to postoperative recurrence in patients with HBV-related HCC.[23] However, it remains unclear whether ELAVL1 is associated with other clinical outcomes and the prognosis of patients, such as overall mortality in all HCC patients. Furthermore, an in vitro study has demonstrated that the long non-coding RNA (lncRNA) CCTA2 enhances HCC progression by interacting with ELAVL1, which in turn facilitates cancer cell migration and invasion.[24] Beyond these findings, there has been limited research on the role of ELAVL1 in HCC. In our study, we observed that higher ELAVL1 predicted higher frequency of TNM III–IV and BCLC stage C–D. Importantly, liver function is a critical factor in determining the prognosis and treatment options for HCC patients, as it significantly influences the functional liver reserve and the ability to tolerate treatments.[25] The BCLC staging system includes assessments of liver function, reflecting its impact on prognosis and treatment decisions. We also found that the serum level of ELAVL1 is associated with poorer prognosis, providing further evidence for the involvement of ELAVL1 in HCC.

Limitations

This study also has several limitations. First, it is a retrospective study, not a prospective one. Second, our focus was only on the value of ELAVL1 at the time of diagnosis. The long-term and dynamic impacts of ELAVL1 levels on the prognosis of HCC patients remain uncertain.

Conclusions

In this retrospective analysis, we showed that higher serum levels of ELAVL1 were associated with worse clinical outcomes and poorer prognosis in patients with HCC. These findings could serve as a novel biomarker and a research target for investigating HCC.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.13868336. The package includes the following files:

Supplementary Table 1. Kolmogorov–Smirnov test results for data distribution in continuous variables.

Supplementary Table 2. Results of the unpaired Student's t-test with Levene's test in Table 2.

Supplementary Table 3. Results of the Box–Tidwell test for Model 2 in Table 4.

Supplementary Table 4. VIF values of variables in Model 1 in Table 4.

Supplementary Table 5. VIF values of variables in Model 2 in Table 4.

Supplementary Table 6. Hosmer–Lemeshow test for Model 1 in Table 4.

Supplementary Table 7. Contingency table for the Hosmer–Lemeshow test for Model 1 in Table 4.

Supplementary Table 8. Casewise List for Model 1 in Table 4.

Supplementary Table 9. Hosmer–Lemeshow test for Model 2 in Table 4.

Supplementary Table 10. Contingency table for the Hosmer–Lemeshow test for Model 2 in Table 4.

Supplementary Table 11. Casewise List for Model 2 in Table 4.

Supplementary Fig. 1. Q–Q plot showed that the residuals are approximately normally distributed in Model 1 in Table 4.

Supplementary Fig. 2. Q–Q plot showed that the residuals are approximately normally distributed in Model 2 in Table 4.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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The causal role of metabolic syndrome components in insomnia: A bidirectional two-sample Mendelian randomization

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Conflict of interest
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Abstract

Background. The neuroendocrine system's role in maintaining bodily homeostasis implicates it in insomnia, suggesting both causal relationships and therapeutic targets. Yet, studies examining the link between metabolic syndrome (MetS) components such as hypertension, elevated blood glucose levels and abnormal cholesterol and insomnia have been inconsistent. Some research suggests a correlation, proposing that metabolic dysfunctions might contribute to sleep disturbances. However, other studies found little to no significant connection, indicating the complexity of this relationship and the potential influence of genetic, lifestyle and environmental factors. These contradictory findings underscore the challenges in fully understanding the intricate interplay between metabolic health and sleep quality.

Objectives. To explore the relationship between MetS and insomnia.

Materials and methods. This study used bidirectional two-sample Mendelian randomization (MR) analysis to determine the causal relationship between the characteristics of MetS components and insomnia. Based on Genome-Wide Association Studies (GWAS) public databases, we explored the causal relationship between waist circumference (WC), hypertension, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), fasting blood glucose (FBG), and the risk of insomnia. Sensitivity analysis was conducted to evaluate the stability, heterogeneity and potential presence of horizontal pleiotropy in the results.

Results. Waist circumference and hypertension were associated with an increased risk of insomnia (WC, odds ratio (OR) = 1.05, 95% confidence interval (95% CI): 1.03–1.06, p = 9.15e–07; hypertension, OR = 1.06, 95% CI: 1.02–1.10, p = 0.005). In the reverse MR analysis, there was no significant causal relationship between insomnia and WC, TG, HDL-C, and FBG.

Conclusions. Our study has demonstrated the close connection between MetS components and insomnia by genetic means, thereby guiding the future research direction of insomnia prevention and treatment.

Key words: metabolic syndrome, insomnia, sensitivity, causal inference, MR analysis

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Background

Insomnia is pervasive sleep disorder characterized by poor sleep quality, difficulty initiating or maintaining sleep, and impaired daytime functioning. It is a significant public health issue, affecting over 27% of the global population,[1] with prevalence rates as high as 50% in developed countries.[2] Chronic insomnia is more than just a sleep problem; it is closely linked to an increased risk of serious mental health conditions, including depression, anxiety and suicidal ideation.[3] The impact of insomnia extends beyond mental health; as it also exacerbates various chronic physical conditions, leading to a diminished quality of life and increased healthcare costs. As such, effective management of insomnia is crucial, yet the current reliance on pharmacological treatments presents challenges, including dependency, withdrawal symptoms and adverse effects.[4] These limitations underscore the urgent need for identifying and addressing modifiable risk factors to develop more sustainable and effective interventions.

Metabolic syndrome (MetS) is a complex cluster of interrelated metabolic abnormalities, including central obesity, dyslipidemia, hypertension, and impaired glucose metabolism.[5] It is a significant contributor to the global burden of cardiovascular disease (CVD), diabetes and mortality.[6] Increasing evidence suggests that MetS is not only a marker of metabolic dysfunction but may also play a crucial role in the pathophysiology of sleep disorders, particularly insomnia. The bidirectional relationship between sleep and metabolic health is well documented; poor sleep can lead to metabolic disturbances, while metabolic dysregulation can impair sleep quality.[7] Central obesity, for example, is associated with increased pro-inflammatory cytokines, which may disrupt sleep architecture. Similarly, hypertension and dyslipidemia can influence autonomic nervous system function, further contributing to sleep disturbances. However, while the association between MetS and insomnia is recognized, the causal pathways remain inadequately understood, with most studies being limited by observational designs that cannot fully account for confounding factors or reverse causality.

Recent advancements in genetic epidemiology, particularly Mendelian randomization (MR), offer a powerful tool to address these limitations. Mendelian randomization uses genetic variants as instrumental variables to infer causal relationships between exposures (e.g., MetS components) and outcomes (e.g., insomnia), leveraging the random allocation of alleles at conception to mimic the conditions of a randomized controlled trial (RCT). This approach minimizes biases from confounding and reverse causality, providing stronger evidence for causal inference in the relationship between MetS and insomnia. Despite its potential, the use of MR to investigate the causal effects of the MetS on sleep disorders is still in its infancy, with existing studies often limited by small sample sizes and inadequate consideration of potential pleiotropy.

This study aimed to fill these gaps by employing a comprehensive bidirectional two-sample MR approach to investigate the causal relationships between key MetS components (central obesity, hypertension, dyslipidemia, and glucose metabolism) and the risk of insomnia. Furthermore, we explored the potential reverse causality to understand whether insomnia might contribute to the development of MetS. By addressing these complex interactions, this study sought to provide robust evidence that can inform more targeted prevention and treatment strategies for insomnia, ultimately contributing to improved public health outcomes.

Objectives

We conducted a comprehensive analysis using a bidirectional two-sample MR approach to gain a thorough understanding of the relationship between insomnia and components of metabolic syndrome. Employing this method, we not only examined the influence of MetS components on insomnia but also explored whether insomnia could potentially influence the development of MetS components. This bidirectional analysis is crucial for verifying the presence of a causal relationship between the exposure (MetS components) and the outcome (insomnia), thus providing a robust framework to explore these complex interactions.

Materials and methods

Study and data sources

The statistics for the components of MetS were obtained from Genome-Wide Association Studies (GWAS) public databases, and data on the waist circumference (WC), hypertension, triglycerides (WC), high-density lipoprotein cholesterol (HDL-C), and fasting blood glucose (FBG) were collected. The meta-analysis conducted by the UK Biobank yielded results for high blood pressure and WC.[8] The WC dataset comprised 462,166 samples and 9,851,867 single-nucleotide polymorphisms (SNPs), whereas the hypertension dataset contained 461,880 samples and 9,851,867 SNPs. Triglycerides and HDL-C were obtained from a study conducted by Richardson et al.,[9] with 441,016 samples and 12,321,875 SNPs in the TG dataset, and 403,932 samples and 12,321,875 SNPs in the HDL-C dataset. Data on FBG were obtained from the meta-analyses performed by the consortium for glucose and insulin-related characteristics, which encompassed a total of 281,416 samples.[10] The GWAS summary statistics for insomnia were obtained from the GWAS public databases,[11] which included 462,341 European individuals with 9,851,867 SNPs. Insomnia was diagnosed using the criteria from the International Classification of Diseases, 11th edition (ICD-11). The qualified SNPs are summarized in Table 1.

Table 1. The R^2 and F-statistics for the genetic instruments in the MR analyses

Exposure		Outcome	SNPs, n	R^2 [%]	F-statistic
MR	WC	insomnia	339	9.84	103.63
	hypertension	insomnia	217	7.89	72.35
	TG	insomnia	313	9.05	95.69
	HDL-C	insomnia	280	8.11	35.57
	FBG	insomnia	21	1.18	55.92
Re-MR	insomnia	WC	39	1.73	69.24
	insomnia	hypertension	39	1.77	53.98
	insomnia	TG	34	1.38	23.31
	insomnia	HDL-C	34	1.34	42.54
	insomnia	FBG	15	0.57	21.11

MetS – metabolic syndrome; MR – Mendelian randomization; WC – waist circumference; TG – triglycerides; HDL-C – high-density lipoprotein cholesterol; SNPs – single nucleotide polymorphisms.

Statistical methods main analysis

Supplementary techniques such as MR-Egger, MR-PRESSO (Mendelian Randomization Pleiotropy RESidual Sum and Outlier), weighted median (WM), simple mode, and weighted mode were used to evaluate possible diversity and pleiotropy, thereby ensuring the reliability of the primary analysis. MR-Egger provides a dependable estimation of the causal impact by incorporating the intercept term, while also indicating the existence of directional pleiotropy. A significant intercept term indicates the presence of horizontal pleiotropy.[12,13] The MR-PRESSO tool was used to detect and eliminate any influential outliers, thereby enhancing the heterogeneity and horizontal pleiotropy of the analysis.[14] The combination of multiple estimates using WM enables the determination of a single overall effect, which remains reliable even if half of the SNPs are considered invalid.[15] To explore the potential variation among the chosen independent variables (IVs), the Cochran's Q test was conducted. In cases where heterogeneity was found in the study, the MR analysis was performed with inverse-variance weighted (IVW) method.

Sensitivity analyses

The leave-one-out analysis was performed to demonstrate the resilience of the findings through sensitivity analyses and examining each SNP,[16] which enabled the identification and exclusion of variables that could potentially influence the results. The MR study overview diagram is presented in Fig. 1.

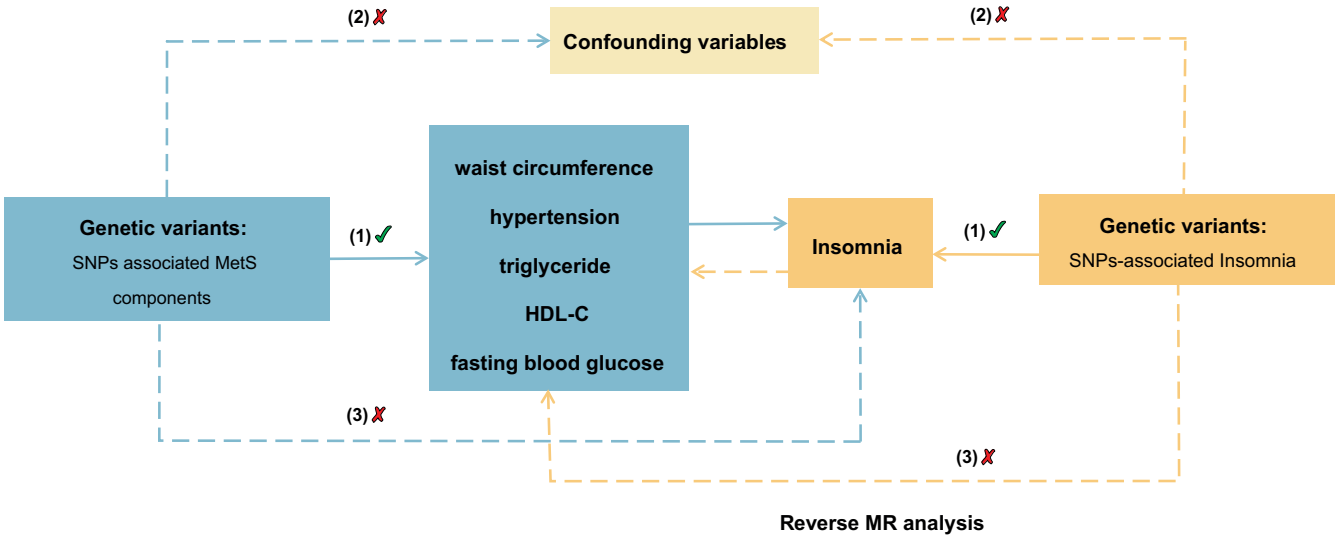

Fig. 1. The overview of Mendelian randomization (MR) analysis. 1. Independent variables are directly associated with exposure; 2. Independent variables are not associated with any confounders between exposure and outcome; 3. Independent variables do not affect the outcome via pathways unrelated to the exposure

HDL-C – high-density lipoprotein cholesterol; SNPs – single nucleotide polymorphisms; MetS – metabolic syndrome.

Software

All statistical analyses were conducted using the "TwoSampleMR", "vroom", "tidyr", and "forest plot" packages within R v. 4.2.3 (R Foundation for Statistical Computing in Vienna, Austria).

Results

Investigating the impact of components of MetS on insomnia

Table 2 displays the findings of MR analyses, indicating that genetically predicted WC has a 1.05-fold association with insomnia risk. Furthermore, hypertension also contributes to an increased risk of insomnia (odds ratio (OR) = 1.06, 95% confidence interval (95% CI): 1.02–1.10, p = 0.005, Table 2). The scatter plot illustrates that as the influence of SNPs on insomnia grows, the impact of SNPs on WC and hypertension becomes stronger, suggesting that WC and hypertension contribute to an increased risk of insomnia (Fig. 2). However, no association was found between the genetically instrumented TG, HDL-C, FBG, and the risk of insomnia ($p_{all} > 0.05$, Table 1, Fig. 2). In addition, the MR results exhibited a symmetry, suggesting the absence of heterogeneity (Fig. 3). Neither the MR-Egger nor the MR-PRESSO analyses identified any notable outliers. Furthermore, the exclusion of individual SNPs during the leave-one-out analysis did not significantly impact the results (Fig. 4).

Investigating the impact of insomnia on components of MetS

The reverse MR analyses indicated insomnia leads to a rise in hypertension levels (OR = 1.10, 95% CI: 1.01–1.19, p =0.031; Supplementary Table 1 and Supplementary Fig. 1). However, the sensitivity analysis did not support a causal association. There was no significant causal relationship observed between insomnia and WC, TG, HDL-C, and FBG ($p_{all} > 0.05$; Supplementary Table 1). No heterogeneity was

Table 2. The causal effect of MetS components on insomnia in the MR analysis

Exposure	Outcome	Methods	SNPs, n	p-value	OR (95% CI)	Cochran's p-value	MR-Egger p-value
WC	insomnia	MR-Egger	339	0.3400	0.98 (0.93,1.03)	<0.001	0.848
		weighted median	339	0.0075	1.04 (1.01, 1.06)	–	–
		IVW	339	<0.001	1.05 (1.03, 1.06)	<0.001	–
		simple mode	339	0.0535	1.09 (1.00, 1.19)	–	–
		weighted mode	339	0.1470	1.04 (0.99, 1.09)	–	–
Hypertension	insomnia	MR-Egger	217	0.8800	1.01 (0.91, 1.13)	<0.001	0.372
		weighted median	217	0.0300	1.05 (1.01, 1.10)	–	–
		IVW	217	0.0050	1.06 (1.02, 1.10)	<0.001	–
		simple mode	217	0.3800	1.07 (0.92, 1.24)	–	–
		weighted mode	217	0.4800	1.03 (0.94, 1.13)	–	–
TG	insomnia	MR-Egger	313	0.1900	1.01 (0.99, 1.03)	<0.001	0.369
		weighted median	313	0.6800	1.00 (0.99, 1.01)	–	–
		IVW	313	0.2900	0.99 (0.98, 1.00)	<0.001	–
		simple mode	313	0.2400	0.99 (0.96, 1.01)	–	–
		weighted mode	313	0.4300	1.00 (0.98,1.01)	–	–
HDL-C	insomnia	MR-Egger	280	0.2800	0.99 (0.98, 1.01)	<0.001	0.464
		weighted median	280	0.2300	0.99 (0.98, 1.00)	–	–
		IVW	280	0.1400	1.01 (1.00, 1.02)	<0.001	–
		simple mode	280	0.6300	1.01 (0.98, 1.04)	–	–
		weighted mode	280	0.4800	1.00 (0.98, 1.01)	–	–
FBG	insomnia	MR-Egger	21	0.5200	1.02 (0.97, 1.07)	0.07	0.973
		weighted median	21	0.4400	1.01 (0.98, 1.04)	–	–
		IVW	21	0.1500	1.02 (0.99, 1.04)	0.09	–
		simple mode	21	0.4000	1.02 (0.98, 1.07)	–	–
		weighted mode	21	0.3600	1.01 (0.99, 1.04)	–	–

MetS – metabolic syndrome; MR – Mendelian randomization; IVW – inverse-variance weighted method; WC – waist circumference; TG – triglycerides; HDL-C – high-density lipoprotein cholesterol; OR – odds ratio; 95% CI – 95% confidence interval; SNPs – single nucleotide polymorphisms.

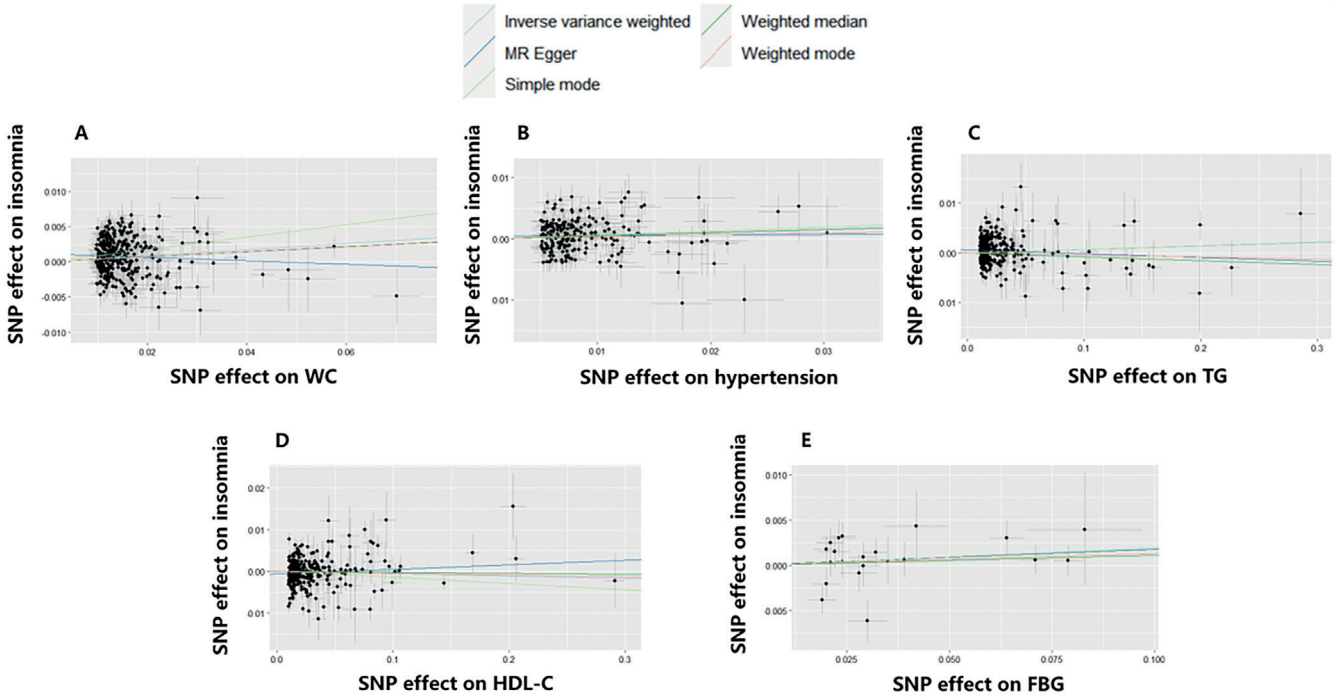

Fig. 2. The scatter plots of the association between genetically predicted in the Mendelian randomization (MR) analysis. A. Waist circumference (WC); B. Hypertension; C. Triglycerides (TG); D. High-density lipoprotein cholesterol (HDL-C); E. Fasting blood glucose (FBG) on insomnia in the MR analysis

SNP – single nucleotide polymorphism.

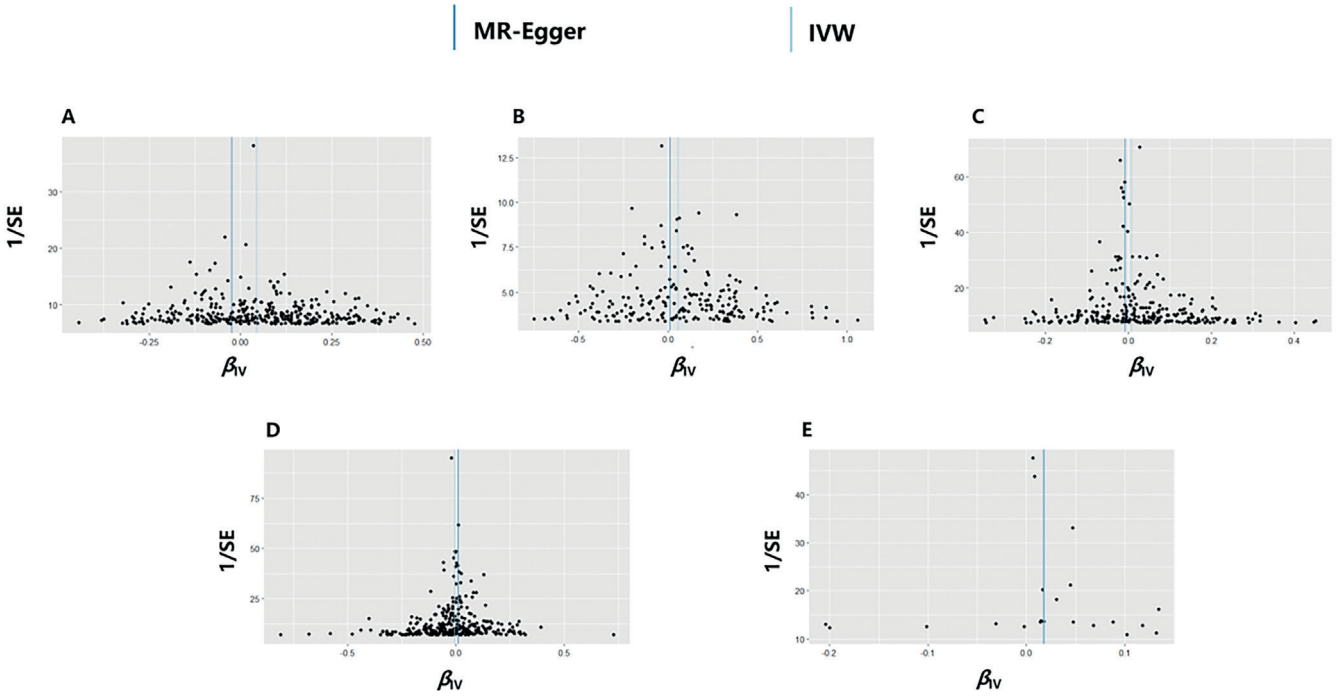

Fig. 3. The funnel plots of the association between metabolic syndrome (MetS) components and insomnia genetically in the Mendelian randomization (MR) analysis. A. Waist circumference (WC); B. Hypertension; C. Triglycerides (TG); D. High-density lipoprotein cholesterol (HDL-C); E. Fasting blood glucose (FBG)

IVW – inverse-variance weighted method.

detected in the Cochran's Q test (Supplementary Fig. 2), and no remarkable anomalies were found in the MR-Egger and MR-PRESSO analysis ($p_{all} > 0.05$). The leave-one-out analysis exhibited no substantial influence when each SNP was individually removed (Supplementary Fig. 3).

Discussion

Our bidirectional MR study presents innovative genetic evidence suggesting that WC and hypertension are causally associated with an increased risk of insomnia.

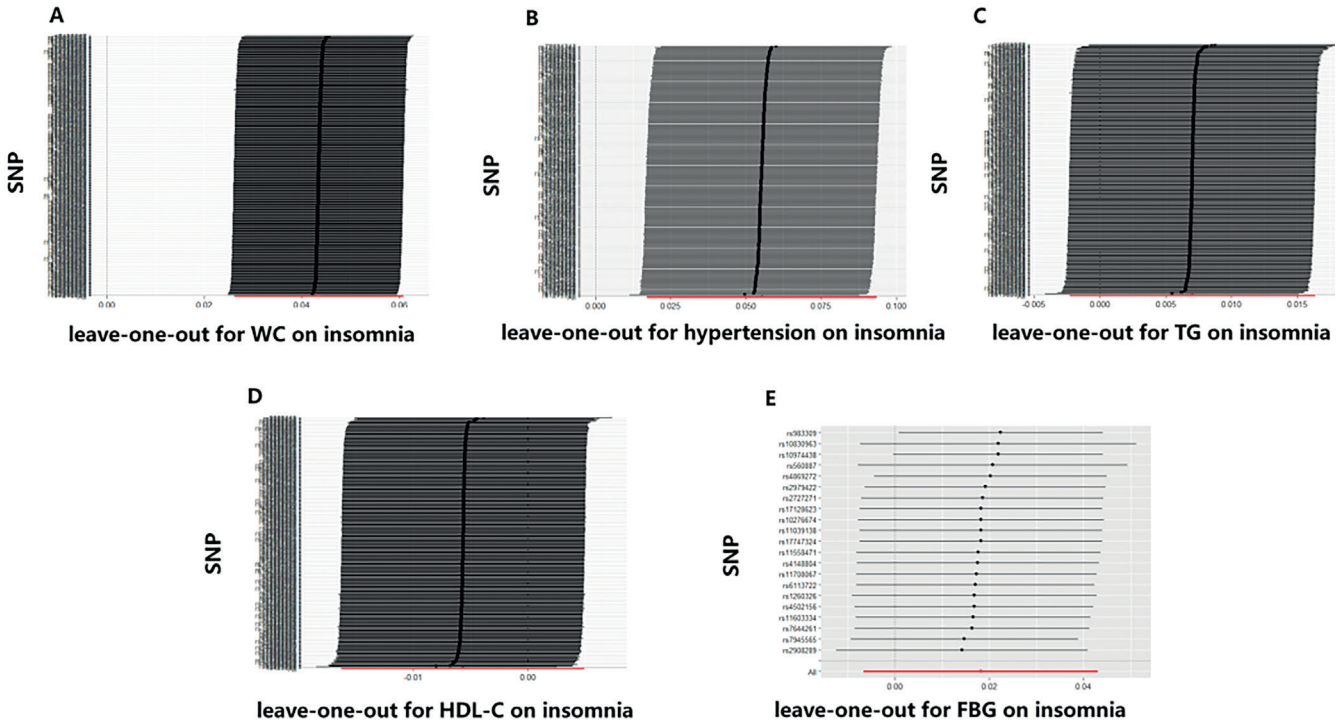

Fig. 4. The leave-one-out analysis of the association between metabolic syndrome components and insomnia genetically in the Mendelian randomization (MR) analysis. A. Waist circumference (WC); B. Hypertension; C. Triglycerides (TG); D. High-density lipoprotein cholesterol (HDL-C); E. Fasting blood glucose (FBG)

SNP – single nucleotide polymorphism.

Nevertheless, the reverse MR analysis did not find any causal relationship between insomnia and the components of MetS, suggesting that the observed associations in epidemiological studies may not be bidirectional.

Insomnia and MetS are both significant public health concerns with substantial global burdens. The linkage between these 2 conditions has been highlighted in various epidemiological studies, where patients with insomnia frequently present with metabolic disorders.[17,18] For instance, central obesity, a key component of MetS, is commonly observed among individuals with insomnia. A large-scale study from the EpiHealth cohort in Sweden, involving 18,823 participants, reported that individuals with larger WC had a 2.44 times higher risk of developing insomnia, emphasizing the potential role of obesity in sleep disturbances.[19] The underlying biological mechanisms may involve leptin resistance, which is prevalent in centrally obese individuals and disrupts energy balance regulation via the hypothalamus.[20] This disruption can activate the hypothalamic–pituitary–adrenal (HPA) axis, leading to elevated levels of corticotropin-releasing hormone (CRH) and chronic stress responses that interfere with sleep.[21–23]

Hypertension, another critical component of MetS, also shows a strong association with insomnia.[24–27] Chronic activation of the sympathetic nervous system and the HPA axis in hypertensive individuals may lead to elevated cortisol levels, which are known to disrupt circadian rhythms and impair sleep quality.[28,29] Our findings corroborate previous studies, such as the meta-analysis that found hypertensive patients are at a 1.41 times higher risk of insomnia.[30] This is consistent with the pathophysiological understanding that hypertension-induced cortisol elevation can delay sleep onset and reduce deep sleep stages, thereby contributing to insomnia.[31,32]

Despite these associations, the relationship between other components of the MetS, such as dyslipidemia and insulin resistance, and insomnia is less clear. Our MR study did not find any causal link between FBG, TG, HDL-C, and insomnia. This contrasts with some observational studies, such as a Hong Kong-based study that suggested a higher FBG level in insomniacs.[33] However, the inconsistency may be due to methodological limitations of observational studies, such as confounding and reverse causality, which MR is specifically designed to address. It is also possible that the metabolic pathways linking these factors to insomnia are more complex and involve intermediate variables not captured in our analysis.

Inflammation may play a crucial role in the connection between MetS and insomnia, as both conditions are associated with elevated levels of pro-inflammatory cytokines like C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6).[34–36] These cytokines can influence both metabolic processes and sleep regulation. For example, IL-6 has been implicated in both pancreatic islet resistance and sleep disruption.[37–39] The inflammatory hypothesis is further supported by the observation that antihypertensive drugs like eprosartan, which reduce

inflammation, can also improve sleep quality in hypertensive patients, suggesting that controlling inflammation could be a potential therapeutic target for insomnia, particularly in patients with MetS.[40]

Limitations

Several limitations of the current study should be acknowledged. First, despite conducting multiple sensitivity analyses, the presence of horizontal pleiotropy cannot be entirely ruled out, which may have influenced the observed associations. Second, the genetic databases utilized, while comprehensive, lacked the granularity needed for detailed stratified analyses. This limitation restricts our ability to examine how the impact of MetS on insomnia may vary across different demographic groups, such as by age or gender. This could result in an incomplete understanding of how different subgroups might be differentially affected by the relationships between MetS components and insomnia. Additionally, our study focused exclusively on populations of European descent, which raises concerns about the generalizability of the findings to other ethnic groups. Future research should focus on obtaining more detailed genetic and phenotypic data to enable comprehensive subgroup analyses. This will help to unravel the complex and potentially differential causal relationships between MetS and insomnia across diverse populations. Understanding these nuances could lead to more personalized approaches in both prevention and treatment, ultimately improving patient outcomes.

Conclusions

Our bidirectional MR study highlights the complex pattern of interactions between metabolic components and insomnia. The MR analysis supports that WC and hypertension are the risk factors for insomnia, and early intervention could potentially mitigate the risk and severity of insomnia.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.14844660. The package includes the following files:

Supplementary Table 1. The causal effect of insomnia on MetS components in the reverse MR analysis.

Supplementary Fig. 1. The scatter plots of the association between genetically predicted in the reverse MR analysis. A. Insomnia on WC B. Hypertension; C. TG; D. HDL-C; E. FBG in the MR analysis.

Supplementary Fig. 2. The funnel plots of the association between insomnia and MetS components genetically in the reverse MR analysis. A. WC; B. Hypertension; C. TG; D. HDL-C; E. FBG.

Supplementary Fig. 3. The leave-one-out analysis of the association between insomnia and MetS components genetically in the reverse MR analysis. A. WC; B. Hypertension; C. TG; D. HDL-C; E. FBG.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Preliminary exploration of the potential role of salvianolic acid F in regulating ovarian cancer cell proliferation, migration, invasion, and apoptosis and its association with the EP300/PI3K/AKT pathway

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Conflict of interest
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Abstract

Background. Salvianolic acid F (SAF), an important water-soluble monomeric component, is derived from the herbal medicine *Salvia miltiorrhiza* (SM) Bunge. Although SAF has been suggested to suppress various cancers, its role in ovarian cancer (OC) and the underlying mechanisms remain largely unclear.

Objectives. This study aimed to investigate the effects of SAF on OC cell growth, invasion, migration, and apoptosis, as well as to elucidate the underlying mechanisms, including those involving the EP300/PI3K/AKT signaling pathway.

Materials and methods. In vitro cell culture experiments were conducted to assess the effects of SAF on the proliferation, migration, invasion, and apoptosis of OVCAR-3 (Ovarian Cancer Cell Line 3) and SK-OV-3 (Sloan-Kettering Ovarian Cancer 3) cells. Network pharmacology was further employed to explore SAF's impact on OC and to elucidate the potential underlying mechanisms. The EP300-mediated PI3K/AKT signaling pathway was selected for validation to confirm SAF's effects on inducing apoptosis and inhibiting cell proliferation in these OC cell lines.

Results. Salvianolic acid F suppressed the growth, invasion and migration of SK-OV-3 and OVCAR-3 cells, and induced apoptosis. A network pharmacology analysis of SAF's effects on OC identified core targets, TP53, EP300, STAT3, MMP9, NFKB1, HIF1A, and PTGS2, through protein–protein interaction (PPI) network analysis using the STRING database. Salvianolic acid F inhibited EP300 expression in SK-OV-3 cells, reduced the p-PI3K/PI3K ratio, and increased both the Bax/Bcl-2 ratio and the cleaved caspase-3/caspase-3 ratio in OVCAR-3 and SK-OV-3 cells. However, the addition of A485, an EP300 inhibitor, did not further enhance the effects of SAF.

Conclusions. Salvianolic acid F inhibited OC cell growth, migration and invasion while promoting apoptosis. The EP300/PI3K/AKT pathway is a key mechanism through which SAF regulates OC progression. Additionally, SAF may represent a promising candidate drug for treating OC.

Key words: apoptosis, migration, ovarian cancer, proliferation, salvianolic acid F

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Highlights

- SAF Inhibits Ovarian Cancer Cell Growth, Migration, and Invasion: This study is the first to demonstrate that Salvianolic acid F (SAF) significantly inhibits the growth, migration, and invasion of ovarian cancer cell lines SK-OV-3 and OVCAR-3, while inducing apoptosis, providing experimental evidence for its anti-tumor effects.
- Network Pharmacology Reveals Core Targets: Through network pharmacology analysis and protein-protein interaction (PPI) network analysis using the String database, core targets such as TP53, EP300, STAT3, MMP9, NFKB1, HIF1A, and PTGS2 were identified, offering important insights into the mechanisms of SAF.
- Key Role of the EP300/PI3K/AKT Pathway: Mechanistic research indicates that SAF promotes ovarian cancer cell apoptosis by regulating the EP300-mediated PI3K/AKT signaling pathway, revealing its potential molecular mechanisms.
- SAF as a Novel Candidate Drug for Ovarian Cancer Treatment: The results demonstrate that SAF exhibits significant anti-ovarian cancer activity, making it a potential candidate drug for ovarian cancer treatment and providing new directions for clinical research.
- Multidisciplinary Approach to Validate Mechanisms: This study combines in vitro cell experiments and network pharmacology analysis to systematically explore the anti-tumor effects and mechanisms of SAF, offering a scientific methodological reference for research on active components of natural medicines.

Background

Ovarian cancer (OC) is a leading cause of mortality among gynecological malignancies.[1] It ranks 3rd in incidence among cancers of the female reproductive system, following cervical and uterine cancers.[2] Due to its subtle early symptoms and atypical late symptoms, OC exhibits the highest mortality and recurrence rates among gynecological cancers.[3]

Combined surgery and chemotherapy remain the standard approach for treating OC. However, severe complications, high recurrence rates and resistance to platinum-based drugs (e.g., cisplatin and carboplatin) are frequently observed during treatment.[4] Additionally, tumor metastasis further complicates OC management.[5] Thus, there remains an unmet need to optimize OC treatment.

As demonstrated in numerous studies, traditional Chinese medicine (TCM) brings about antitumor effects in the treatment of OC by regulating cancer cell growth and apoptosis, inhibiting angiogenesis and reducing resistance to platinum-based chemotherapeutics.[6] *Salvia miltiorrhiza* (SM), a TCM herb, has been extensively used to treat various conditions, including cardiovascular diseases and cancer.[7–9] According to some pharmacological studies, SM achieves its activity via 2 compounds, namely, hydrophilic phenolic acids and lipophilic diterpenoids.[10] Typically, phenolic acid possesses various bioactivities, such as anti-coagulation, anti-oxidation and anticancer.[11]

Salvianolic acids are a group of polyphenolic compounds predominantly found in the roots of SM, also known as danshensu (DSU).[12] Among them, salvianolic acid B (SAB) and salvianolic acid F (SAF) are 2 distinct compounds with different chemical structures and potential bioactivities. Salvianolic acid B contains multiple phenolic rings and carboxylic acid groups, giving it a more complex structure compared to SAF, which also contains phenolic rings but is structurally simpler. Their concentration in SM can vary significantly based on the geographical location, cultivation methods and extraction techniques.[13] Typically, the concentration ranges from a few micrograms to several milligrams per gram of dried root material. The extent of absolute oral bioavailability of SAB in dogs was calculated to be only 1.07 ±0.43%.[14] Due to its simple structure, SAF might have higher bioavailability than SAB; however, further experimentation is required to verify this. The mechanism of action of SAF has not yet been fully elucidated. Salvianolic acid F exhibits strong antioxidant capacity and mitigates oxidative injury by scavenging hydroxyl radicals.[15] It has also been shown to suppress the proliferation of lung cancer cells.[16]

Objectives

The antitumor effects of SAF and its underlying mechanisms in OC remain unclear. This study aimed to evaluate the sensitivity of OC cells to SAF in vitro, assess its effects on cell growth, invasion and migration, and investigate the underlying molecular mechanisms.

Materials and methods

Chemicals

Salvianolic acid F was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (purity, ≥98%; Shanghai, China). Cisplatin and A485 were provided by Sigma-Aldrich (St. Louis, USA). Cell Counting Kit-8 (CCK-8) was provided by Dojindo Molecular Technologies (Kumamoto, Japan). The Click-iT® EdU Alexa Fluor® 594 Imaging Kit was

purchased from Thermo Fisher Scientific (Waltham, USA). Antibodies against P300, caspase-3, cleaved caspase-3, Bcl-2, Bax, PI3K, phospho-PI3K, AKT, phospho-AKT, and GAPDH were supplied by Cell Signaling Technology (CST; Danvers, USA). Goat anti-rabbit and anti-mouse IgG (H+L) secondary antibodies were provided by Abcam (Cambridge, UK). All remaining chemicals were of reagent-grade purity.

Cells and cell treatment

Ovarian cancer cell lines (OVCAR-3 (Ovarian Cancer Cell Line 3) and SK-OV-3 (Sloan-Kettering Ovarian Cancer 3)) were obtained from Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (China). Of them, SK-OV-3 cells were cultivated within McCoy's 5A that contained 10% fetal bovine serum (FBS; Gibco, Waltham, USA) and 1% penicillin-streptomycin (Invitrogen, Waltham, USA), whereas OVCAR-3 cells were cultivated within Roswell Park Memorial Institute Medium (RPMI)-1640 medium that contained 10% FBS (Gibco) and 1% penicillin–streptomycin (Invitrogen). The above cell lines were subject to incubation under 37°C and 5% CO_2 conditions.

Cell viability assay

Cell viability was measured using the CCK-8 assay. In brief, cells (1×10^4/well) were inoculated in 96-well microtiter plates and incubated overnight. Thereafter, different dose (0, 5, 10, 20, 40, 60, 80, and 100 μM) of SAF was added to treat cells for 24, 48 and 72 h. Then, every well was introduced with CCK-8 (10 μL) for extract 4-h incubation in dark under 37°C. Absorbance value measured at 450 nm was used to analyze cell viability.

Cell proliferation assay

OVCAR-3 and SK-OV-3 cells (5×10^3/well) were inoculated into 96-well plates for a 24-h period, followed by another 48-h SAF treatment (0, 20 and 40 μM). In line with specific instructions, we utilized Click-iT® 5-ethynyl-2'-deoxyuridine (EdU) imaging kit for the detection of cell proliferation. Proliferation was detected through EdU (red) staining, whereas DAPI (4′,6-diamidino-2-phenylindole) staining (blue) was performed for nuclear counter-staining. ImageJ v. 1.54f (National Institutes of Health (NIH), Bethesda, USA) was employed for calculating the EdU-positive cell percentage.

Cell apoptosis assay

In line with specific protocols, we adopted Annexin V-FITC/PI kit (Beyotime Biotechnology, Shanghai, China) for detecting cell apoptosis and used the flow cytometer (BD LSRFortessa™; BD Biosciences, Franklin Lakes, USA) for measuring the apoptosis rate. To be specific, 2 OC cell lines were inoculated into the 6-well plates at 1×10^6/well density for a 24-h period prior to 48-h SAF treatment (0, 20 and 40 μM). After cell suspension using 1X binding buffer within the 5-mL culture tube at the 1×10^6 cells/mL density, cells were subjected to Annexin V-FITC staining and PI counterstaining for a 15-min period under ambient temperature. The flow cytometer (BD FACSCalibur™; BD Biosciences) was used for determining apoptotic cell. Data were analyzed using FlowJo software (TreeStar Inc., Ashland, USA).

Transwell assay

Cell migration and invasion were evaluated using transwell assays. Briefly, the 2 OC cell lines were subjected to resuspension within the serum-free medium. Transwell chambers (8-μm pore size) with/without Matrigel® coating were then seeded with 500 μL cell suspension containing 5 × 105 cells/mL. Additionally, 1 mL medium that contained 10% FBS was added into bottom chambers, while SAF (0, 20 and 40 μM) was introduced to the top chamber. At 48-h post-culture, cotton swabs were utilized to discard cells on membrane surface, whereas 4% paraformaldehyde was added to fix cells penetrating lower membrane surface, and then 0.1% cresyl violet was applied in cell staining. Phase-contrast microscopy (Nikon Eclipse Ti2; Nikon Corp., Tokyo, Japan) was employed to obtain images. Cells that invaded bottom membrane were counted.

Salvianolic acid F and OC-target screening

The chemical structure and Simplified Molecular Input Line Entry System (SMILES) format of SAF were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The targets of SAF were screened by searching its SMILES format in the SwissTargetPrediction database (www.swisstargetprediction.ch/), PharmacoPhoric Bit (PPB) database (gdb.unibe.ch/) and SEA database (sea.bkslab.org/). The disease targets of OC were screened from the Genecards database (https://www.genecards.org) by searching "ovarian cancer".

Build protein–protein interaction networks

To clarify the relationship between OC-related targets and SAF-related targets, the 2 target sets were intersected. To further explore interactions between target genes, the statistically identified disease-drug target genes were uploaded to STRING (http://string-db.org) to construct a protein–protein interaction (PPI) network. The species was set to human (*Homo sapiens*), and default parameters were used. The "string_interactions_short.TSV" file was then downloaded and uploaded into Cytoscape 3.6.1 (Cytoscape Consortium, San Diego, USA) for topological analysis using the NetworkAnalyzer tool. For visualization, degree score was used as the reference standard, where protein size and color were proportional to their degree

scores. Proteins were arranged from the inside out based on their degree scores. Core proteins were filtered using a degree score threshold >15.

Western blotting analysis

In line with specific instructions, western blotting analysis was carried out. To be specific, both OC cells were processed with 48-h SAF treatment (0, 20 and 40 μM), and harvested for lysis with RIPA lysis buffer. The protein contents were measured by bicinchoninic acid (BCA) protein detection kit. A total of 30 μg protein aliquots from total cell lysates were exposed to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation prior to wet transfer on the polyvinylidene difluoride (PVDF) membrane. Thereafter, the membrane was blocked using 5% defatted milk within tris-buffered saline (TBS; consisting of 0.1% Tween 20, pH 7.6) for a 1-h period under ambient temperature, incubated using primary antibodies (EP300, caspase-3, cleaved caspase-3, Bcl-2, Bax, PI3K, phospho-PI3K, AKT, phospho-AKT, p-AKT, and GAPDH form CST) overnight and later incubated using corresponding secondary antibodies (goat anti-rabbit and anti-mouse IgG (H+L) secondary antibodies from Abcam) for a 1-h period under ambient temperature. Protein bands were exposed using the ECL imaging system (Clinx, Shanghai, China), while Image Lab v. 3.0 (BioRad Laboratories, Hercules, USA) was adopted for intensity examination.

Statistical analyses

We performed an experiment on at least 3 independent samples. We used GraphPad v. 6 (GraphPad Software, La Jolla, USA) for statistical analysis. The value was expressed in the form of all data points. We used a nonparametric test due to small sample sizes. The comparison among groups was performed using the nonparametric Kruskal–Wallis test, followed by Dunn's test to compare variables among the groups. A $p < 0.05$ was considered to be statistically significant.

Results

Salvianolic acid F suppressed cell proliferation

To evaluate the effect of SAF on suppressing proliferation, we treated OVCAR-3 and SK-OV-3 OC cells with SAF. Following exposure, CCK-8 assays were conducted at various concentrations and time points, revealing that SAF inhibited OC cell proliferation in a dose- and time-dependent manner. In addition, SAF inhibited OVCAR-3 and SK-OV-3 cell viability, with superior inhibitory effects observed at 72 h in a dose-dependent manner (Fig. 1). Cisplatin was selected as the positive control due to its established approval as a systemic therapy for OC.[17] After 48 h of SAF treatment, the 50% inhibitory concentrations (IC_{50}) for OVCAR-3 and SK-OV-3 cells were determined to be 28.89 μM (27.20–30.65 μM) and 29.94 μM (27.79–32.22 μM), respectively. Furthermore, the EdU assay demonstrated a significant reduction in EdU-positive OVCAR-3 and SK-OV-3 cells following 48-h SAF exposure at 20 and 40 μM (Fig. 2). These findings indicate that SAF effectively reduces OC cell viability.

Salvianolic acid F inhibited cell migration and invasion

To evaluate the effect of SAF on OC cell mobility, we treated OVCAR-3 and SK-OV-3 cells with 40 μM SAF while performing the Transwell assay. The results demonstrated that SAF at 20 and 40 μM effectively inhibited OC

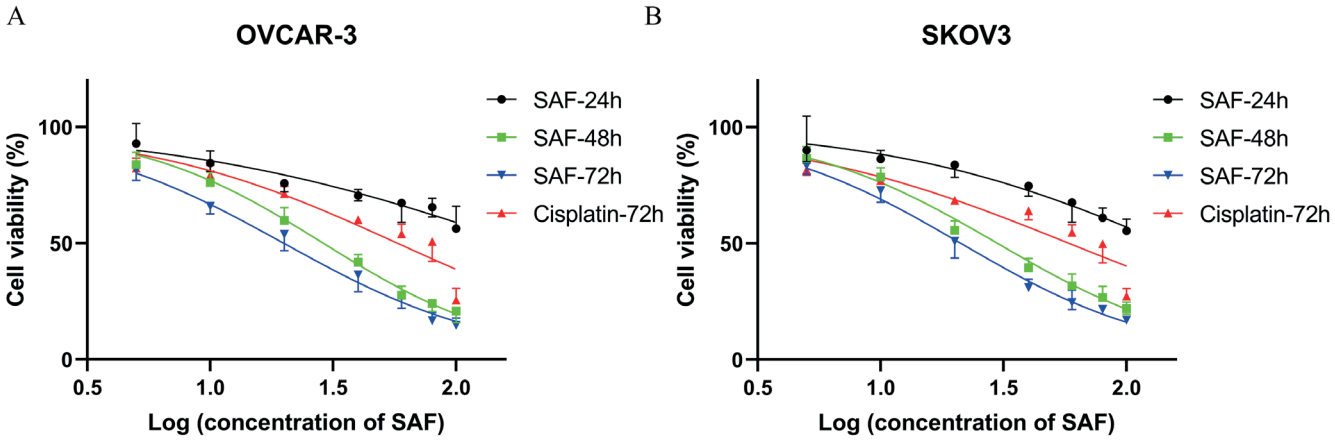

Fig. 1. Suppression of cell viability by SAF. OVCAR-3 (A) and SK-OV-3 (B) cell viability under treatment at different SAF concentrations for 24, 48 and 72 h, with cisplatin being the positive reference. Data were transformed using the formula $\log_{10}$(data) and analyzed with nonlinear regression using a dose-response-inhibition model (log(inhibitor) vs normalized response–variable slope). Results are presented as median and quartiles

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3.

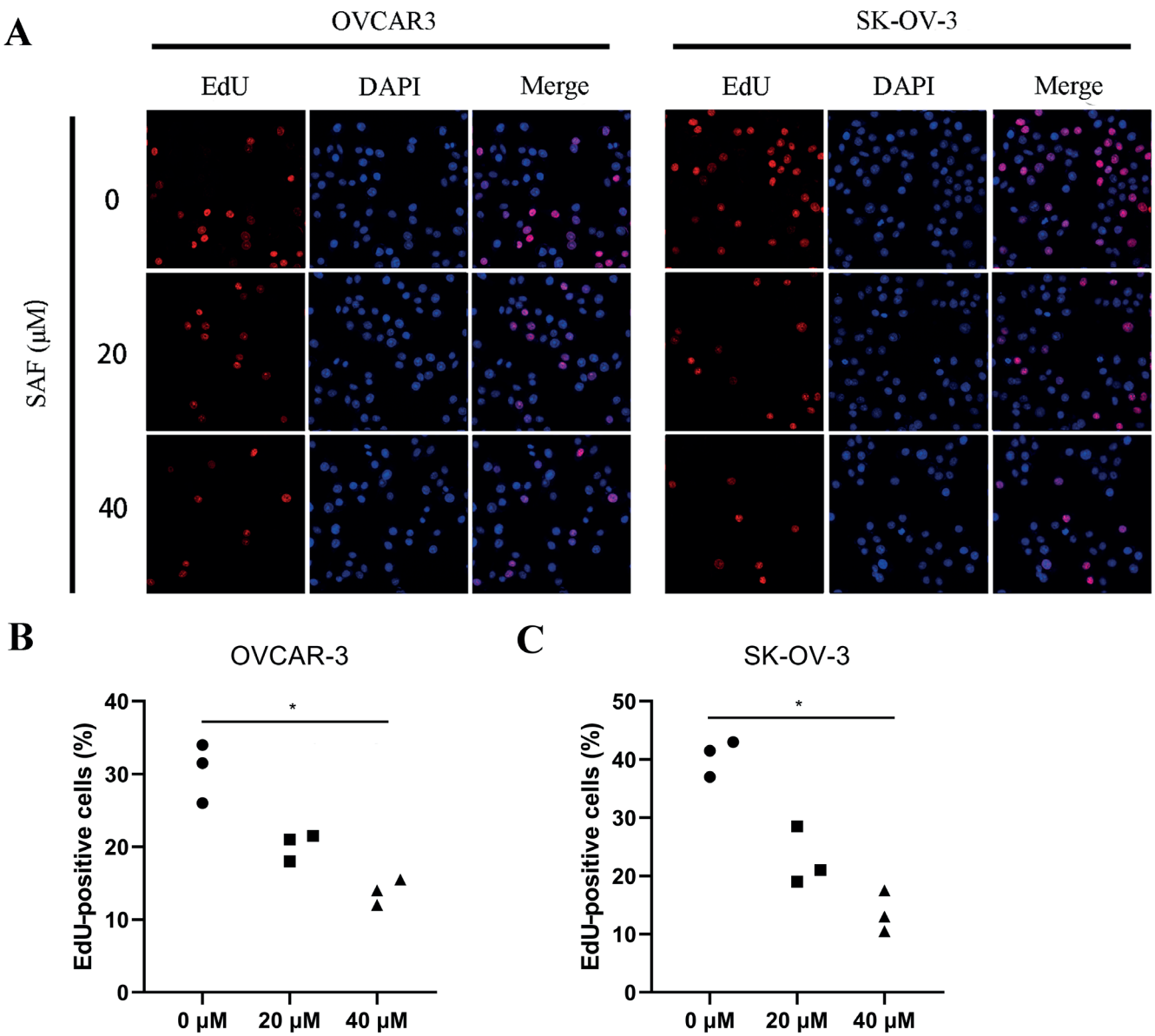

Fig. 2. Suppression of cell proliferation by SAF. The ratio of EdU-positive cells in OVCAR-3 (p = 0.032 vs 20 μM SAF; p = 0.010 vs 40 μM SAF) and SK-OV-3 (p = 0.011 vs 20 μM SAF; p = 0.001 vs 40 μM SAF) cells after 48 h of SAF treatment was visualized through EdU staining. The data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test. Results are presented as individual data points; *p < 0.05; scale bar: 50 μm

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3; EdU – 5-ethynyl-2′-deoxyuridine.

cell migration in vitro (Fig. 3) Additionally, in vitro invasion assays revealed that SAF at 40 μM significantly suppressed the invasive capacity of OVCAR-3 and SK-OV-3 cells (Fig. 4) Collectively, these findings indicate that SAF markedly impairs the migration and invasion of OC cells.

Salvianolic acid F promoted cell apoptosis

Flow cytometry was performed to examine the role of SAF in apoptosis using Annexin V-FITC/PI double staining in OVCAR-3 and SK-OV-3 cells. After 48 h of SAF treatment (40 μM), treated cells exhibited a significantly elevated apoptosis rate in a dose-dependent manner compared to untreated cells (Fig. 5) To investigate the mechanism by which SAF induces apoptosis, we performed western blot analysis to assess caspase-3 expression. The results showed that SAF significantly increased caspase-3 cleavage in a dose-dependent manner compared to control cells (Fig. 6A–C). Moreover, SAF treatment led to a dose-dependent decrease in Bcl-2 expression and an increase in Bax expression relative to control cells (Fig. 6A,D,E) These findings suggest that SAF promotes apoptosis by enhancing caspase-3 cleavage.

The target prediction of SAF in OC

The chemical structure of SAF is shown in Fig. 7A. A total of 132 target proteins were predicted for SAF, while 2,158

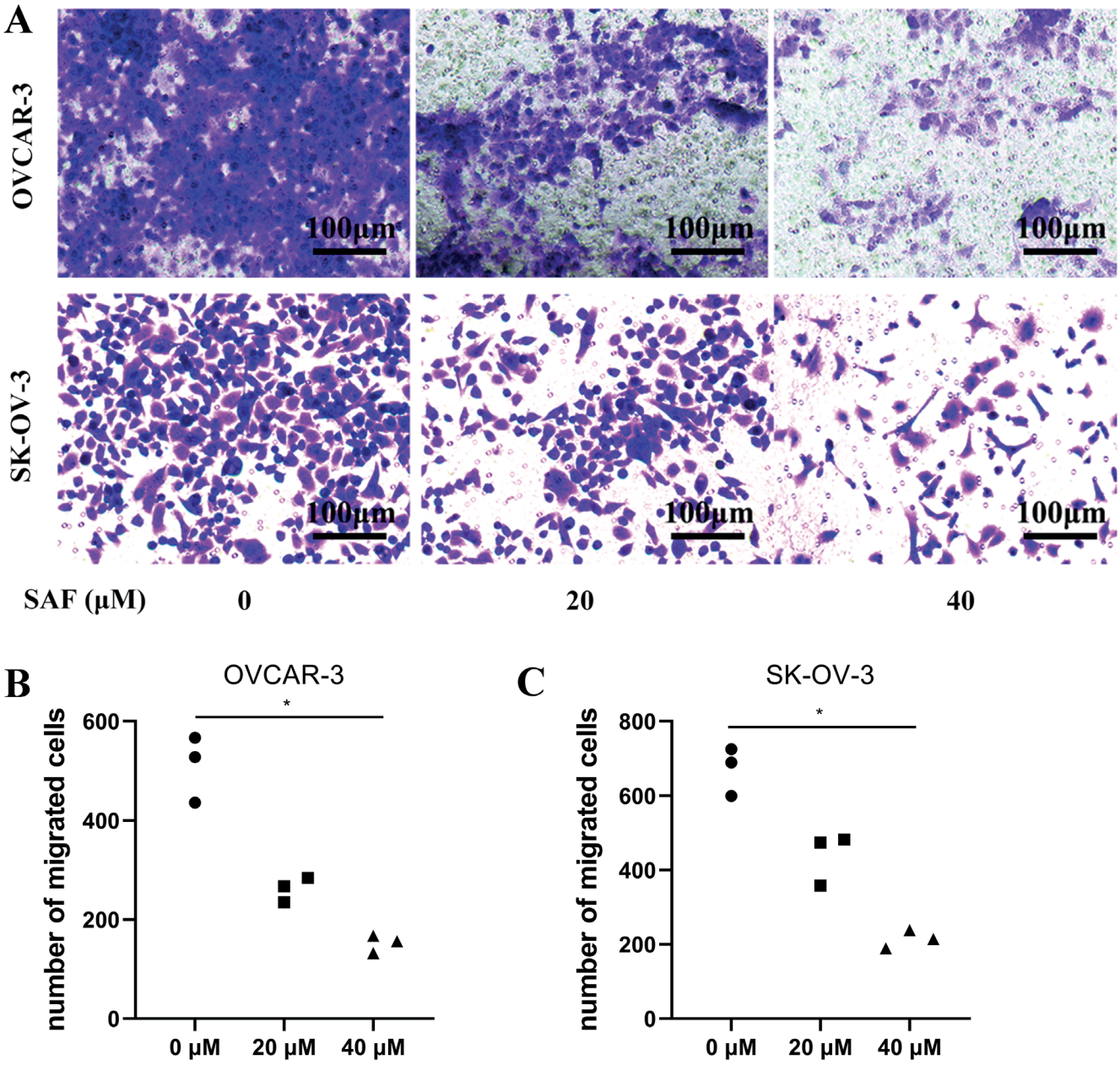

Fig. 3. Suppression of cell migration and invasion by SAF. The Transwell assay was used to assess cellular motility in OVCAR-3 ($p = 0.015$ vs 20 μM SAF; $p = 0.008$ vs 40 μM SAF) and SK-OV-3 ($p = 0.013$ vs 20 μM SAF; $p = 0.003$ vs 40 μM SAF) cells after 48 h of treatment with SAF at 0, 20 and 40 μM. Data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test and are presented as individual data points; *$p < 0.05$; scale bars = 100 μm

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3.

disease-associated targets were identified for OC. Interaction analyses between these datasets identified 43 common targets, which were considered potential anti-OC targets of SAF (Fig. 7B). Further network analysis using Cytoscape 3.6.1 identified key core targets, including TP53, EP300, STAT3, MMP9, NFKB1, HIF1A, and PTGS2 (Fig. 7C).

Salvianolic acid F suppressed EP300/PI3K/AKT pathway

EP300 has been reported to regulate tumor cell growth, differentiation and migration through the PI3K/AKT signaling pathway. To explore the mechanism by which SAF suppresses cell progression, we performed western blot analysis to assess the EP300/PI3K/AKT pathway. The results showed that EP300, p-PI3K/PI3K, Bax/Bcl-2, and cleaved caspase-3/caspase-3 ratios were significantly reduced in 40 μM SAF-treated cells compared to untreated controls (Fig. 8A,B, Fig. 9) Furthermore, the addition of A485 significantly enhanced the inhibitory effect of SAF on cell viability (Fig. 9F). These findings suggest that SAF suppresses the EP300/PI3K/AKT signaling pathway, and the combination of A485 and SAF may further enhance SAF-induced cytotoxicity.

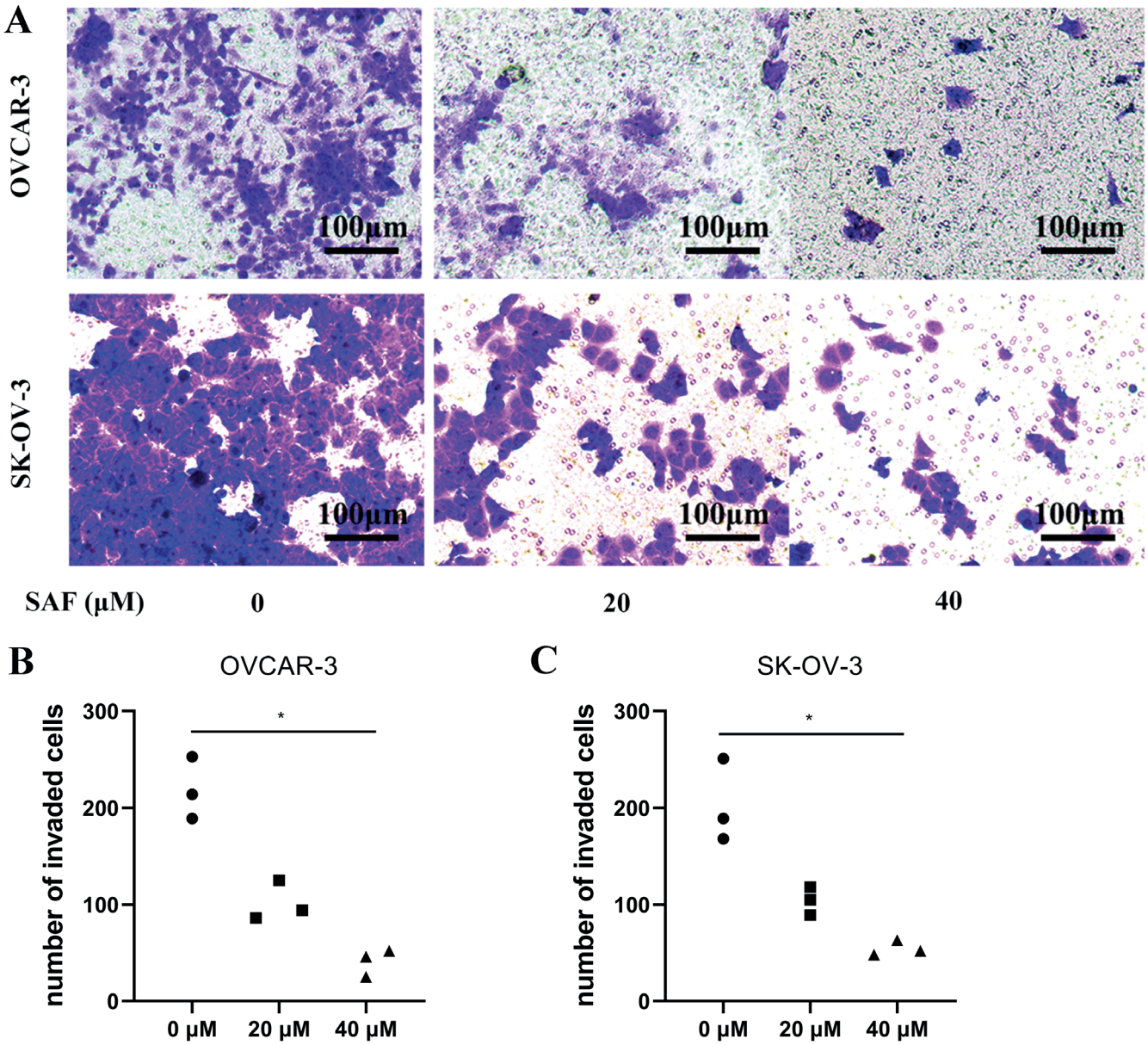

Fig. 4. Suppression of cell invasion by SAF. The Matrigel-coated Boyden chamber assay was used to evaluate cell invasion in OVCAR-3 (p = 0.010 vs 20 μM SAF; p = 0.004 vs 40 μM SAF) and SK-OV-3 (p = 0.047 vs 20 μM SAF; p = 0.024 vs 40 μM SAF) cells. Representative photomicrographs show invaded cells on the membrane, stained with 0.1% cresyl violet. Data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test and are presented as individual data points; *p < 0.05; scale bars = 100 μm

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3.

Discussion

Platinum-based chemotherapy remains the primary treatment for advanced or relapsed OC. However, clinical studies over the past 3 decades suggest that it has not significantly improved overall survival rates. In clinical practice, many patients develop relapse disease following first-line chemotherapy, suffer from severe chemotherapy-related side effects and exhibit poor prognostic outcome.[18,19] An essential aspect of chemotherapy is that the incorporation of targeted therapy drugs can enhance its efficacy or mitigate side effects.

Salvia miltiorrhiza has been used in TCM for thousands of years. Among its extensively studied phenolic acids are DSU, salvianolic acid A (SAA), SAB, and salvianolic acid C (SAC).[20] Salvianolic acid A extracted from SM was found in experiments in vitro to suppress A549 cell viability.[21] Salvianolic acid B could activate renal autophagy through microRNA-145-5p/PI3K/AKT pathway to attenuate membranous nephropathy.[22] Notably, SAB demonstrated a significant inhibitory effect on the growth of OC SKOV3 cells while promoting their apoptosis. This effect may be mediated through downregulation of livin expression, upregulation of caspase-3 expression and cell cycle arrest.[23]

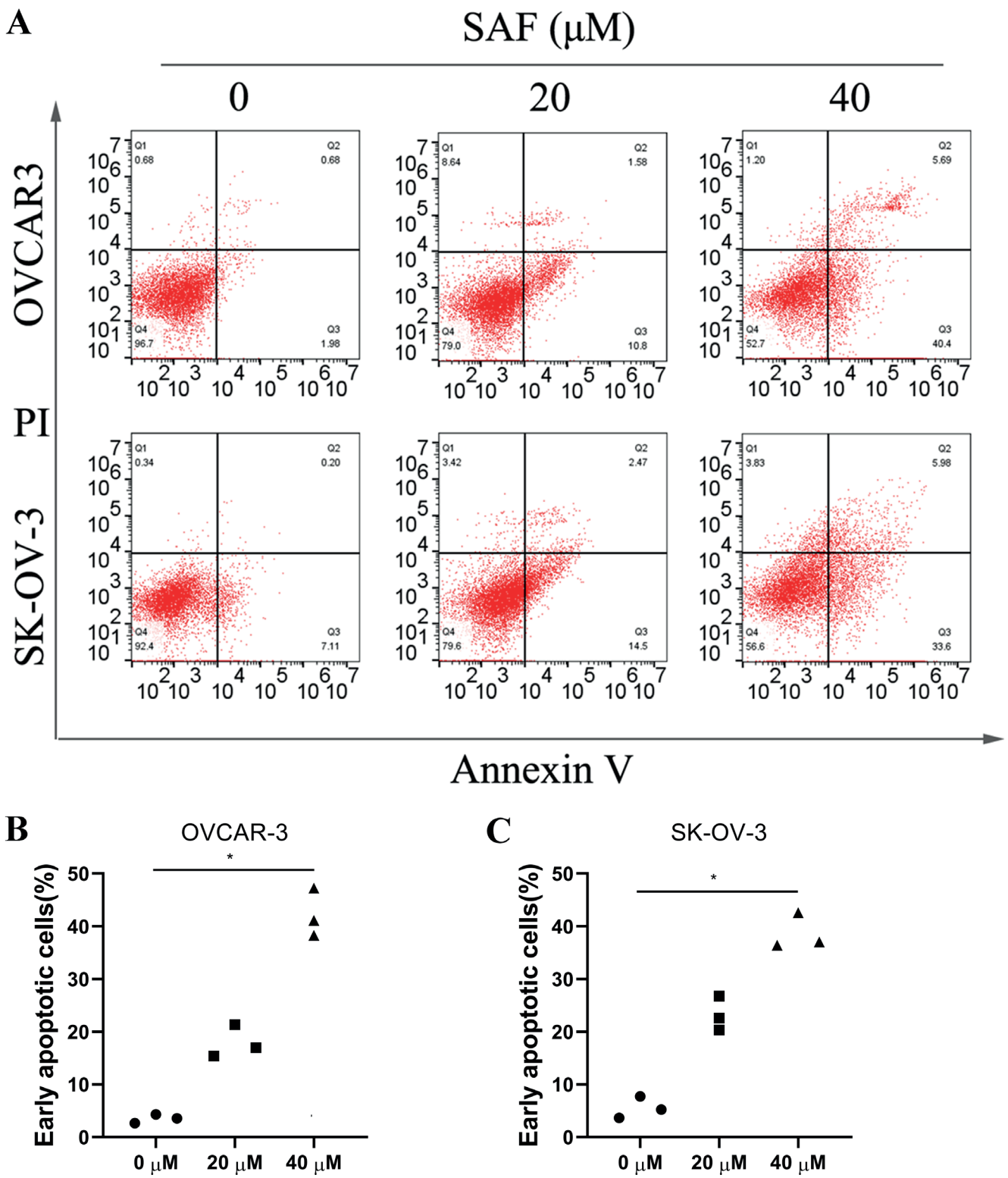

Fig. 5. Salvianolic acid F induces cell apoptosis. A. Cell apoptosis in OVCAR-3 and SK-OV-3 cells was measured with flow cytometry following 48 h of SAF exposure; B,C. Quantification of apoptosis showed significant differences in OVCAR-3 ($p = 0.011$ vs 20 μM SAF; $p = 0.004$ vs 40 μM SAF) and SK-OV-3 ($p = 0.003$ vs 20 μM SAF; $p = 0.001$ vs 40 μM SAF) cells. Data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test and are presented as individual data points; *$p < 0.05$

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3.

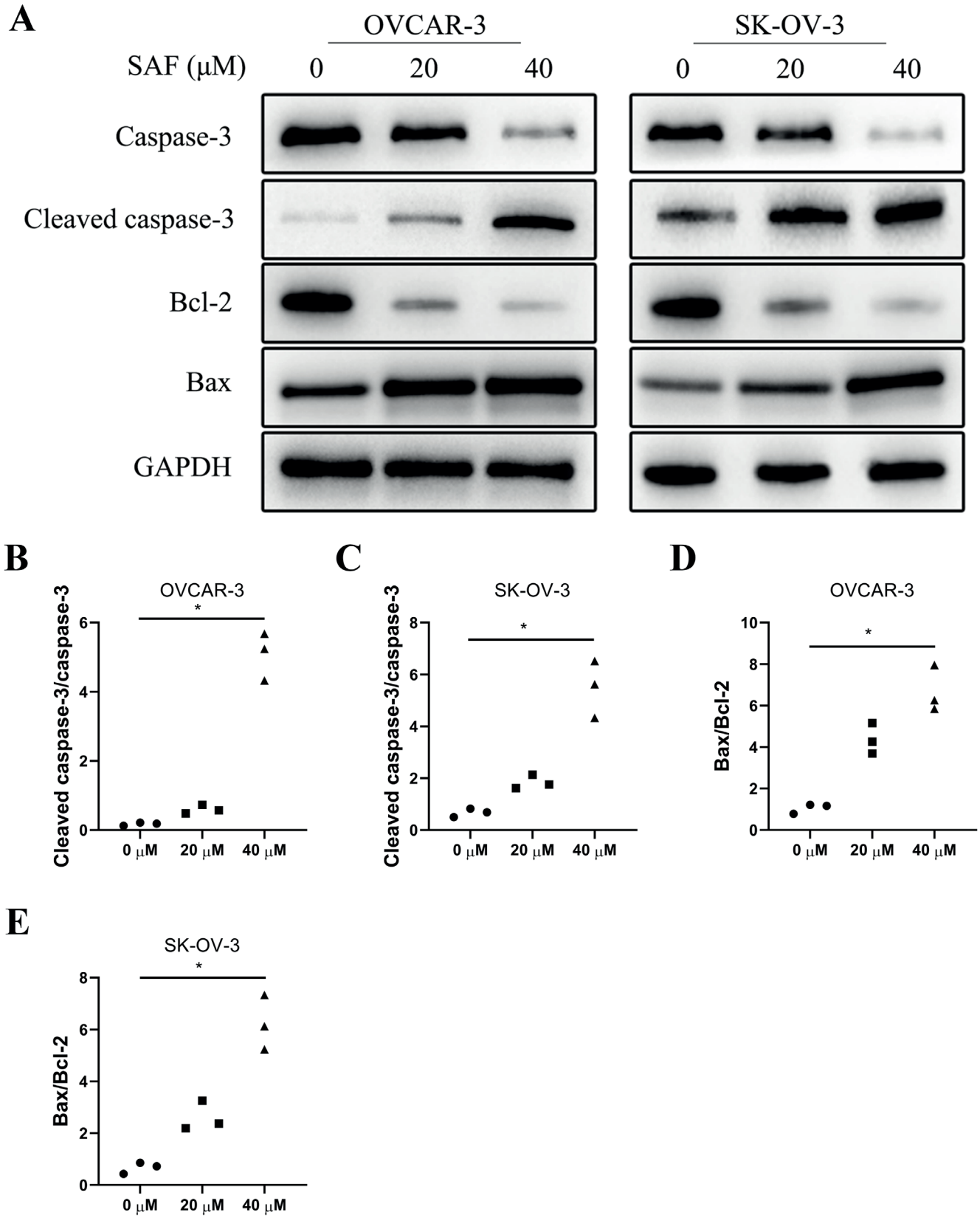

Fig. 6. SAF inhibited apoptotic signaling. A. Apoptosis-related protein levels in OVCAR-3 and SK-OV-3 cells were measured with western blot analysis following 48 h of SAF exposure. B,C. Cleaved caspase-3/caspase-3 ratio from (A) in OVCAR-3 ($p = 0.019$ vs 20 μM SAF; $p = 0.006$ vs 40 μM SAF) and SK-OV-3 ($p = 0.006$ vs 20 μM SAF; $p = 0.015$ vs 40 μM SAF). Data are presented as mean ± standard deviation (SD) of 3 replicates ($p < 0.05$). D,E. Bax/Bcl-2 ratio from (A) in OVCAR-3 ($p = 0.011$ vs 20 μM SAF; $p = 0.010$ vs 40 μM SAF) and SK-OV-3 ($p = 0.018$ vs 20 μM SAF; $p = 0.009$ vs 40 μM SAF). Data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test and are presented as individual data points; *$p < 0.05$

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3.

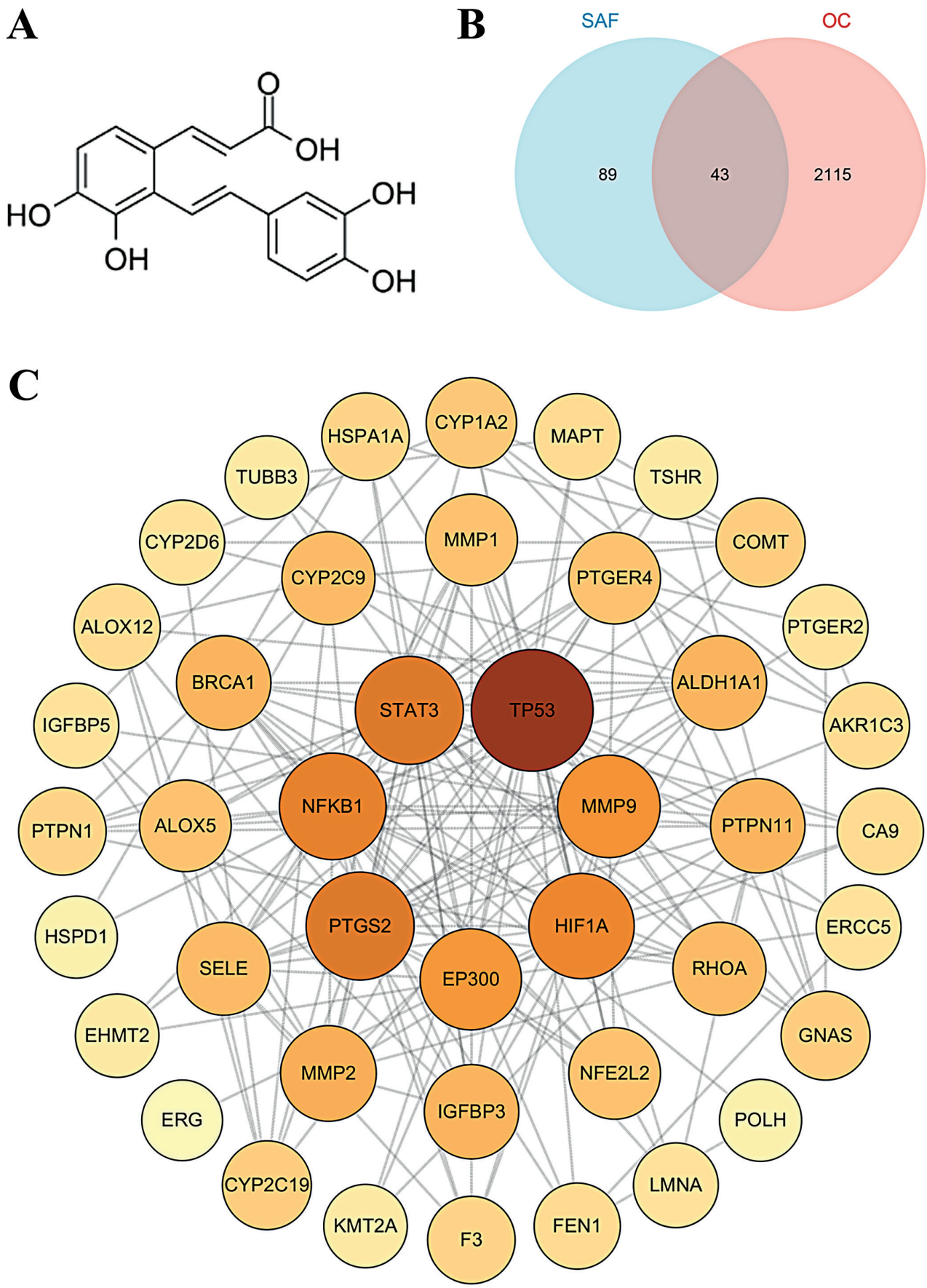

Fig. 7. Screening of potential targets of salvianolic acid F (SAF) in ovarian cancer (OC). A. The chemical structure of SAF is shown; B. Venn analysis was performed to identify the intersection between potential SAF targets and disease targets in OC; C. Protein–protein interaction (PPI) analysis was conducted on the intersecting proteins to identify core targets. Node size represents the degree value, while lines indicate relationships between targets

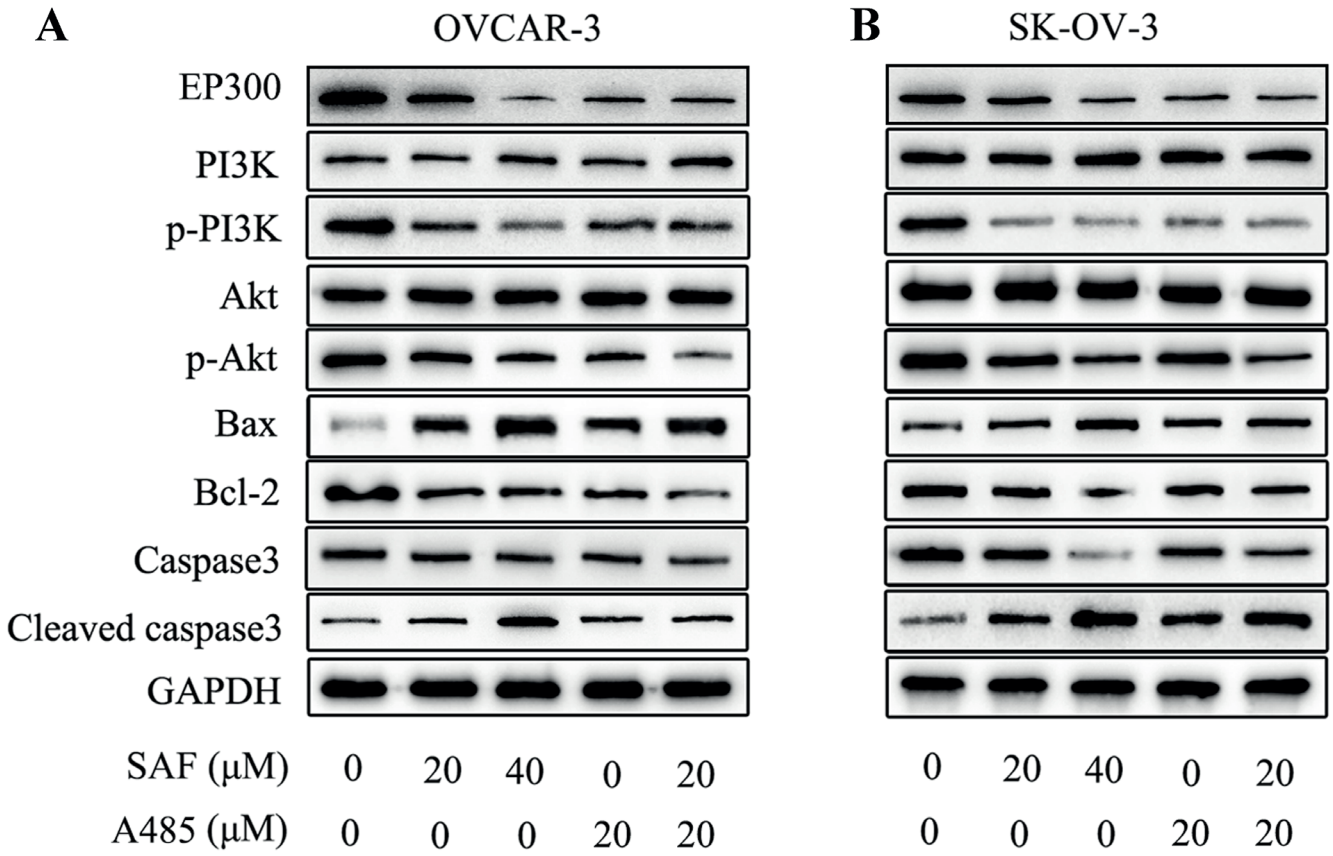

Fig. 8. Salvianolic acid F inhibits the EP300/PI3K/AKT pathway. Western blot analysis was performed to measure the protein expression levels of EP300, PI3K, AKT, p-PI3K, p-AKT, Bax, Bcl-2, caspase-3, and cleaved caspase-3 in OVCAR-3 and SK-OV-3 cells

SAF – salvianolic acid F; OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3; EP300 – E1A binding protein P300; PI3K – phosphoinositide 3-kinase; AKT – protein kinase B; Bax – BCL2-associated X protein; Bcl-2 – B-cell lymphoma 2.

In addition, the chemical shifts observed in ^{1}H-NMR and ^{13}C-NMR for salvianolic acid Y (SAY) are similar to those of SAB. Moreover, the cell protection rate of SAY was 54.2% in rat pheochromocytoma line PC12, significantly higher than the 35.2% of SAB.[24] Hence, there is a need and necessity to further study the role of SAY in OC. Salvianolic acid C was discovered to enhance bone marrow mesenchymal stem cells (BMMSCs) osteogenic differentiation among osteoporotic rats through activation of AMPK/SIRT1 pathway.[25] Salvianolic acid F was also the major effective component with promising antitumor activity.[16] However, its function in OC is still unclear.

Cell apoptosis is a fundamental process of cell death and is closely linked to tumorigenesis.[26] The Bcl-2 family of proteins plays a pivotal role in regulating apoptosis, with Bax and Bcl-2 being key mediators of this process.[27] Specifically, Bax can activate or inhibit Bcl-xL and Bad, while Bcl-2 functions to suppress Bax, thereby preventing apoptosis. The Bax/Bcl-2 ratio, rather than the individual protein levels, is a critical factor in determining apoptosis susceptibility.[28] The caspase family plays a critical role in regulating the above process.[29] According to our results, Bax and cleaved-caspase-3 expression were elevated, whereas Bcl-2 expression was decreased after SAF treatment, while the Bax/Bcl-2 ratio was evidently increased in vitro. Therefore, the SAF-mediated cell death was partly controlled by Bax/Bcl-2 and caspase-dependent apoptosis.

To investigate the potential mechanism of SAF in anti-OC, we conducted a network pharmacological analysis and screened 7 central proteins (TP53, EP300, STAT3, MMP9, NFKB1, HIF1A, and PTGS2), which might be involved in the process of SAF inhibiting OC. These proteins have been validated to participate in the regulation of OC cells proliferation, migration and apoptosis.[30–35] E1A-binding protein p300 (EP300) can regulate cell survival, proliferation and apoptosis through multiple pathways, including the PI3K/AKT signaling pathway.[36,37] E1A-binding protein p300 is a transcription coactivator that regulates gene expression through acetylation of histones and non-histone targets. It plays a crucial role in cell cycle regulation, DNA repair and apoptosis.[37] Studies have shown that EP300-mediated acetylation inhibits the activity of FOXO transcription factors, thereby regulating gene expression and influencing cell survival and apoptosis through the PI3K/AKT signaling pathway.[38,39]

In this study, SAF significantly suppressed EP300 expression and the PI3K/AKT pathway, with its inhibitory effect further enhanced by the addition of A485, an EP300 inhibitor. Additionally, SAF markedly upregulated Bax and cleaved caspase-3 expression in OVCAR-3 and SK-OV-3

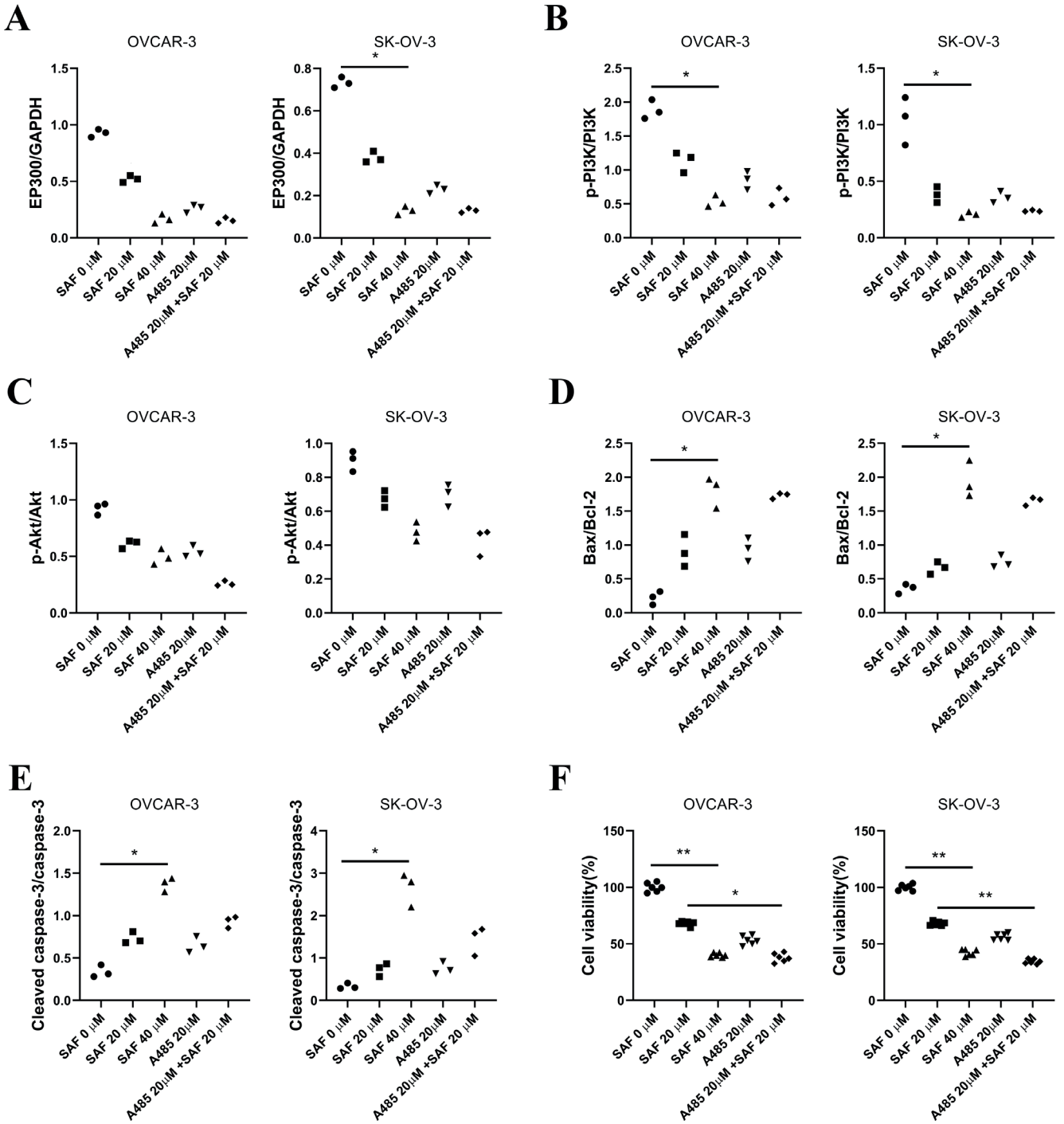

Fig. 9. Quantification of western blot results. A. EP300/GAPDH ratio in OVCAR-3 and SK-OV-3 cells; B. p-PI3K/PI3K ratio in OVCAR-3 and SK-OV-3 cells; C. p-AKT/AKT ratio in OVCAR-3 and SK-OV-3 cells; D. Bax/Bcl-2 ratio in OVCAR-3 and SK-OV-3 cells; E. Cleaved caspase-3/caspase-3 ratio in OVCAR-3 and SK-OV-3 cells; F. OVCAR-3 and SK-OV-3 cell viability after 72 h of treatment across different groups. Data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's post hoc test and are presented as individual data points; *$p < 0.05$, **$p < 0.01$

OVCAR-3 – Ovarian Cancer Cell Line 3; SK-OV-3 – Sloan-Kettering Ovarian Cancer 3; EP300 – E1A binding protein P300; PI3K – phosphoinositide 3-kinase; AKT – protein kinase B; Bax – BCL2-associated X protein; Bcl-2 – B-cell lymphoma 2; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

cells, a process further facilitated by EP300 inhibition. Moreover, the combination of SAF and EP300 inhibitors significantly enhanced the suppression of cell viability. These findings suggest that the EP300/PI3K/AKT signaling pathway plays a pivotal role in the anti-OC effects of SAF.

Limitations

Although this study revealed that SAF inhibits ovarian cancer cell growth, migration and invasion, and promotes cell apoptosis by suppressing the EP300-mediated PI3K/AKT pathway, there are still some limitations. First,

the study is primarily based on in vitro cell experiments, lacking support from in vivo experimental data. Therefore, the anti-tumor effects of SAF in a living organism and its potential toxicity require further validation. Second, while the core targets identified through network pharmacology analysis (such as TP53, EP300, STAT3, etc.) provide direction for mechanistic research, not all targets have been experimentally validated, leaving potential mechanisms undiscovered. Additionally, this study only selected 2 cell lines, SK-OV-3 and OVCAR-3, for experiments, which does not account for the heterogeneity of ovarian cancer. Future research should expand to include more cell lines and clinical samples to validate the generalizability of the results. Finally, the specific dosage, administration methods and clinical application potential of SAF need further exploration. These limitations provide directions for future research to more comprehensively evaluate the application value of SAF in ovarian cancer treatment.

Conclusions

Our research initially suggests that SAF plays a potential role in regulating tumor cell proliferation, migration, invasion, and apoptosis, particularly through its possible association with the EP300/PI3K/AKT pathway. These findings provide important insights for further in-depth studies.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.13956748. The package includes the following files:

Supplementary Table 1. The analysis results of data from Fig. 1 through nonlinear regression by dose-response-Inhibition (log(inhibitor) vs normalized response–variable slope).

Supplementary Table 2. The analysis results of data from Fig. 2 through nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test with Bonferroni correction.

Supplementary Table 3. The analysis results of data from Fig. 3 through nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test with Bonferroni correction.

Supplementary Table 4. The analysis results of data from Fig. 4 through nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test with Bonferroni correction.

Supplementary Table 5. The analysis results of data from Fig. 5 through nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test with Bonferroni correction.

Supplementary Table 6. The analysis results of data from Fig. 6 through nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test with Bonferroni correction.

Supplementary Table 7. The analysis results of data from Fig. 9 through nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's post hoc test with Bonferroni correction.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Long-term exposure of indoxyl sulfate induces mesothelial-to-mesenchymal transition of peritoneal mesothelial cells via β-catenin-involved signaling pathway

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Abstract

Background. Long-term peritoneal dialysis (PD) leads to peritoneal injury, with mesothelial-to-mesenchymal transition (MMT) potentially serving as an initial and reversible stage of this process. Indoxyl sulfate (IS), a protein-bound uremic toxin that accumulates in patients with declining renal function, is known to be associated with epithelial–mesenchymal transition (EMT) in proximal renal tubular cells. However, its effects on peritoneal mesothelial cells, which serve as the first-line barrier during PD, have not yet been investigated.

Objectives. This study aimed to evaluate whether IS induces MMT in human peritoneal mesothelial cells during PD through the β-catenin signaling pathway.

Materials and methods. A human peritoneal mesothelial cell line (HMrSV5) was used for this in vitro study. Cells were treated with IS or combined with β-catenin inhibitor ICG-001, and high glucose PD fluid (PDF) served as a positive control. Morphology, proliferation and adhesion were assessed, while the expression of β-catenin and α-smooth muscle actin (α-SMA) as mesenchymal markers, along with E-cadherin as a mesothelial marker, were analyzed at both RNA and protein levels using real-time polymerase chain reaction (PCR) and western blot, respectively.

Results. The number of viable and adherent cells was significantly increased in the IS and PDF groups compared to the control ($p < 0.05$). Treatment with ICG-001 significantly reduced both viable and adherent cell numbers compared to cells treated with IS or PDF alone ($p < 0.05$). At the RNA level, IS treatment significantly decreased E-cadherin expression ($p = 0.002$) while significantly increasing β-catenin ($p = 0.001$) and α-SMA ($p = 0.002$) expression compared to the control group. These changes were reversed by ICG-001 treatment. Protein expression showed similar trends.

Conclusions. Indoxyl sulfate induces MMT in human peritoneal mesothelial cells, and these changes can be reversed by the specific β-catenin inhibitor ICG-001. This suggests that IS may be considered as another inducer of MMT during PD through the β-catenin signaling pathway.

Key words: indoxyl sulfate, β-catenin, epithelial–mesenchymal transition, peritoneal dialysis, mesothelial-to-mesenchymal transition

Introduction

Peritoneal dialysis (PD) is one of the renal replacement therapies (RRTs) administered to patients with end-stage renal disease (ESRD). Peritoneal dialysis uses the peritoneum as a living dialysis membrane to clear waste and extra fluid. Compared to hemodialysis (HD), PD offers a lower risk of infection, greater patient mobility and an improved quality of life. Additionally, PD accounts for more than 10% of RRTs worldwide.[1–3] However, long-term PD leads to peritoneal injury, resulting in structural and functional deterioration of the peritoneum, including peritoneal fibrosis and a progressive loss of ultrafiltration. These complications are the primary reasons for PD discontinuation.[4–6] Furthermore, peritoneal fibrosis has been associated with increased morbidity and mortality in patients who have undergone PD.[7–9]

Peritoneal fibrosis is a progressive and complex pathological process involving multiple cell types, signaling pathways and molecular interactions. In response to inflammatory or profibrotic stimuli, peritoneal and immune cell populations expand, triggering specific signaling pathways that drive inflammation and ultimately lead to fibrosis. Among these intricate processes, mesothelial cells play a central role in peritoneal membrane alterations through mesothelial-to-mesenchymal transition (MMT), a key mechanism contributing to fibrosis.[3]

Mesothelial-to-mesenchymal transition is a special type of epithelial–mesenchymal transition (EMT) that occurs in mesothelial cells; it is a process in which epithelial cells lose polarity and adhesion and acquire mesenchymal features, including infiltration and migration ability, accompanied by downregulated epithelial markers such as E-cadherin, and upregulated mesenchymal markers such as α-smooth muscle actin (α-SMA).[10] The EMT has been known as the initial change of fibrosis that starting by transforming growth factor beta (TGF-β)-induced signaling pathway in several organs, including the heart, liver and kidney.[11–13] In addition, MMT in peritoneal mesothelial cells has been observed in peritoneal injury cell model using primary and immortal peritoneal mesothelial cell lines in vitro, chronic kidney disease (CKD) and peritoneal injury animal models in vivo, and clinically in PD patients.[14–16]

The definite inducer of MMT and sequential peritoneal injury in PD patients is still uncertain. Currently, long-term PD fluid (PDF) exposure has mainly been considered to cause peritoneal injury in PD patients because of its hyperosmotic, hyperglycemic and acidic features.[4,15] In addition to PDF, we hypothesized that long-term exposure of uremic toxins may be also one cause of MMT of peritoneal mesothelial cells based on previous studies. Indoxyl sulfate (IS) is one of the protein-bound uremic toxins that accumulate in patients with declining renal function because of impaired clearance from the circulation. The removal of protein-bound uremic toxins is more challenging than that of other nitrogenous wastes due to their strong affinity for proteins, regardless of whether elimination occurs via HD or PD.[17,18] In addition to a sign of declining renal function, mounting evidence demonstrates that IS has a direct pathogenic impact on proximal tubular cells, leading to tubular atrophy and renal fibrosis.[19] Indoxyl sulfate is shown to be associated with EMT and induces the expression of pro-fibrotic markers such as TGF-β, fibronectin and α-SMA in proximal renal tubular cells.[20] Furthermore, downregulated E-cadherin and upregulated α-SMA were observed in renal tissue in a CKD mouse model, and the fibrotic area was suppressed in mice treated with AST-120, which inhibits the absorption of indole, the IS precursor, indicating the possible role of IS in the process of EMT and renal fibrosis.[21] Evidence demonstrates that EMT combined with chronic inflammation and oxidative stress caused by IS may lead to CKD and progress further to renal fibrosis. In addition, the IS level may serve as a prognostic predictor for CKD, given that the relationship between CKD complications and poor patient outcomes has been demonstrated.[22–24]

The possibility of the abovementioned alteration may also exist in the case of MMT in peritoneal mesothelial cells of patients who underwent long-term exposure of IS due to PD. The EMT is an early and reversible process in the development of organ fibrosis, including kidney fibrosis.[25] Since fibrosis and subsequent organ failure currently lack effective treatments, developing interventions that target critical signaling molecules involved in EMT, based on our understanding of potential inducers and underlying mechanisms, could help treat or prevent fibrosis. Such approaches may prolong PD technique survival and improve outcomes for patients.

Objectives

The present study evaluated whether IS induced MMT in peritoneal mesothelial cell line (HMrSV5). Since TGF-β, Wnt and Notch extracellular domain (NECD) signaling pathways play important roles in the fibrosis process,[25] β-catenin as the common molecule of both TGF-β and Wnt signaling pathways was observed to determine the possible molecular mechanism of IS-induced MMT in peritoneal mesothelial cells.

Materials and methods

Cell culture and treatment

A human peritoneal mesothelial cell line (HMrSV5) was purchased from Shanghai Guandao Bio Co., Ltd (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, USA) with 10% fetal bovine serum (FBS; Gibco)

at 37°C with 5% CO_2 until 80% confluence. Cells were transferred to serum-free DMEM medium overnight before treatment. Cells in the control group were maintained in serum-free DMEM. Cells in the IS and IS+ICG-001 groups were treated with 1 mmol/L IS (Sigma-Aldrich, Shanghai, China) with or without 5 μmol/L ICG-001 (Meilunbio, Dalian, China), respectively, for 12, 24, 48, or 72 h. Cells in the PDF and PDF+ICG-001 groups were treated with PD fluid with 4.25% dextrose (Baxter Healthcare, Shanghai, China) with or without 5 μmol/L ICG-001, respectively, for 12, 24, 48, or 72 h. The PDF served as a positive control. The concentrations and treatment periods of IS and ICG-001 were chosen according to the results of our preliminary test based on previous studies.[26,27] Cell morphology was observed under an inverted phase-contrast microscope (model BX43; Olympus Corp., Tokyo, Japan).

Cell proliferation assay

Cells were seeded (2×10^3 per well) into a 96-well plate and cultured in serum-free DMEM medium overnight before treatment. After treatment, the proliferation of treated cells in each group was assessed using Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. Absorbance was measured at 450 nm.

Adhesion assay

Cells were treated as described above for 48 h and then resuspended in serum-free RPMI-1640 (Hyclone, Logan, USA) to 2×10^4 per well and plated in Matrigel® (BD Biosciences, San Jose, USA)-coated 96-well plates at 37°C for 1 h. Non-adherent cells were removed by washing the plates with phosphate-buffered saline (PBS). Adherent cells were fixed with 4% phosphate-buffered paraformaldehyde for 30 min, stained with 0.5% crystal violet for 15 min and counted under an inverted phase-contrast microscope (model BX41; Olympus Corp.).

RNA extraction and RT-PCR

The isolation of RNA was carried out using the TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The preparation of cDNA was accomplished by employing the Prime-Script RT Master Mix (TaKaRa, Kusatsu, Japan). Transcript levels were quantified on the 7900 HT Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR Premix Ex Taq (TaKaRa). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method and normalized to the housekeeping gene *GAPDH*. Primers used were as follows:

E-cadherin, forward: GCCGAGAGCTACACGTTCAC, reverse: GTCGAGGGAAAAATAGGCTG;
β-catenin, forward: AAAGCGGCTGTTAGTCACTGG, reverse: CGAGTCATTGCATACTGTCCAT;
α-SMA, forward: CTATGAGGGCTATGCCTTGC, reverse: GCTCAGCAGTAGTAACGAAGGA;
GAPDH, forward: GGAGCGAGATCCCTCCAAAAT, reverse: GGCTGTTGTCATACTTCTCATGG.

Western blot

Treated cells were harvested and lysed with phenylmethylsulfonyl fluorid (PMSF)-contained radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) on ice for 30 min. Lysed cells were centrifuged at 4°C for 20 min, supernatants were collected. Protein was quantified using the Bradford method, equal amount of protein of each group was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to 0.2 μm polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, USA). The membrane was blocked in 5% dried skimmed milk powder in tris-buffered saline with Tween (TBST) solution at room temperature for 2 h and probed with primary antibodies against E-cadherin (1:1000; Cell Signalling Technology (CST), Danvers, USA), α-SMA (1:1000; Abcam; Cambridge, USA), β-catenin (1:5000; Abcam), or GAPDH (1:1000; Abcam) overnight at 4°C, followed by incubated in appropriate secondary antibodies conjugated with horseradish peroxidase (HRP; 1:2,000; ABclonal, Wuhan, China) at room temperature for 1 h. Protein bands were visualized by electrochemiluminescence (ECL) western blotting detection system (GE Healthcare Life Sciences, Logan, USA) and the intensity was measured using Image Pro Plus v. 6.0 software (Media Cybernetics, Rockville, USA).

Statistical analyses

All data were entered into IBM SPSS v. 22.0 statistical software (IBM Corp., Armonk, USA) for statistical analysis, while GraphPad Prism 6 (GraphPad Software, San Diego, USA) and Adobe Photoshop (Adobe Inc., Mountain View, USA) were used for chart making. The Shapiro–Wilk test was used to assess the normality of the distribution for the data. Due to the non-normal distribution of data, the Kruskal–Wallis test was used for group comparisons, followed by Dunn's post hoc test. A p-value <0.05 was considered statistically significant. Results were presented as box-and-whisker plots, with all data points displayed as individual dots. The sample size for each experiment ranged from 3 to 4, as indicated in the corresponding figure legends.

Results

MMT-related cell characteristics

Indoxyl sulfate induced morphological changes in HMrSV5 cells, shifting from the rounded appearance observed in the control group to an elongated, fibroblast-like

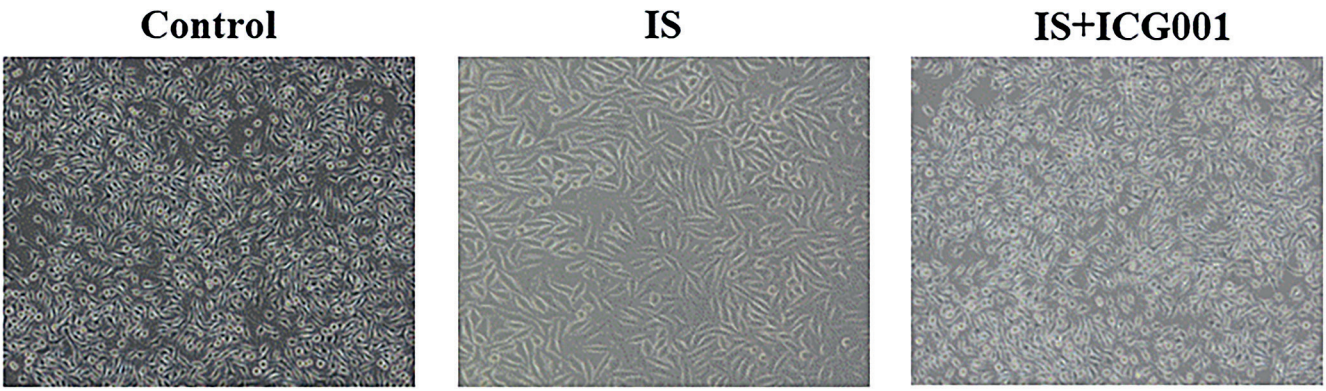

Fig. 1. Morphologic changes induced by IS were eliminated by β-catenin inhibitor ICG-001

IS – indoxyl sulfate.

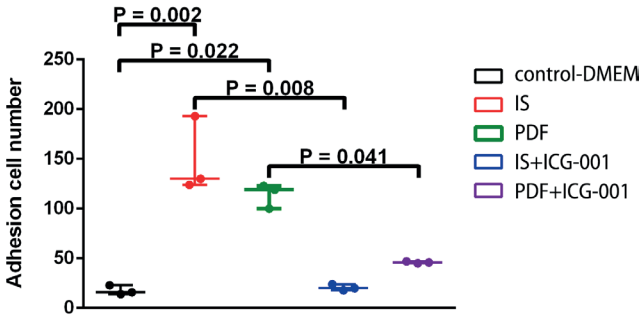

Fig. 2. Significantly increased cell proliferation was found after exposure of indoxyl sulfate or peritoneal dialysis fluid with 4.25% dextrose for 72 h, and eliminated by β-catenin inhibitor ICG-001. The Kruskal–Wallis test showed a significant difference among groups (df = 4, H = 13.033, p = 0.011); p-values examined using the Dunn's post hoc test were presented for significant difference between 2 groups; n = 3

IS – indoxyl sulfate; df – degrees of freedom; PDF – peritoneal dialysis fluid; DMEM – Dulbecco's modified Eagle's medium.

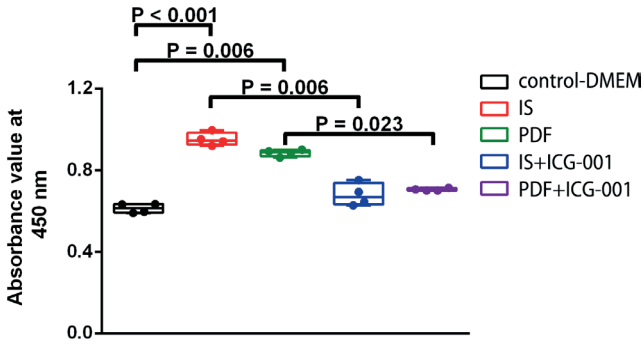

Fig. 3. Significantly increased adherence capability was found after exposure of indoxyl sulfate or peritoneal dialysis fluid with 4.25% dextrose for 48 h, and eliminated by β-catenin inhibitor ICG-001. The Kruskal–Wallis test showed a significant difference among groups (df = 4, H = 17.0857, p = 0.002); p-values examined using the Dunn's post hoc test were presented for significant difference between 2 groups; n = 4

IS – indoxyl sulfate; df – degrees of freedom; PDF – peritoneal dialysis fluid; DMEM – Dulbecco's modified Eagle's medium.

morphology. These changes were mitigated by the addition of the β-catenin inhibitor ICG-001, as evidenced by the cuboidal shape of cells in the IS+ICG-001 group (Fig. 1).

Cell Counting Kit-8 assay was used for evaluating proliferation status after IS treatment. Viable cell numbers showed significant differences between the control group and IS or PDF groups (p = 0.002 and p = 0.022, respectively), and between the IS and IS+ICG-001 and the PDF and PDF+ICG-001 groups (p = 0.008 and p = 0.041, respectively) at 72 h. No significant differences were found between the control group, IS+ICG-001 or PDF+ICG-001 groups at 72 h (Fig. 2).

Matrigel was used for evaluating the adherence ability of cells after IS treatment. Adhesion cell numbers significantly increased after IS or PDF treatment compared with those of the control group (p < 0.001 and p = 0.006, respectively). ICG-001 significantly reduced the adhesion cell numbers compared with IS or PDF treatment only (p = 0.006 and p = 0.023, respectively) (Fig. 3).

RNA expression of MMT markers

The present study sought to evaluate the RNA expression of E-cadherin, β-catenin and α-SMA in order to determine whether MMT was induced by IS and the related signaling pathway in HMrSV5 cells. After being treated by IS or PDF, E-cadherin RNA expression was significantly reduced compared with that of the control group from 12 to 72 h (data not shown). Adding ICG-001 reversed the change of E-cadherin RNA expression after IS or PDF treatment, shown by the significant differences in E-cadherin RNA expression between the IS and IS+ICG-001 groups at 48 h (Fig. 4A, H = 17.5, p = 0.002). After being treated by IS or PDF, β-catenin RNA expression was significantly increased compared with that of the control group from 12 to 72 h (data not shown). Adding ICG-001 reversed the change of β-catenin RNA expression, as shown by the significant differences in β-catenin RNA expression between the IS and IS+ICG-001 groups, and PDF and PDF+ICG-001 groups at 72 h (Fig. 4B, H = 18.0714,

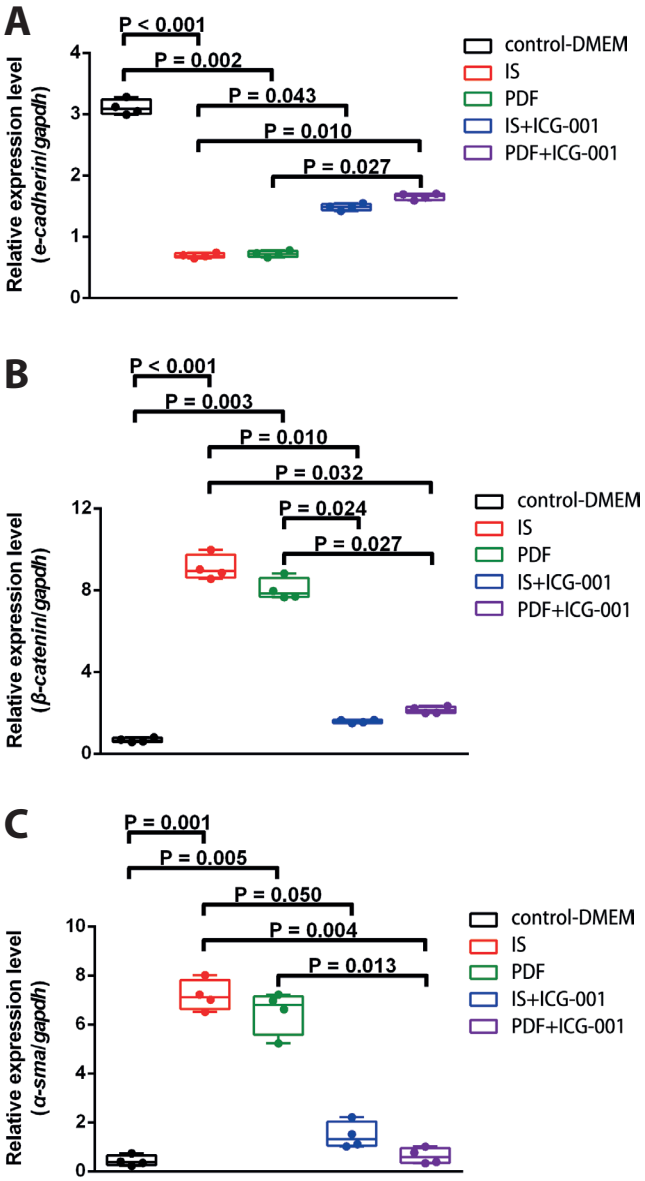

Fig. 4. Mesothelial-to-mesenchymal transition (MMT) of human peritoneal mesothelial cell line HMrSV5 was induced by indoxyl sulfate or peritoneal dialysis fluid with 4.25% dextrose and eliminated by β-catenin inhibitor ICG-001, indicated by the changes of RNA expression of E-cadherin at 48 h (A), β-catenin at 72 h (B) and α-SMA at 48 h (C). RNA level was normalized by GAPDH expression. The Kruskal–Wallis test showed a significant difference among groups (df = 4, H = 17.5, p = 0.002 for E-cadherin; df = 4, H = 18.0714, p = 0.001 for β-catenin; df = 4, H = 16.7733, p = 0.002 for α-SMA); p-values examined using the Dunn's post hoc test were presented for significant difference between 2 groups; n = 4

IS – indoxyl sulfate ; df – degrees of freedom; PDF – peritoneal dialysis fluid; DMEM – Dulbecco's modified Eagle's medium; α-SMA – α-smooth muscle actin.

p = 0.001). After being treated by IS or PDF, α-SMA RNA expression was significantly increased compared with that of the control group from 24 to 72 h (data not shown). Adding ICG-001 reversed the change of α-SMA RNA expression, shown by the significant differences in α-SMA RNA expression between the IS and IS+ICG-001 groups and PDF and PDF+ICG-001 groups at 48 h (Fig. 4C, H = 16.7733, p = 0.002).

Protein expression of MMT markers

The protein expression of E-cadherin, β-catenin and α-SMA was evaluated. The representative images and quantitative data are shown in Fig. 5A and 5B–5D, respectively. After being treated by IS or PDF, E-cadherin protein expression was significantly reduced compared with that of the control group from 24 to 72 h. Adding ICG-001 reversed the change of E-cadherin protein expression, shown by the significant differences in E-cadherin protein expression between the IS and IS+ICG-001 groups and PDF and PDF+ICG-001 groups at 48 h (Fig. 5B, H = 16.4552, p = 0.002). After being treated by IS or PDF, β-catenin protein expression was significantly increased compared with that of the control group from 24 to 72 h. Adding ICG-001 reversed the change of β-catenin protein expression, shown by the significant differences in β-catenin protein expression between the IS and IS+ICG-001 groups, and PDF and PDF+ICG-001 groups at 48 h (Fig. 5C, H = 15.744, p = 0.003). After being treated by IS or PDF, α-SMA protein expression was significantly increased compared with that of the control group from 24 to 72 h. Adding ICG-001 reversed the change of α-SMA protein expression, shown by the significant differences in α-SMA protein expression between the IS and IS+ICG-001 and PDF and PDF+ICG-001 groups at 24, 48 and 72 h (Fig. 5D, H = 17.4119, p = 0.002).

Discussion

In the present study, IS was found to have the same capability as high-glucose PDF to induce MMT in human peritoneal mesothelial cells, which was presented by exhibiting a fibroblast-like appearance and functionally gaining the abilities of adhesion and proliferation. In addition, other MMT features including upregulated α-SMA and β-catenin, and downregulated E-cadherin in both protein and RNA levels were also found. These changes were reversed by the specific β-catenin inhibitor ICG-001, indicating that in peritoneal mesothelial cells, the β-catenin signaling pathway was not only involved in PDF-induced MMT, but IS-induced MMT was also activated through the β-catenin pathway.

Besides the convenience of implementation because it does not require frequent visits to hospital or medical facility, as well as assures greater patient mobility and higher quality of life for patients, PD is selected as the modality for RRT for better preservation of residual renal function, reduction of the risk of cardiovascular comorbidities or infection, and improvement of the prognosis of patients with ESRD rather than HD.[16] The peritoneal cavity is covered by semipermeable peritoneum, which is composed of a sub-mesothelial zone, a basement membrane and a single layer of mesothelial cells as the first-line barrier. However, the integrity of the peritoneal mesothelial barrier

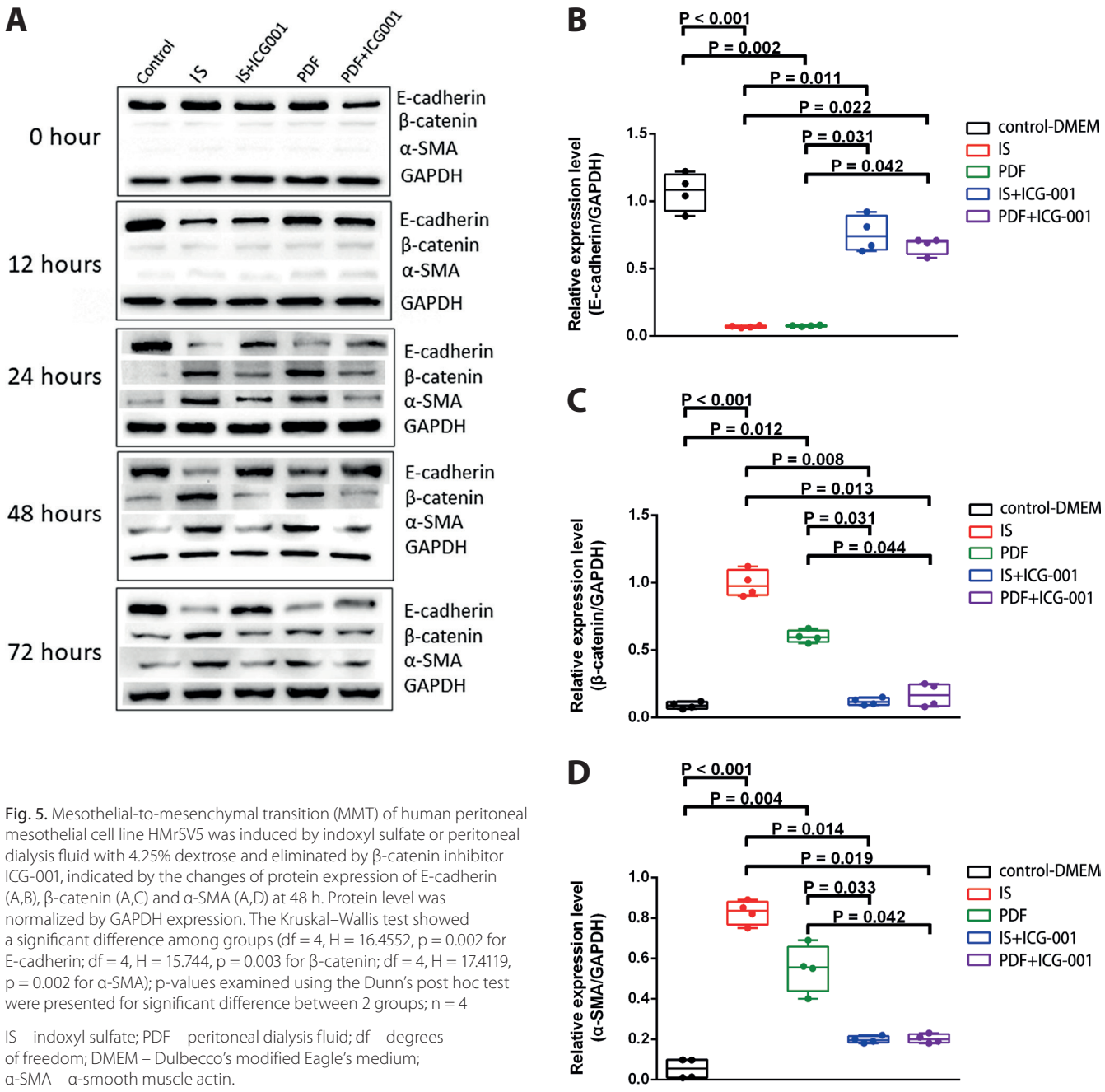

Fig. 5. Mesothelial-to-mesenchymal transition (MMT) of human peritoneal mesothelial cell line HMrSV5 was induced by indoxyl sulfate or peritoneal dialysis fluid with 4.25% dextrose and eliminated by β-catenin inhibitor ICG-001, indicated by the changes of protein expression of E-cadherin (A,B), β-catenin (A,C) and α-SMA (A,D) at 48 h. Protein level was normalized by GAPDH expression. The Kruskal–Wallis test showed a significant difference among groups (df = 4, H = 16.4552, p = 0.002 for E-cadherin; df = 4, H = 15.744, p = 0.003 for β-catenin; df = 4, H = 17.4119, p = 0.002 for α-SMA); p-values examined using the Dunn's post hoc test were presented for significant difference between 2 groups; n = 4

IS – indoxyl sulfate; PDF – peritoneal dialysis fluid; df – degrees of freedom; DMEM – Dulbecco's modified Eagle's medium; α-SMA – α-smooth muscle actin.

can be disrupted after PD. The pathogenetic mechanisms are recognized including angiogenesis, hyalinizing vasculopathy and fibrosis.[28,29] Significant crosstalk and interplay between these changes and the associated signaling pathways have been demonstrated.[30]

The EMT plays a key role in embryogenesis and wound healing under normal physiological conditions and is typically activated following injury, where it is associated with organ fibrosis. Renal fibrosis-associated diseases include CKD, polycystic kidney disease and diabetic nephropathy. Peritoneal fibrosis due to PD can be considered as one of the consequences of renal fibrosis-associated diseases.[25] Podocytes, endothelium and tubular epithelial cells in the kidney, and peritoneal mesothelial cells have been identified as sources of EMT/MMT during the renal fibrosis-associated disease progression, as described previously.[25] The EMT can be triggered by various signaling pathways, many genes and protein may involve in this process, and these pathways crosstalk intensively and intricately. Transforming growth factor beta, Wnt and NECD are recognized as the main activated receptors of the injury stimuli, and they sequentially activate specific transcriptional molecules for the expression of mesenchymal and inhibitory function of endothelial proteins.[25] Beta-catenin is the downstream molecule of both TGF-β and Wnt signaling pathways. As shown in a peritoneal fibrosis animal model, TGF-β treatment increased the expressions of WNT2 and WNT4, and also of β-catenin. Transforming

growth factor beta treatment increased the expressions of WNT2 and WNT4, and also of β-catenin. When WNT antagonist DKK-1 or β-catenin inhibitor ICG-001 was treated, both resulted in attenuation of peritoneal angiogenesis; in addition, DKK-1 increased E-cadherin level and inhibited MMT, which all indicates the intensive crosstalk of the signaling pathways.[31] Thus, β-catenin was chosen to be monitored in the present study. Transforming growth factor beta is a key contributor to peritoneal injury and is associated with the induction of collagen deposition, fibroblast activation and angiogenesis of the peritoneum.[32] Signaling pathways induced by TGF-β divide into Smad-dependent and non-Smad pathways. In the Smad-dependent pathway, Smad2/3 is phosphorylated by PKC and activated by TGF-β receptor 1 and activin I-β; then, it is released from the receptor complex and binds to Smad4. This complex translocates to the nucleus to regulate the transcription of target genes involved in fibrosis. Via the non-Smad pathway, TGF-β activation leads to the inactivation of β-catenin inhibitor GSK-3β by phosphorylation. Elevated expressions of WNTs and β-catenin have been observed in isolated peritoneal mesothelial cells from PD patients, and are strongly associated with MMT.[33] Through the same process of inactivation of GSK-3β by TGF-β or WNTs, released β-catenin translocates to the nucleus and activates the transcription of target genes including *fibronectin*, *Twist*, *MMPs*, *Jagged1*, *LEF1*, and *Snail*, which are the key factors during EMT/MMT development.[25] For example, activated Snail directly inhibits E-cadherin expression, and matrix metalloproteinase-9 (MMP-9) cleaves E-cadherin.[34,35] Besides signaling transduction, β-catenin can bind with E-cadherin, and this complex is associated with actin filaments to form a dynamic link with the actin cytoskeleton, which is important for the maintenance of epithelial cell layers.[33] Isolated peritoneal mesothelial cells from the effluent of patients underwent PD for more than 1 year had higher expression levels of β-catenin than that from patients who just started with PD for less than 1 month, and it was accompanied by expressional changes of E-cadherin and α-SMA, which are well-known markers of EMT/MMT, indicating the importance of β-catenin in MMT during PD.[33] Thus, β-catenin was selected for the initial step to clarify the involved signaling pathway of IS-induced MMT in peritoneal mesothelial cells in the present study. In addition, in a peritoneal fibrosis mouse model, inhibition of β-catenin by ICG-001 showed reduced high-glucose induced angiogenesis severity and VEGF level of the peritoneum,[31] so ICG-001 was used in the present study to confirm the involvement of β-catenin.

Indoxyl sulfate is an end product of dietary tryptophan, which is formed in liver and then enters systemic circulation.[36] Indoxyl sulfate has been thought of as a consequent sign of declining renal function initially; however, emerging data indicate that IS has direct cytotoxicity on proximal tubular epithelial cells and negatively impacts the kidney, and IS exposure may be associated with CKD progress.[22] Under healthy conditions, IS is trapped by organic anion transporters (OATs) at the basolateral membrane of proximal tubular epithelial cells and then excreted by active secretion, which may explain why previous studies have focused mainly on this type of renal cell. In the present study, the effects of IS on the peritoneum of patients with PD was evaluated since the peritoneum is used as dialysis membrane during PD, intensive contact of IS may be associated with MMT and fibrosis of the peritoneum. Despite this, IS has been found to be involved in fibrosis, a long-term process involving several cell types. For example, Nakano et al. demonstrated that IS induces EMT in the human tubular epithelial cell line HK-2, promotes the differentiation of the fibroblast cell line NRK-49F into myofibroblasts, and triggers an inflammatory response in the macrophage cell line THP-1, all through the mTORC1 signaling pathway.[21] It remains unknown whether a specific cell type in the peritoneum selectively responds to IS. In this study, we provide evidence that exposure to IS triggers MMT in peritoneal mesothelial cells, potentially contributing to peritoneal injury during PD.

Based on preliminary test results, we selected a treatment concentration of 1 mmol/L for IS. Although this concentration was considerably lower than the serum levels observed in HD patients and even in healthy study participants, significant MMT features were still evident. Previous studies have indicated that serum IS levels in HD patients were more than 50 times higher than those in individuals with normal renal function (2.99 ±0.18 mg/dL or 140 mmol/L pre-HD vs 0.05 ±0.01 mg/dL or 2.34 mmol/L).[37] As the primary contact layer, peritoneal mesothelial cells may activate signaling pathways in response to IS as an injury-induced stimulus. Consequently, the impact of IS exposure on these cells cannot be overlooked.

For effective treatment and prevention of peritoneal dysfunction in patients undergoing PD, it is essential to identify both the underlying mechanisms and the key inducers. Currently, high glucose exposure of peritoneal mesothelial cells during PD is the only known inducer, which has led to the development of glucose-sparing dialysis solutions to mitigate the risk of high glucose-induced MMT.[38] Indoxyl sulfate, a uremic toxin, was identified as another potential inducer of MMT via the β-catenin signaling pathway during PD in the present study.

Therefore, routine monitoring of serum and peritoneal IS levels, along with the development of strategies to remove or reduce IS levels in the peritoneal cavity, could provide an alternative approach to prolonging PD use in patients with ESRD. Additionally, the use of specific inhibitors, such as ICG-001 (employed in this study to block the MMT signaling pathway), may offer potential benefits. The definition of abnormal serum and peritoneal IS level should be established by a larger survey including ESRD patients under HD or PD, and individuals with normal renal function. Additionally, effective and safe techniques for the repeated assessment of morphological and molecular

changes in the peritoneum, such as endoscopic methods in both animal and clinical studies, should be developed to enable comprehensive and regular evaluations.

Limitations

The primary limitation of this study is that it was conducted exclusively in vitro. Additionally, to highlight the maximum pharmacological effects of the treatments investigated, results were presented at a single time point. More in vitro and in vivo studies should be done to overcome the limitations of the present study. For example, data of direct assessment of cell viability and vitality should be provided. Furthermore, to strengthen the association between IS and MMT, evidence from PD animal models and clinical patients are needed. Assessments of IS levels in the peritoneal cavity, morphological changes at the cellular and tissue levels, and the expression of molecules involved in β-catenin-associated signaling pathways should be performed in the future.

Conclusions

Indoxyl sulfate is known to be associated with EMT in proximal renal tubular cells. However, its impact on peritoneal mesothelial cells, which serve as the first-line barrier in PD, remains unexplored. Indoxyl sulfate induces MMT of human peritoneal mesothelial cells morphologically and functionally and these changes can be reversed by specific β-catenin inhibitor ICG-001, indicating β-catenin signaling pathway plays an important role in the process of MMT in peritoneal mesothelial cells. Routine monitoring of serum IS may be necessary to prolong PD survival and improve the outcome. Further investigation of the complete mechanism for developing strategy focused on IS-induced MMT might be worthy for patients who underwent PD.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.14032147. The package includes the following files:

Supplementary Table 1. The results of post hoc Dunn's test of the variables presented in Fig. 2.

Supplementary Table 2. The results of post hoc Dunn's test of the variables presented in Fig. 3.

Supplementary Table 3. The results of post hoc Dunn's test of the variables presented in Fig. 4A.

Supplementary Table 4. The results of post hoc Dunn's test of the variables presented in Fig. 4B.

Supplementary Table 5. The results of post hoc Dunn's test of the variables presented in Fig. 4C.

Supplementary Table 6. The results of post hoc Dunn's test of the variables presented in Fig. 5B.

Supplementary Table 7. The results of post hoc Dunn's test of the variables presented in Fig. 5C.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Circular RNA hsa_circ_0008433 drives vascular smooth muscle cell modulation in intracranial aneurysm pathogenesis

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Conflict of interest
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Abstract

Background. Intracranial aneurysm (IA) is a serious condition that can lead to a life-threatening rupture, often resulting in a hemorrhagic stroke. Vascular smooth muscle cell (VSMC) dysfunction is a critical factor in the pathogenesis of IA, yet the molecular mechanisms underlying this relationship are not yet fully understood. Recent studies suggest that circular RNAs (circRNAs) are involved in various vascular diseases. High-throughput sequencing identified hsa_circ_0008433 as significantly upregulated in IA tissues, especially in ruptured cases, suggesting a role in IA progression.

Objectives. To further investigate the potential effects of hsa_circ_0008433 on the rupture of human IA.

Materials and methods. This study aimed to investigate the effects of hsa_circ_0008433 on IA rupture. We validated the expression of hsa_circ_0008433 in IA patient tissue samples through reverse transcription quantitative polymerase chain reaction (RT-qPCR), comparing ruptured and unruptured aneurysms. Human brain vascular smooth muscle cells (HBVSMCs) were utilized to establish overexpression and knockdown models for hsa_circ_0008433. Cell Counting Kit-8 (CCK-8) and wound healing assays were conducted to assess cell proliferation and migration, while western blotting was employed to measure VSMC phenotype markers including α-smooth muscle actin (α-SMA), smooth muscle protein 22-alpha (SM22α), matrix metalloproteinase-2 (MMP-2), and matrix metalloproteinase-9 (MMP-9).

Results. The RT-qPCR analysis confirmed that hsa_circ_0008433 was significantly upregulated in IA tissues, especially in ruptured samples ($p < 0.05$). Overexpression of hsa_circ_0008433 in HBVSMCs promoted proliferation, migration and phenotype switching, indicated by increased expression of MMPs and decreased contractile proteins. The effects were reversed by the knockdown of hsa_circ_0008433.

Conclusions. We have shown that hsa_circ_0008433 regulates vascular smooth muscle cell function and promotes behaviors that may lead to intracranial aneurysm instability. This study advances the understanding of the role of circRNAs in vascular pathology and identifies hsa_circ_0008433 as a potential therapeutic target for IA. These findings open opportunities for targeted treatments and broader applications in vascular disease research.

Key words: circular RNA, intracranial aneurysm, vascular smooth muscle cell, phenotype, rupture

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Highlights

- First study to demonstrate the upregulation of hsa_circ_0008433 in intracranial aneurysm tissues, particularly in ruptured cases.
- Overexpression of hsa_circ_0008433 promotes vascular smooth muscle cell proliferation, migration and phenotype switching.
- Knockdown of hsa_circ_0008433 reduces HBVSMC proliferation and migration, suggesting its pivotal role in aneurysm instability.
- hsa_circ_0008433 is identified as a potential biomarker and therapeutic target for intracranial aneurysm rupture prevention.
- Findings provide new insights into circRNA regulation of vascular smooth muscle cells in cerebrovascular diseases.

Background

Intracranial aneurysms (IAs) are arterial swellings within the cerebral vasculature, often resulting from congenital anomalies or acquired injuries, and are a leading cause of aneurysmal subarachnoid hemorrhage.[1,2] Intracranial aneurysm is the 3rd most common cerebrovascular disease after cerebral thrombosis and hypertensive hemorrhage.[3] When an IA ruptures, blood enters the subarachnoid space or brain parenchyma, leading to hemorrhagic stroke.[4,5] This abrupt bleeding elevates intracranial pressure, causing cerebral edema, hypoxia and ischemic injury to brain cells.[6,7] The hemorrhage-induced inflammatory response further exacerbates brain by disrupting the blood–brain barrier and triggering secondary injuries, often resulting in severe neurological deficits or death.[8,9] Consequently, IA rupture remains a critical concern in neurosurgical research due to its high fatality rate.[10]

While IA has been extensively studied, the mechanisms underlying its formation and rupture are not fully understood. Emerging research highlights the crucial role of vascular smooth muscle cells (VSMCs) in IA pathophysiology.[11,12] Under healthy conditions, VSMCs maintain a contractile phenotype, supporting vascular stability. However, in pathological states, VSMCs can switch from a contractile to a pro-inflammatory phenotype.[13,14] This phenotypic switch involves decreased expression of contractile proteins, such as α-smooth muscle actin (α-SMA), and increased expression of matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM).[15,16] These transformations can destabilize the vascular structure, heightening the risk of IA rupture. The phenotypic diversity of VSMCs is therefore central to the formation, progression and rupture of IAs.[17,18]

Circular RNA (circRNA)s represents a novel class of non-coding RNA characterized by a stable closed-loop structure, which makes it highly stable compared to linear RNA.[19,20] Unlike linear RNAs, circRNA lacks a 5' cap and 3' poly(A) tail, making it resistant to exonucleases and thus more stable.[21,22] Recent studies show that circRNAs can act as "sponges" for microRNAs (miRNAs) and modulate target gene expression through a competing endogenous RNA (ceRNA) mechanism.[23,24] Circular RNAs have been implicated in several diseases, including diabetes, neurological disorders, cardiovascular disease (CVD), and cancer.[25–28] Within the cerebrovascular system, circRNAs have been associated with key processes such as angiogenesis, autophagy, apoptosis, and inflammation.[29,30] They are also believed to regulate VSMC phenotype and might even act as miRNA carriers within IAs.[31,32] Recent studies indicate that circRNAs are essential in regulating the phenotype of human brain vascular smooth muscle cells (HBVSMCs).[33] Changes in circRNA expression may influence IA formation and rupture by modulating the proliferation, migration and phenotype switching of HBVSMCs. Although circRNAs hold significant potential in vascular diseases, their specific role in IA formation and rupture has not been thoroughly studied.[27] Furthermore, the relationship between circRNA-regulated HBV-SMC proliferation and phenotype switching, as well as its role in the overall pathophysiology of IA, remains unclear. This gap provides a rationale and direction for this study.

In our previous research, high-throughput sequencing identified hsa_circ_0008433 as significantly upregulated in human IA tissues, especially in ruptured aneurysms.[34–37] This finding suggests that hsa_circ_0008433 may play an important role in IA pathology, particularly in the critical and life-threatening event of aneurysm rupture. Based on these preliminary findings, we hypothesize that hsa_circ_0008433 may influence VSMC behavior under pathological conditions, potentially promoting VSMC proliferation, migration and phenotype switching, thereby affecting IA stability and ultimately contributing to aneurysm rupture.

Objectives

This study aims to further investigate the role of hsa_circ_0008433 in HBVSMCs, specifically in promoting cell proliferation, migration and phenotype switching. By gaining a deeper understanding of the molecular mechanisms

underlying hsa_circ_0008433's involvement in IA pathology, we hope to provide new insights for future therapeutic strategies, supporting clinical interventions for cerebrovascular diseases.

Materials and methods

Study design

By analyzing tissue samples from IA patients and healthy volunteers, combined with in vitro transfection experiments, we compared the expression levels of hsa_circ_0008433 and its effects on HBVSMC proliferation, migration and phenotype switching.[32]

Participants

Healthy volunteers (n = 15) and patients with IA (n = 15) who were admitted to our Department of Neurosurgery of the Second Affiliated Hospital of Fujian Medical University (Quanzhou, China) in 2019 were included in the analysis. Tissues from the aneurysm wall of IA patients were considered the IA wall group, while tissues from the superficial temporal artery of healthy volunteers served as the control group. Additionally, the IA tissues were categorized into 2 subgroups based on the aneurysmal state: the ruptured group (n = 7) and the unruptured group (n = 8). The study was approved by the Ethics Committee of The Second Affiliated Hospital of Fujian Medical University (approval No. 2021-434).

Cell cultivation and group divide

For in vitro experiments, HBVSMCs (cat. No. PCS-100-012) were purchased from the American Type Culture Collection (ATCC; Manassas, USA) and cultured in a special complete medium (CM-H116; Procell, Wuhan, China) at 37°C and 5% CO_2. Human brain vascular smooth muscle cells transfected with negative siRNA and hsa_circ_0008433 siRNA were designated as the si-NC group and the si-hsa_circ_0008433 group, respectively. Besides, pLC5-circ plasmid was used to transfect HBVSMCs to over-express hsa_circ_0008433. The normal HBVSMCs without any treatment and HBVSMCs transferred with pLC5 circ plasmid were named the blank group and the vector group, respectively. Both pLC5 circ plasmid and siRNA were designed and purchased from Geneseed Biotech Co., Ltd. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to themanufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction

The HBVSMCs were split using TRIzol (Thermo Fisher Scientific, Waltham, USA) and total RNA was separated according to themanufacturer's instructions. Then, 1 μg RNA was reverse transcribed using reverse transcriptase kits of miRNA or M-MLV (Moloney murine leukemia virus reverse transcriptase; Thermo Fisher Scientific, again following the manufacturer'sinstructions. QuantStudio5™ Real-Time PCR System (Applied Biosystems, Foster City, USA) was adopted for a quantitative reverse transcription polymerase chain reaction (RT-qPCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control and a $2^{-\Delta\Delta Ct}$ method was used to measure relative expression. The sequence results for circRNA were derived from circBase (http://circrna.org). The primer pairs were synthesized by Sangon Biotech (Shanghai, China).

Cell Counting Kit-8

After the above grouping and treatment, HBVSMCs were seeded into 96 well plates at a density of 5×10^4 cells/well at 37°C overnight. Subsequently, 10 μL Cell Counting Kit-8 (CCK-8) solution (Abcam, Cambridge, USA) was added to each well for 4 h of incubation at 37°C. Then, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, USA). Each sample was tested independently in triplicate.[38]

Wound healing assay

Cell migration was observed using wound healing assay. Cells (2×10^5) were seeded in a 6-well plate after the above-mentioned grouping and arrangement. When the cells reached 90% confluence, a sterile micropipette tip was used to scratch the cell monolayer, forming the wound. Simultaneously, the cells were cultured in a serum-free medium. After 24 h, the wound area was examined using a light microscope (CKX41; Olympus Corp., Tokyo, Japan). Additionally, ImageJ software v. 1.41 (National Institutes of Health (NIH), Bethesda, USA) was used to measure the wound width. Finally, cell migration capacity was expressed as a percentage of original wound distance.

Western blotting

Cells were lysed using radio immunoprecipitation assay buffer (RIPA; Beyotime, Shanghai, China), followed by centrifugation at 10,000 × g for 5 min. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific). Then, 25 μg of protein per sample was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis. Following separation, the proteins were transferred onto a polivinylidene difluoride (PVDF) membrane. The membrane was subsequently blocked with 5% skimmed milk powder (Solarbio, Beijing, China) for 2 h to prevent nonspecific binding. It was then incubated overnight at 4°C with the following primary antibodies: anti-α-SMA (ab7817, 1:10,000; Abcam), anti-smooth muscle protein 22-α (SM22α) (ab1410 6,

1:10,000; Abcam), anti-MMP-9 (ab76003, 1:500; Abcam), anti-MMP-2 (ab92536, 1:1,000; Abcam), and anti-GAPDH (ab37168, 1:10,000; Abcam). Next, the membrane was washed and incubated with horseradish peroxidase (HRP)-linked secondary antibody (Abcam) at ambient temperature for 2 h. Protein bands were then analyzed using the BeyoECL Plus kit (Solarbio) and quantified using ImageJ. For blot analysis, GAPDH was used as a standardized reference.

Statistical analyses

Statistical analysis was conducted using IBM SPSS v. 26.0 (IBM Corp., Armonk, USA). Data are presented as median (interquartile range (IQR)). The Mann–Whitney U test was used to compare significant differences between the 2 groups. For comparisons involving 3 or more groups, the Kruskal–Wallis test was applied, followed by Dunn's post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

Upregulation of hsa_circ_0008433 in intracranial aneurysm tissues and intracranial aneurysm rupture tissues

To confirm the expression of hsa_circ_0008433, we performed RT-qPCR on aneurysm wall samples. As shown in Fig. 1A, Table 1 and Supplementary Table 1, hsa_circ_0008433 expression levels were notably higher in the IA wall group compared to the control group. Additionally, Fig. 1B and Table 2 show that hsa_circ_0008433 expression was increased in the ruptured group compared to the unruptured group. These findings suggest a close association between high hsa_circ_0008433 expression and IA pathogenesis.

Overexpression of hsa_circ_0008433 promotes the proliferation and migration of HBVSMCs

Table 1. The comparison of circ_0008433 expression levels between the control group and the intracranial aneurysm (IA) wall group using the Mann–Whitney (M–W) U test, n, Me (Q1–Q3) (Fig. 1A)

Group		U	p-value
control	IA wall		
15, 1.04 (1.02–1.15)	15, 2.23 (2.07–2.29)	225.000	<0.001

n – number of observations; Me (Q1–Q3) – median (Q1–Q3); U – value of the M–W test statistic.

Table 2. Comparison of the expression levels of circ_0008433 between the unruptured and ruptured groups using the Mann–Whitney (M–W) U test, n, Me (Q1–Q3, *min–max) (Fig. 1B)

Group		U	p-value
unruptured	ruptured		
8, 1.05 (1.02–1.15, 1.01–1.18)	6, 1.77 (1.65–2.00, 1.58–2.01)	0	<0.001

n – number of observations; Me (Q1–Q3, *min–max) – median (Q1–Q3, * minimum–maximum for n < 8); U – value of the M–W test statistic.

Table 3. Comparison of the relative expression levels of circ_0008433 among groups using the Kruskal–Wallis test (Fig. 2A), n, Me (min–max)

Group	Me (min–max)	H	df	p-value
Blank	3, 1.15 (1.04–1.19)	12.83	3	0.012
Vector	3, 0.95 (0.94–0.99)			
Circ_0008433	3, 1.95 (1.93–1.99)			
si_NC	3, 1.08 (1.04–1.18)			
si_circ_0008433	3, 0.34 (0.32–0.36)			

n – number of observations; Me (min–max) – median (minimum–maximum); H – the value of the Kruskal–Wallis test statistic; df – degrees of freedom.

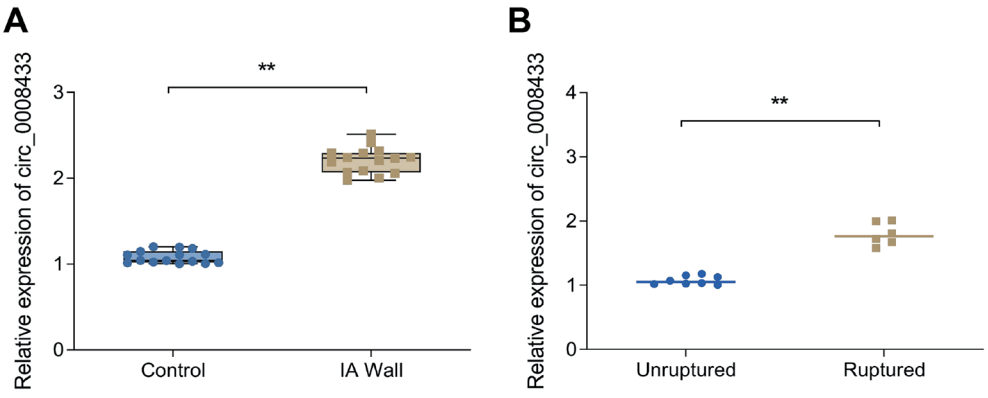

Fig. 1. Expression differences of hsa_circ_0008433 between IA and healthy tissues as well as ruptured IA and unruptured IA tissues using RT-qPCR **$p < 0.01$

IA – intracranial aneurysm; RT-qPCR – quantitative reverse transcription polymerase chain reaction.

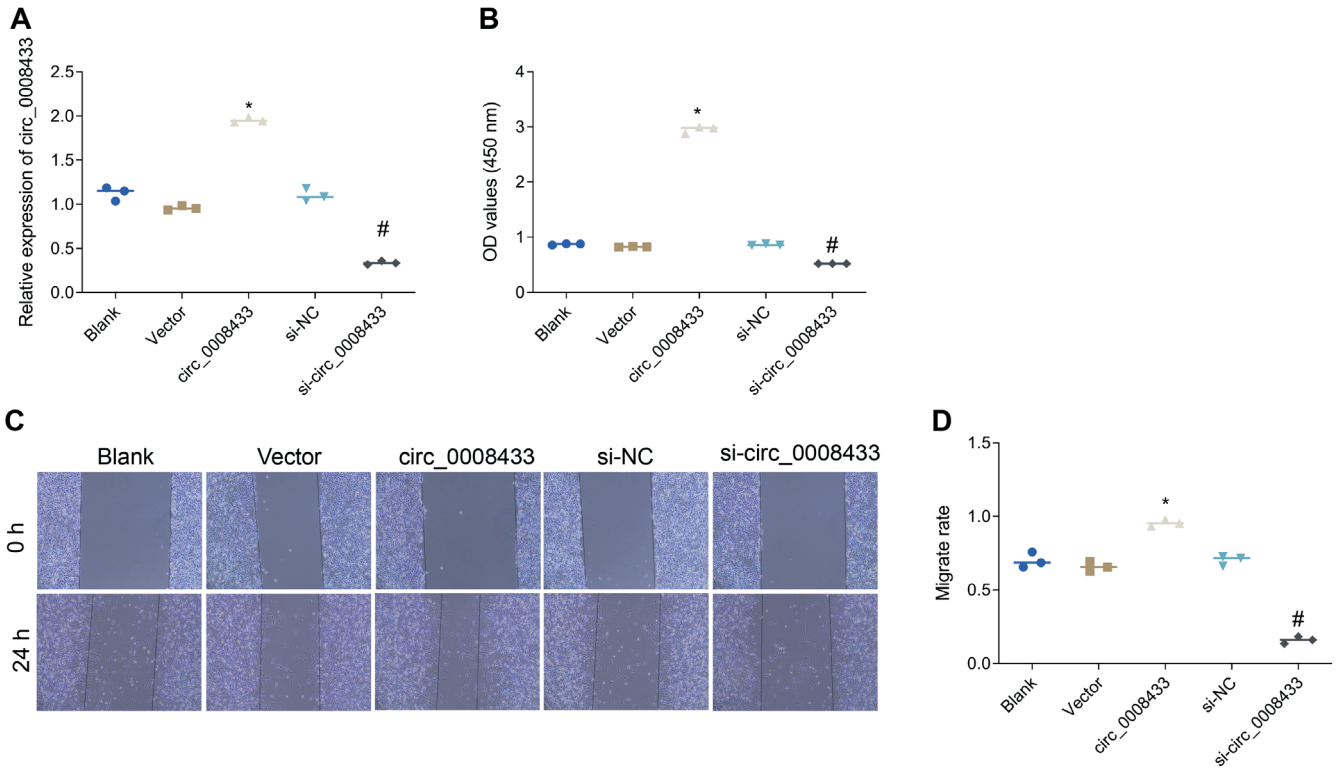

Fig. 2. Effects of hsa_circ_0008433 expression on the proliferation and migration of human brain vascular smooth muscle cell s(HBVSMCs). A. RT-qPCR was used to verify the transfection of vectors; B. Cell viability assay was performed in the blank group, the vector group, the hsa_circ_0008433 group, the si-NC group, and the si-hsa_circ_0008433 group to evaluate cell proliferation capacity; C. The scratch experiment was performed to show residual injury areas at 0 and 24 h after scratching, followed by the evaluation of migration capacity of cells in the blank group, the vector group, the hsa_circ_0008433 group, the si-NC group, and the si-hsa_circ_0008433 group; D. The migration rate of each group was assessed; *p < 0.05 vs vector group; **p < 0.01 vs vector group; #p < 0.05 vs si-NC group; ##p < 0.01 vs si-NC group.

RT-qPCR – quantitative reverse transcription polymerase chain reaction.

Table 4. Comparison of the median expression levels of circ_0008433 among groups using the Kruskal–Wallis test (Fig. 2B)

Group	Me (min–max)	H	df	p-value
Blank	3, 0.88 (0.86–0.88)	12.83	3	0.012
Vector	3, 0.83 (0.82–0.84)			
Circ_0008433	3, 2.98 (2.88–3.00)			
si_NC	3, 0.86 (0.85–0.88)			
si_circ_0008433	3, 0.521 (0.521–0.522)			

n – number of observations; Me (min–max) – median (minimum–maximum); H – the value of the Kruskal–Wallis test statistic; df – degrees of freedom.

Table 5. Analysis comparing the relative expression levels of the circ_0008433 between groups (Fig. 2D) using the Kruskal–Wallis test to evaluate the differences between the groups. The results show an H value of 11.50 and a p-value of 0.021, indicating significant differences between the groups

Group	Me (min–max)	H	df	p-value
Blank	0.69 (0.66–0.76)	11.50	3	0.021
Vector	3, 0.66 (0.63–0.69)			
Circ_0008433	3, 0.95 (0.93–0.98)			
si_NC	3, 0.72 (0.66–0.73)			
si_circ_0008433	3, 0.16 (0.14–0.18)			

n – number of observations; Me (min–max) – median (minimum–maximum); H – the value of the Kruskal–Wallis test statistic; df – degrees of freedom.

Overexpression of hsa_circ_0008433 significantly upregulated its expression levels and enhanced cell viability, indicating a promote effect on cell proliferation (Fig. 2, Tables 3–5 and Supplementary Tables 2–4). Conversely, knocking down hsa_circ_0008433 reduced its expression levels and decreased cell viability, underscoring its essential role in cell survival. Wound healing assays further demonstrated that hsa_circ_0008433 overexpression significantly enhanced HBVSMCs' migratory capacity, crucial for vascular repair, while knockdown reduced migration rates. Overall, these findings indicate that hsa_circ_0008433 promotes HBVSMC proliferation and migration, highlighting its potential importance in vascular repair and remodeling mechanisms.

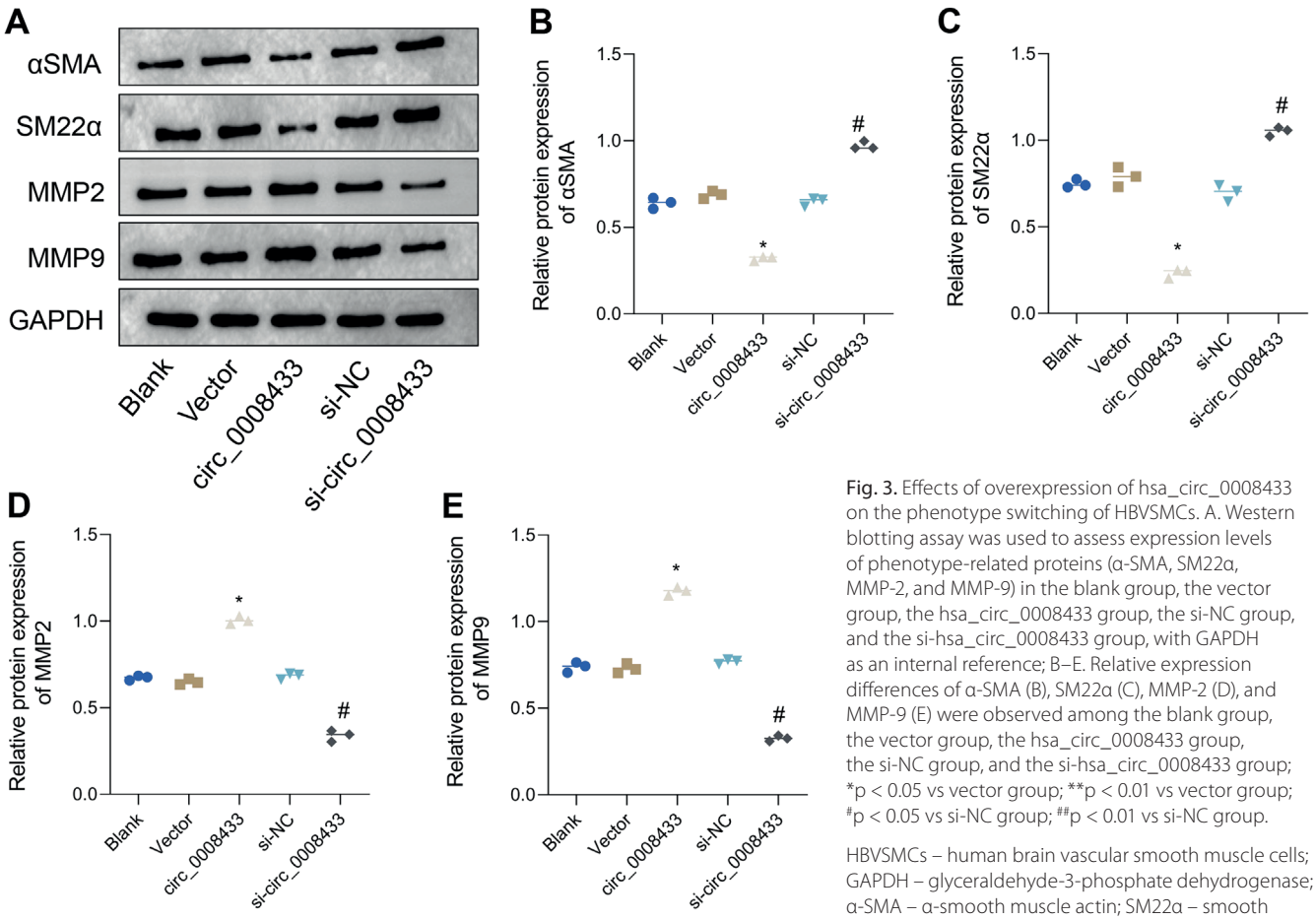

Fig. 3. Effects of overexpression of hsa_circ_0008433 on the phenotype switching of HBVSMCs. A. Western blotting assay was used to assess expression levels of phenotype-related proteins (α-SMA, SM22α, MMP-2, and MMP-9) in the blank group, the vector group, the hsa_circ_0008433 group, the si-NC group, and the si-hsa_circ_0008433 group, with GAPDH as an internal reference; B–E. Relative expression differences of α-SMA (B), SM22α (C), MMP-2 (D), and MMP-9 (E) were observed among the blank group, the vector group, the hsa_circ_0008433 group, the si-NC group, and the si-hsa_circ_0008433 group; *p < 0.05 vs vector group; **p < 0.01 vs vector group; #p < 0.05 vs si-NC group; ##p < 0.01 vs si-NC group.

HBVSMCs – human brain vascular smooth muscle cells; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; α-SMA – α-smooth muscle actin; SM22α – smooth muscle protein 22-α; MMP – matrix metalloproteinase.

Table 6. Comparison of the relative protein expression levels of αSMA among groups using the Kruskal–Wallis test (Fig. 3B)

Group	Me (min–max)	H	df	p-value
Blank	0.64 (0.61–0.67)	12.40	3	0.015
Vector	3, 0.69 (0.67–0.71)			
Circ_0008433	3, 0.33 (0.31–0.33)			
si_NC	3, 0.66 (0.62–0.66)			
si_circ_0008433	3, 0.96 (0.96–1.00)			

n – number of observations; Me (min–max) – median (minimum–maximum); H – the value of the Kruskal–Wallis test statistic; df – degrees of freedom.

Overexpression of hsa_circ_0008433 promotes phenotype switching of HBVSMCs

To investigate the effect of hsa_circ_0008433 on the phenotype switching of HBVSMCs, we assessed the expression levels of key proteins associated with this process. There were no significant differences in α-SMA, SM22α, MMP-2, and MMP-9 expression levels among the blank, vector and si-NC groups. However, the overexpression of hsa_circ_0008433 led to a significant decrease in α-SMA and SM22α levels and an increase in MMP-2 and MMP-9 levels, as compared to the vector group (Fig. 3A–C, Table 6 and Supplementary Tables 5–7). Conversely, knockdown of hsa_circ_0008433 resulted in increased α-SMA and SM22α levels and decreased MMP-2 and MMP-9 levels, compared to the si-NC group (Fig. 3A,D,E and Supplementary Tables 8–11). These findings suggest that hsa_circ_0008433 overexpression promotes phenotype switching in HBVSMCs, favoring a shift towards a pro-inflammatory state.

Discussion

This study provides a comprehensive investigation into the role of hsa_circ_0008433 in IA pathogenesis, a condition characterized by high morbidity and mortality due to its potential for rupture, which can lead to life-threatening subarachnoid hemorrhage.[39,40] Prior high-throughput sequencing indicated that hsa_circ_0008433 is significantly upregulated in IA tissues, especially in ruptured cases, suggesting its involvement in IA pathology.[27] Our study builds on these findings by verifying the overexpression of hsa_circ_0008433 through RT-qPCR, particularly

in aneurysm wall tissues, confirming its close association with IA. Through in vitro experiments, we reveal the functional role of hsa_circ_0008433 in promoting HBVSMC proliferation, migration and phenotype switching, highlighting its potential as a key factor in IA development and instability. There is an increasing body of literature indicating that various circRNAs are involved in regulating HBVSMCs in IA and other cerebrovascular diseases,[41] underscoring the relevance of our findings. For example, hsa_circ_002039 has been shown to be downregulated in IA patient tissues and to promote VSMC proliferation.[42,43] Similarly, hsa_circ_0021001 is regarded as a potential biomarker for IA, with low expression levels correlating with poorer clinical outcomes.[44] Another circRNA, hsa_circ_0031608, is highly expressed in proinflammatory VSMCs and aortic dissection, where it promotes HBVSMC migration and proliferation, contributing to vascular remodeling.[45] However, these studies often do not delve into the precise mechanisms of action for these circRNAs. By contrast, our study provides a systematic analysis of hsa_circ_0008433, demonstrating not only its expression in IA tissues but also its functional impact on HBVSMCs, thus shedding light on its potential mechanisms of action.

Our in vitro experiments show that knocking down hsa_circ_0008433 significantly reduces HBVSMC proliferation and migration, emphasizing its essential role in maintaining these functions under pathological conditions. Previous studies have highlighted the importance of VSMC phenotype switching in the formation and rupture of IAs.[46] Under normal physiological conditions, VSMCs adopt a contractile phenotype that supports vascular stability. However, in response to pathological stimuli, VSMCs can shift to a pro-inflammatory or matrix-remodeling phenotype, characterized by an upregulation of MMPs (MMP-2 and MMP-9) and a downregulation of contractile proteins such as α-SMA and SM22α.[15,47–51] This switch is associated with increased proliferative and migratory capacities, which are more commonly observed in ruptured IA tissues than in unruptured ones,[51,52] suggesting a link to aneurysm wall remodeling and rupture. Our findings indicate that hsa_circ_0008433 may be instrumental in facilitating this phenotype switching. Specifically, silencing hsa_circ_0008433 led to reduced MMP-2 and MMP-9 expression and increased α-SMA and SM22α levels, suggesting that hsa_circ_0008433 drives HBVSMCs towards a pro-inflammatory phenotype that compromises aneurysm wall stability.

These findings imply that hsa_circ_0008433 plays a modulatory role in vascular cell behavior within the aneurysmal environment, providing new insights into IA pathophysiology. Understanding hsa_circ_0008433's influence on HBVSMCs facilitates novel therapeutic strategies. If hsa_circ_0008433 indeed promotes HBVSMC proliferation, migration and phenotype switching, then targeting this circRNA could help stabilize aneurysmal walls and potentially prevent rupture. Additionally, given its upregulation in IA tissues, hsa_circ_0008433 may serve as a diagnostic or prognostic biomarker, aiding in early detection and risk assessment in high-risk IA patients.

Looking ahead, future research should focus on several key areas. First, validating these findings in vivo would provide a clearer picture of the role of hsa_circ_0008433 in IA stability and rupture risk. Developing animal models that can manipulate hsa_circ_0008433 levels would help determine whether modifying its expression could reduce aneurysm rupture risk or prevent aneurysm formation altogether. Second, a deeper exploration of the molecular pathways associated with hsa_circ_0008433's effects on HBVSMC behavior is necessary. By investigating its interactions with pathways such as mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transforming growth factor beta (TGF-β), we could better understand how this circRNA influences HBVSMC function and IA pathophysiology. Third, hsa_circ_0008433's potential as a therapeutic target warrants further exploration. If effective delivery systems can be developed to modulate hsa_circ_0008433 levels or inhibit its function in HBVSMCs, it may be possible to stabilize aneurysmal walls in high-risk IA patients, offering a non-surgical alternative for those unable to undergo traditional interventions.

This study underscores the importance of translational research by linking hsa_circ_0008433 mechanisms in HBVSMCs to potential clinical applications, thereby accelerating lab-to-patient translation.[53] Additionally, exploring neural mechanisms in IAs may clarify cognitive impairments in patients, as studies indicate reduced local gyrification, cognitive deficits and white matter lesions in unruptured IA cases.[54–56] Integrating neuroimaging data with circRNA research could deepen our understanding of IA's impact on brain function.

Limitations

While our findings are promising, this study has several limitations. First, the relatively small sample size of clinical IA tissues may limit the generalizability of our results. Larger studies across multiple centers are necessary to confirm these findings and to validate the robustness of hsa_circ_0008433 as a biomarker.[16] Second, this research is limited to in vitro experiments, which, while valuable for understanding molecular mechanisms, do not fully capture the complex in vivo environment of the human brain. The pathology of IA in living organisms involves a multitude of factors – including hemodynamic forces, inflammatory mediators and immune cells – that are absent in cell culture models. Therefore, animal models or clinical studies would be essential for confirming the pathophysiological relevance of hsa_circ_0008433 in IA.[17] Third, this study focuses exclusively on a single circRNA, hsa_circ_0008433, without considering potential

interactions with other circRNAs that may contribute to IA pathogenesis. Future studies should adopt a more comprehensive approach, examining multiple circRNAs to develop a fuller understanding of the regulatory networks at play in IA.[18,19] Other confounding factors may also influence hsa_circ_0008433 expression and IA risk, such as patient age, gender and pre-existing conditions like hypertension or diabetes. Future research should consider these variables in study design and analysis to better understand how they may modulate circRNA expression and impact IA pathology. Additionally, while this study demonstrated the impact of hsa_circ_0008433 on MMP-2, MMP-9, α-SMA, and SM22α levels, the underlying molecular pathways remain unclear. It is essential to explore potential interactions with major signaling pathways, such as MAPK, NF-κB and TGF-β, which are known to play roles in vascular inflammation and remodeling.[57–59] Understanding these interactions could reveal the precise mechanisms by which hsa_circ_0008433 regulates HBVSMC phenotype and IA formation.

Conclusions

Overall, our study demonstrates that hsa_circ_0008433 plays a crucial role in the proliferation and migration of VSMCs in IA patients and may be involved in the phenotype modulation of HBVSMCs. These findings suggest that hsa_circ_0008433 is associated with the pathological processes of IAs. The upregulation of hsa_circ_0008433 in IA tissues indicates its potential as a biomarker for the diagnosis and prognosis of IAs. Clinically, targeting hsa_circ_0008433 could lead to novel therapeutic strategies to prevent the progression or rupture of IAs. Future research should aim to elucidate the detailed molecular mechanisms by which hsa_circ_0008433 regulates HBVSMCs behavior, confirm these findings in vivo, and explore the broader network of circRNAs involved in cerebrovascular diseases. These efforts will contribute to a more comprehensive understanding of IA pathophysiology and support the development of targeted therapies to improve patient outcomes.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.14997708. The package includes the following files:

Supplementary Table 1. Checking the assumption of normality (using the Shapiro–Wilk test).

Supplementary Table 2. Dunn's post hoc test results for group comparisons of circ_0008433 expression in Fig. 2A.

Supplementary Table 3 Dunn's post hoc comparison of cell viability between groups in Fig. 2B.

Supplementary Table 4. Dunn's post hoc test results, pairwise comparison of migration capacity between groups in Fig. 2D.

Supplementary Table 5. Dunn's post hoc test results, pairwise comparison of α-SMA protein expression levels between groups in Fig. 3B.

Supplementary Table 6. In the analysis comparing the relative expression levels of SM22α protein among groups (Fig. 3C), the Kruskal–Wallis test was used to evaluate the differences between the groups. The results showed an H value of 12.23 and a p-value of 0.016, indicating significant differences between the groups.

Supplementary Table 7. Dunn's post hoc test results, pairwise comparison of SM22α protein expression levels between groups in Fig. 3C.

Supplementary Table 8. In the analysis comparing the relative expression levels of MMP-2 protein among groups (Fig. 3D), the study used the Kruskal–Wallis test to evaluate the differences between the groups.

Supplementary Table 9. Dunn's post hoc test results, pairwise comparison of MMP-2 protein expression levels between groups in Fig. 3D.

Supplementary Table 10. In the analysis comparing the relative expression levels of MMP-9 protein among groups (Fig. 3E), the Kruskal–Wallis test was used to evaluate the differences between the groups.

Supplementary Table 11. Dunn's post hoc test results, pairwise comparison of MMP-9 protein expression levels between groups in Fig. 3E.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Enhancing Castor stent graft placement: A novel approach using direction-turnover and unwrapping techniques

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Conflict of interest
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Abstract

Background. Patients with aortic arch lesions involving the left subclavian artery (LSA) usually require endovascular surgery. The Castor single-branch stent graft provides a targeted solution for such cases, with potential benefits enhanced by the direction-turnover and unwrapping techniques.

Objectives. To evaluate the safety, efficacy and short-term outcomes of the direction-turnover joint unwrapping techniques combined with a Castor single-branch stent graft in treating aortic arch lesions involving the LSA.

Materials and methods. From January 2022 to June 2023, 18 patients with aortic arch and LSA lesions underwent Castor stent graft placement at Binzhou Medical University Hospital (Shangdong, China). Preoperative and postoperative imaging with computed tomography angiography (CTA) was used to assess stent placement and efficacy at 1, 3, 6, and 12 months post-surgery.

Results. All 18 patients successfully received the stent graft without intraoperative complications, stent migration or residual shunting. Postoperative imaging confirmed accurate stent placement with no evidence of thrombosis or endoleaks.

Conclusions. The direction-turnover and unwrapping techniques improve the accuracy and safety of Castor stent graft placement, effectively reducing guidewire entanglement and enhancing procedural success. These techniques show promise for broader application in complex aortic arch interventions.

Key words: Castor stent graft, direction-turnover, unwrapping, aortic arch lesions, left subclavian art

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Highlights

- This study demonstrates the effectiveness of the Castor single-branch stent graft for treating aortic arch lesions involving the left subclavian artery.
- Direction-turnover and unwrapping techniques significantly improve stent graft placement precision and reduce guidewire entanglement.
- The procedure shows promising safety and efficacy with no complications such as stent migration or endoleaks.
- Postoperative imaging confirms long-term stability of stent placement with minimal complications over a 12-month follow-up.
- These techniques offer a potential improvement in clinical outcomes for complex aortic arch interventions.

Background

Aortic arch diseases, which include aortic dissection, intramural hematoma, penetrating aortic ulcer, traumatic aortic disconnection, aneurysm, and aortic coarctation, pose significant health risks. The global incidence of aortic dissection alone is estimated at 2.8–6.0 cases per 100,000 individuals per year, with mortality rates reaching 50% within 72 h if untreated.[1,2] Similarly, aortic aneurysms have an annual incidence of approx. 5.9 per 100,000, with rupture cases demonstrating mortality rates as high as 90%.[3] These diseases, particularly acute aortic syndromes such as aortic dissection and aneurysm rupture, are characterized by rapid onset, poor prognosis and high mortality, underscoring the need for prompt and effective treatment.

Traditionally, these conditions are treated with open thoracotomy and artificial vascular replacement of the aortic arch, procedures that require general anesthesia and hypothermic cardiopulmonary bypass. However, this approach has significant limitations; it is not suitable for elderly patients or those with underlying health conditions, as hypothermic cardiopulmonary bypass can lead to ischemia/reperfusion (I/R) injuries in the heart and brain. In 1994, Dake et al.[4] introduced thoracic endovascular aortic repair (TEVAR) as a less invasive alternative to open thoracic aortic surgery, utilizing catheter-based techniques within the aortic lumen under imaging guidance.[5] Compared to open and hybrid surgeries, TEVAR offers advantages of reduced trauma, lower risk of complications, better aesthetics outcomes, and favorable prognosis.

However, single-graft TEVAR alone cannot adequately address complex aortic arch diseases, particularly when branch artery reconstruction is necessary.[6] To address this, new methods have emerged, including the chimney,[7] in situ fenestration,[8] in-situ fenestration,[9] and branched stent techniques.[10] Each approach offers unique benefits but also presents distinct limitations. The chimney technique, for instance, involves placing a secondary "chimney" stent alongside the primary graft to maintain blood flow to vital arteries. However, this approach is prone to type I endoleak, as the primary stent graft often has incomplete adhesion to both the chimney graft and the aortic wall.[11] The in situ fenestration technique enables direct creation of fenestrations in the graft within the patient's body but poses a high risk of vascular damage from the needle or laser used.[12] Finally, while the branched stent technique reduces the risk of endoleak, its technical complexity can limit its use.[13] Despite improvements in design and deployment methods, these approaches still face limitations in widespread clinical adoption.

The Castor single-branch stent (Fig. 1) was developed to provide a solution tailored to complex aortic arch pathologies. Jointly developed by Shanghai Changhai Hospital and Shanghai Endovastec Company, the Castor stent received regulatory approval in 2017[14] and has been used in over 5,000 cases as of June 2021.[15] Its single-branch design facilitates the reconstruction of left subclavian artery (LSA) blood flow, improving stent anchoring and stability. Additionally, the proximal "fenestration" enables blood flow to the left common carotid and infracarotid arteries, allowing partial reconstruction of aortic arch branches.

Fig. 1. Castor stent graft

These features make the Castor stent a promising approach in managing aortic arch diseases that require both stability and preservation of branch artery blood flow.[16]

In this study, we further enhanced the application of the Castor stent by introducing direction-turnover and unwrapping techniques. These methods were developed specifically to address the challenges of graft positioning and operational complexity in complex aortic arch reconstructions. The direction-turnover technique utilizes spatial inversion to ensure accurate alignment of the stent fenestration with the aortic arch branches, improving placement precision. Meanwhile, the unwrapping technique mitigates the risk of guidewire entanglement, facilitating a smoother and safer procedure. Together, these techniques enhance procedural efficiency and safety, making the Castor stent a more effective solution for complex aortic arch repairs.

Objectives

The aim of the study was to evaluate the safety, efficacy and prognosis of using the directional turning and unwrapping techniques with the Castor single-branch stent graft in the treatment of lesions of the aortic arch and left subclavian artery based on clinical data and follow-up results from 18 patients.

Materials and methods

Participants

A total of 18 patients with aortic arch disease involving the LSA were successfully treated with a Castor single-branch stent graft in Binzhou Medical University Hospital (Shangdong, China). This cohort included 17 men (94.4%) and 1 woman (5.6%), with an average age of 58.72 ±11.28 years. The demographic and clinical data are presented in Tables 1–3. Written informed consent was obtained from the participants prior to the enrollment of this study.

Inclusion criteria: 1. Patients presented with clinical symptoms of aortic arch disease, such as chest pain and tightness. 2. Aortic arch disease was confirmed using computed tomography angiography (CTA) (Fig. 2). 3. Patients met the Castor stent graft indications: presence of aortic arch disease with proximal lesions situated 15 mm distally

Table 1. Summary of clinical data of 18 patients

Patient No.	Sex	Age [years]	Height [cm]	Weight [kg]	Disease	Operation time [min]	Intraoperative blood loss [mL]	Total time in hospital [days]	Time in hospital after surgery [days]	Length of follow-up [months]
1	male	47	167	82	AD (Stanford type B)	130	20	11	9	18
2	female	67	160	60	AD (Stanford type B)	135	10	10	7	17
3	male	52	175	68	AD (Stanford type B)	140	20	9	6	16
4	male	40	170	80	AD (Stanford type B)	120	20	9	6	15
5	male	56	170	80	aortic intramural hematoma	100	50	12	6	9
6	male	80	170	60	penetrating ulcer of the thoracic aorta with intramural hematoma	120	50	12	6	7
7	male	49	175	90	AD (Stanford type B)	115	50	8	7	7
8	male	69	174	85	Penetrating ulcer of the aortic arch	140	50	10	6	5
9	male	60	167	65	aortic intramural hematoma	160	50	11	5	1
10	male	56	170	80	aortic intramural hematoma	140	20	13	4	4
11	male	72	170	75	AD (Stanford type B)	120	50	9	5	9
12	male	42	170	105	AD (Stanford type B)	120	50	11	8	11
13	male	67	180	70	Penetrating ulcer of the aortic arch	125	50	12	5	5
14	male	69	171	75	AD (Stanford type B)	150	50	10	9	6
15	male	48	175	87	AD (Stanford type B)	180	20	10	6	4
16	male	54	170	105	AD (Stanford type B)	165	50	8	6	2
17	male	59	178	75	AD (Stanford type B)	110	50	13	7	3
18	male	70	184	81	aortic arch aneurysm	125	20	17	5	1

AD – aortic dissection.

Table 2. Supplemental clinical data of 18 patients

Patient No.	Hypertension level	Diabetes	Other complications	D1 [mm]	D2 [mm]	D3 [mm]	L1 [mm]	L2 [mm]	L3 [mm]
1	3	no	no	36	30	12	200	25	10
2	3	no	no	36	30	10	200	25	5
3	2	no	postoperative fracture of the left shoulder and fracture of the left ankle	36	30	12	200	30	10
4	3	no	no	30	24	10	200	25	5
5	3	no	no	32	26	12	200	25	5
6	3	no	old cerebral infarction	30	24	10	200	25	10
7	3	no	no	36	30	12	200	25	15
8	2	no	no	34	28	10	200	25	15
9	3	no	stenosis of the inferior mesenteric artery	36	30	10	200	25	10
10	3	no	after Bentall operation	36	30	12	200	25	10
11	2	no	no	36	30	12	200	25	10
12	2	no	no	36	30	12	200	25	15
13	3	yes	superior mesenteric artery stenosis, left renal artery stenosis, cerebral infarction, cerebral atrophy, hyperlipidemia	34	28	10	200	30	5
14	3	yes	coronary heart disease	38	32	10	200	25	5
15	3	no	after Bentall operation	30	24	10	200	25	5
16	3	yes	hyperlipidemia and fatty liver	36	30	12	200	25	10
17	3	no	no	36	30	12	200	25	10
18	3	yes	lacunar cerebral infarction, coronary heart disease, cervical spine degeneration, emphysema, left pulmonary nodule, fatty liver	34	28	12	200	25	5

D1 – Castor stent proximal diameter; D2 – distal diameter of the Castor stent body; D3 – distal diameter of the Castor stent body; L1 – length of the Castor stent body; L2 – length of the collateral branch; L3 – backward displacement of the collateral branch.

Table 3. Summary of demographic, clinical and surgical data of patients undergoing Castor stent graft procedure for aortic arch disease.

Variable	Mean ± SD/median (Q1–Q3)	Count [%]
Sex (male)	–	17 (94.4%)
Age [years]	58.72 ±11.28	–
Height [cm]	170.0 (170.0–175.0)	–
Weight [kg]	79.06 ±12.73	–
Disease type	–	–
AD (Stanford type B)	–	11 (61.1%)
Aortic intramural hematoma	–	3 (16.7%)
Penetrating ulcer	–	3 (16.7%)
Aortic arch aneurysm	–	1 (5.6%)
Operation time [min]	133.06 ±20.52	–
Intraoperative blood loss [mL]	50.0 (20.0–50.0)	–
Total time in hospital [days]	10.83 ±2.18	–
Time in hospital after surgery [days]	6.0 (5.0–7.0)	–
Follow-up time [months]	7.78 ±5.53	–

Continuous variables are presented as mean ± standard deviation (±SD) or median (Q1–Q3); Q1 represents the 1st quartile, and Q3 represents the 3rd quartile; AD – aortic dissection.

to the left common carotid artery (LCCA) and 20 mm distally to the LSA, with extension into the LSA and a proximal landing zone of at least 15 mm.

Exclusion criteria: 1) patients with severe comorbidities that posed significant surgical risks, including advanced heart failure, significant renal insufficiency or uncontrolled coagulopathies; 2) patients with a life expectancy of less than 6 months due to other critical illnesses; 3) patients with anatomical conditions incompatible with the Castor stent graft, such as extremely large or small vessel diameters that could not accommodate the available stent sizes; 4) patients with a history of previous aortic arch surgeries that could complicate the placement and anchoring of the stent.

Although patients with different types of aortic arch diseases were included, strict inclusion and exclusion criteria ensured a relatively uniform cohort suitable for the Castor stent graft procedure. Consequently, the surgical technique was consistent across cases, with minor adjustments made only for specific anatomical complexities to optimize outcomes.

Surgical methods

Preoperative preparation and anesthesia: After induction of anesthesia using a combination of intravenous and

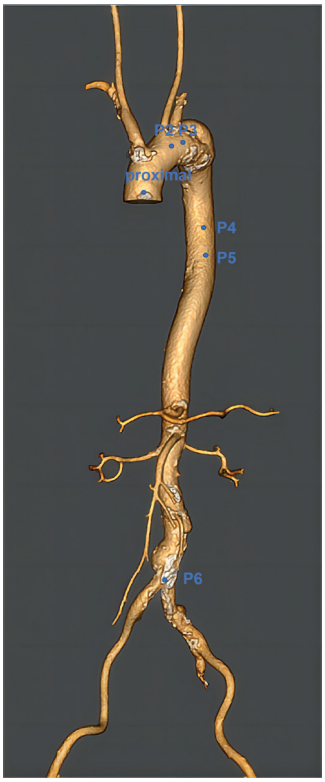

Fig. 2. Preoperative computed tomography angiography (CTA) reconstruction image of one patient

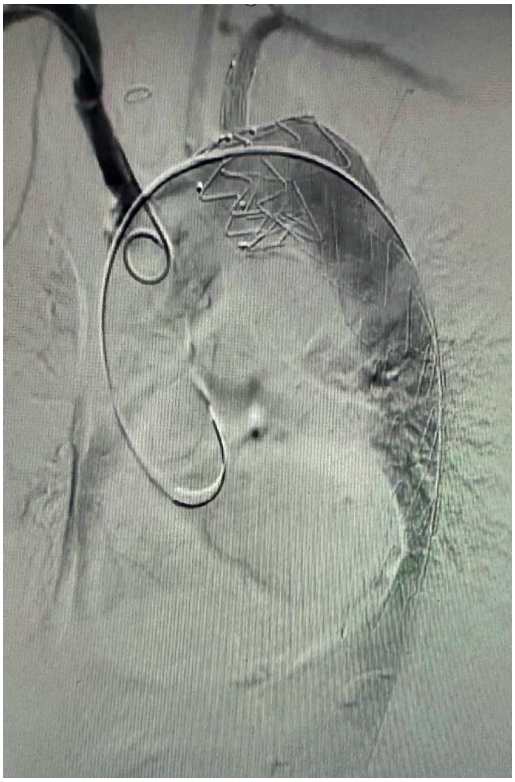

Fig. 3. Intraoperative angiography of Castor stent release

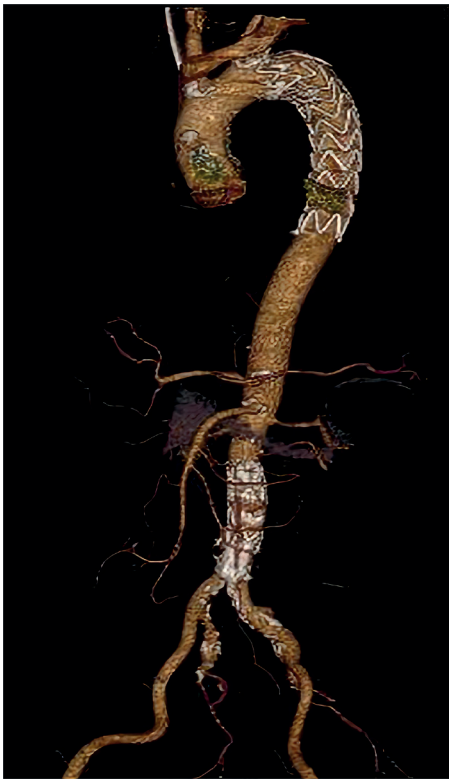

Fig. 4. Postoperative computed tomography angiography (CTA) reconstruction image

inhalation agent, patients were placed in the supine position, disinfected and administered heparin at a dose of 1.0 mg/kg.

Catheter insertion and access: A 7F sheath was inserted through the left brachial artery, and a 5F sheath was inserted via the right brachial artery. An angiography catheter was then introduced through the right brachial artery into the ascending aorta to perform aortography and confirm the precise location of the lesions.

Stent selection criteria: Stent selection was based on preoperative CTA measurements, considering anatomical characteristics such as the proximal and distal landing zones and the orientation of the LSA. The Castor stent model was selected based on the diameter and length required to cover the lesion while ensuring secure anchoring in the landing zones. Measurements were also confirmed intraoperatively using angiography to ensure accurate stent sizing.

Guidewire and stent placement: After establishing vascular access from the left brachial artery to the right femoral artery, a transverse incision was made in the right inguinal region, and the femoral artery was dissected. The selected Castor stent graft was then inserted through the right femoral artery, with placement guided by real-time imaging. The direction-turnover technique was utilized to ensure that the stent fenestration aligned precisely with the aortic arch branches, with the stent automatically flipping to the correct position upon advancement. Re-angiography confirmed the positioning of the stent, showing unobstructed branch arteries and complete lesion coverage (Fig. 3).

Unwrapping technique for guidewire management: To avoid guidewire entanglement, the unwrapping technique was applied when necessary. This involved retracting the stiff guidewire into the stent sheath to reduce tension and then gently guiding the branch wire to the LSA to maintain optimal placement. This step was crucial to ensure proper orientation and secure stent deployment.

Postoperative procedure: After confirming the stent position, all guidewires and catheters were removed, and the right inguinal incision was sutured layer by layer. Postoperatively, aspirin (100 mg) was administered to each patient to reduce the risk of thrombosis.

Equipment and consumables

The procedures used a 7F catheter for brachial artery access, 5F sheaths, and specialized angiography catheters. The Castor stent models were chosen based on individual patient anatomy. Additionally, contrast agents were employed during angiography to facilitate lesion visualization and stent placement.

Variables

The primary outcomes measured included: Operation time: Defined as the time from brachial artery puncture to the removal of all guidewires and catheters. Blood loss: Amount of blood lost during surgery. Complications:

Including residual shunt, arrhythmia, hemolysis, embolism in the aortic arch, stent migration or detachment, and pericardial effusion. Postoperative CTA evaluation: Postoperative CTA was used to assess the morphology and stability of the stent and confirm the absence of endoleaks, displacement or other complications (Fig. 4).

Statistical analyses

Data were collected from surgical records, patient medical records and imaging studies (CTA), with standardized measurement methods applied to ensure accuracy and comparability across all recorded outcomes. Additionally, descriptive statistics were used to summarize patient characteristics and procedural outcomes. Continuous variables were presented as mean ± standard deviation (SD) if normally distributed, or as median with 1st and 3rd quartiles (Q1–Q3) if non-normally distributed. Categorical variables were presented as counts and percentages. No inferential statistical tests were conducted, given the study's preliminary nature and single-arm design.

Results

To evaluate the success of the Castor stent graft placement, we assessed intraoperative and postoperative outcomes related to stent positioning, hemodynamic stability and complication rates.

Intraoperative outcomes

Computer blood tube imaging confirmed that all stents were accurately positioned and completely covered the targeted lesions, with no significant deviation or endoleak observed. Additionally, all 3 branches of the aortic arch remained patent, and no cases required conversion to open thoracotomy. The average duration of surgery was 133.06 ±20.52 min. Intraoperative blood loss had a median of 50.0 mL (Q1–Q3: 20.0–50.0 mL), and no blood transfusions were required. Oxygen saturation remained stable between 95% and 100%, with controlled blood pressure showing no significant fluctuations. Various delivery sheath diameters, ranging from 6F to 10F, were utilized based on the anatomical requirements. Table 2 details the specific Castor stent models used. These findings indicated that the procedure was performed with minimal blood loss and stable patient vital signs, supporting the safety and effectiveness of the surgical approach.

Patient characteristics and hospital stay

The cohort consisted of 18 patients with diverse aortic arch pathologies: 11 patients (61.1%) were diagnosed with Stanford type B dissection, 3 (16.7%) with intramural hematoma, 2 (11.1%) with penetrating ulcers, 1 (5.6%) with a combination of penetrating ulcers and intramural hematoma, and 1 (5.6%) with an aortic arch aneurysm. The mean total hospital stay was 10.83 ±2.18 days, and the time in hospital post-surgery was 6.0 days (Q1–Q3: 5.0–7.0). These results demonstrated a manageable hospital recovery time, indicating that patients tolerated the procedure well and were able to be discharged in a reasonable timeframe.

Follow-up outcomes

At an average follow-up of 7.78 ±5.53 months, CTA re-examinations demonstrated that all Castor stent grafts remained securely positioned, with no evidence of displacement, detachment or occlusion. The lesions continued to be adequately covered, and there was no development of new lesions, endoleaks, or postoperative complications such as infective endocarditis. Notably, all patients reported that chest pain and tightness had either resolved or improved significantly, highlighting the procedure's effectiveness in symptom relief and lesion management.

In summary, the intraoperative and follow-up results underscore the precision and stability of the Castor stent placement, with minimal complications, effective lesion coverage, and promising short-term outcomes for symptom relief and patient recovery.

Discussion

Advantages of Castor single-branch bracket

The Castor single-branch stent was specifically designed to maintain LSA blood flow during endovascular repair of aortic arch diseases, such as type B dissection. Preservation of LSA flow is critical as it reduces the risk of perioperative complications, including cerebral infarction and spinal cord ischemic paraplegia.[17]

The Castor stent offers unique structural benefits compared to traditional straight stents. Its single-branch design, anchored by radial support, provides stability almost perpendicular to aortic blood flow, while simultaneously benefiting from the anchoring force generated by blood flow and blood pressure in the LSA branch. This dual anchoring mechanism enhances stent stability and minimizes migration risks.[18] Moreover, the size and positioning of the Castor stent are customized based on preoperative CTA and intraoperative angiographic measurements.[14] This approach enables precise deployment, making the Castor stent an ideal solution for patients with complex aortic arch lesions who are unsuitable for open surgery.

Application and impact of innovative technologies

To optimize the placement of the Castor stent, we employed 2 innovative techniques: the direction-turnover

technique and the unwrapping technique. The direction-turnover technique, proposed by Professor Qingsheng Lu from Changhai Hospital, leverages spatial inversion to control the stent's fenestration orientation during deployment.[19] As depicted in Fig. 5, the surgeon holds the anterior grip at the 6 o'clock position without rotation, ensuring that the white marker aligns perpendicularly to the ground (Fig. 5A). This alignment ensures that the fenestration remains correctly positioned as the stent advances through the aortic arch (Fig. 5B). Upon reaching the ascending aorta, the fenestration automatically flips to the 12 o'clock position, aligning with the branches of the aortic arch (Fig. 5C). This transformation simplifies the complex 3-dimensional alignment challenge, enabling a more intuitive and accurate stent placement.

The unwrapping technique, used to address guidewire entanglement, involves retracting the stiff guidewire into the stent sheath while maintaining gentle tension on the branch guidewire (Fig. 6). This method allows the branch stent to enter the LSA unobstructed, thus preventing potential cerebral ischemia and reducing the risk of deployment issues. These complementary techniques reduce procedural complexity, improve safety and enhance the reliability of Castor stent implantation, thereby making the procedure more accessible to surgeons across a range of experience levels.

Postoperative outcomes and complications

The short-term outcomes observed in this study demonstrate the effectiveness and safety of the combined approach. All 18 patients successfully underwent Castor stent implantation without the need for conversion to open surgery. Over a follow-up period of 7.78 ±5.53 months, CTA imaging confirmed stable stent positioning with no migration, endoleaks or significant complications. Patients also reported relief from chest pain and tightness, with

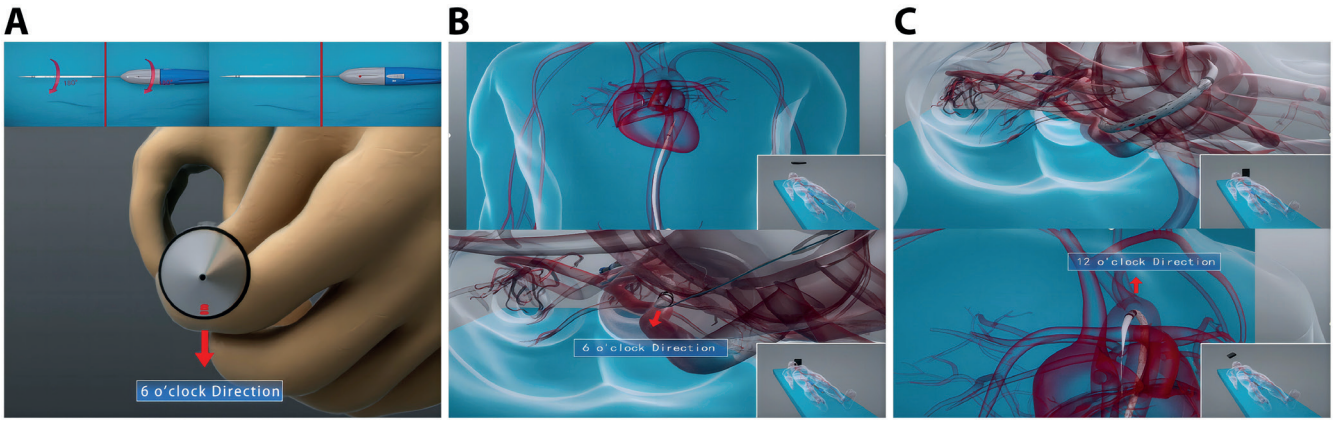

Fig. 5. Schematic diagram of the direction-turnover technique. A. Initial setup of the stent delivery system, with the anterior grip firmly held to maintain a 6 o'clock direction for accurate positioning; B. Advancing the stent through the aortic arch while maintaining the 6 o'clock orientation to ensure proper alignment; C. Upon reaching the desired position, the stent fenestration automatically flips to the 12 o'clock direction, aligning with the aortic arch branches and facilitating precise deployment

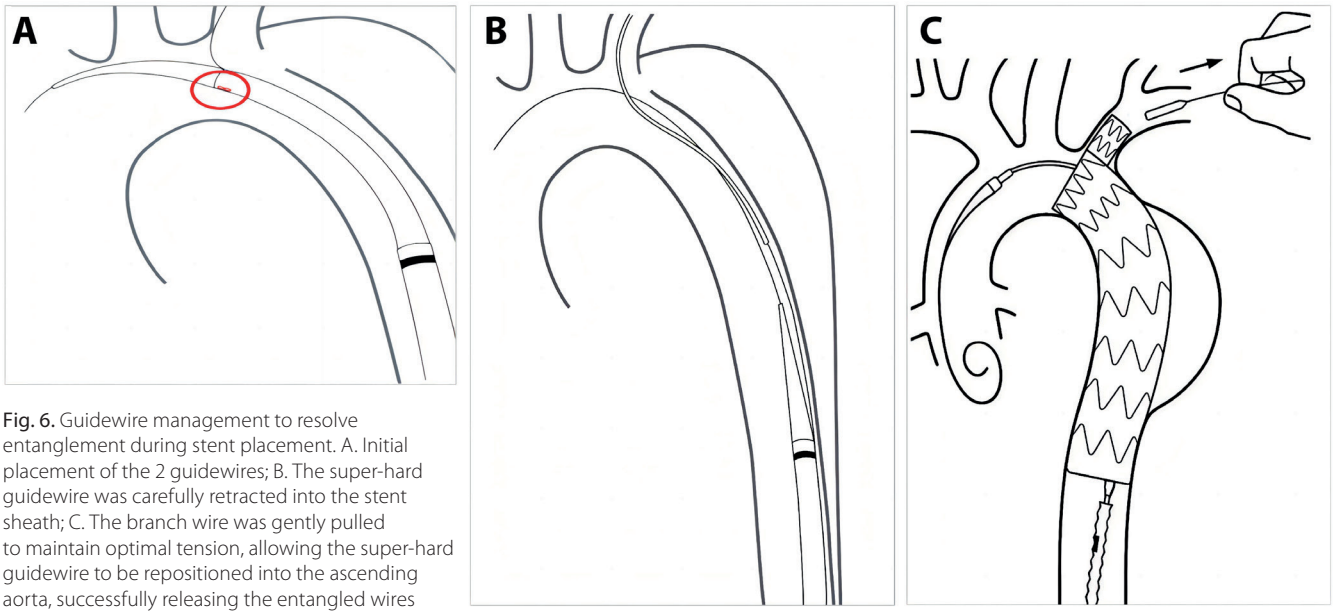

Fig. 6. Guidewire management to resolve entanglement during stent placement. A. Initial placement of the 2 guidewires; B. The super-hard guidewire was carefully retracted into the stent sheath; C. The branch wire was gently pulled to maintain optimal tension, allowing the super-hard guidewire to be repositioned into the ascending aorta, successfully releasing the entangled wires

a mean hospital stay of 10.83 ±2.18 days and a discharge period averaging 6.28 ±1.36 days, suggesting a favorable recovery profile.

Compared to recent studies, the Castor stent approach demonstrates potential advantages. For instance, a study by Shu et al.[20] reported an 11% incidence of type I endoleaks using the chimney technique, whereas no endoleaks were observed in our study. These findings suggest that the Castor stent approach may provide a safer and more efficient alternative for complex aortic arch repairs; however, larger, long-term studies are needed to validate these results.

Noteworthy, factors such as patient age, disease severity and anatomical complexity may influence surgical outcomes, with older or high-risk patients potentially facing different recovery trajectories and higher risks of complications, such as postoperative cerebral infarction or spinal cord ischemia. Future studies should further investigate these variables to refine patient selection and develop effective prevention strategies for such complications. Moreover, as this technique becomes more widely adopted, challenges related to procedural complexity and operator training will need to be carefully addressed. Successful implementation requires expertise in spatial orientation and guidewire manipulation, underscoring the need for comprehensive training and advanced imaging. Addressing these factors will support broader clinical application, especially in high-risk cases.

Limitation of the study and future research directions

While this study provides promising preliminary data, it has several limitations. First, as a single-arm study without a control group, we were unable to perform comparative analyses or assess the relative efficacy of the direction-turnover and unwrapping techniques against alternative methods like the chimney or fenestration techniques. Future studies with a comparative or controlled design would provide stronger evidence for the efficacy of these techniques.

Second, the small sample size (18 patients) limits the generalizability of our findings. Although no major adverse events or failure cases were observed in this cohort, the small sample may not capture the full spectrum of potential complications, especially in more diverse patient populations. Expanding the sample size in future studies would enhance the external validity and reliability of the results and allow for a more comprehensive assessment of safety.

Additionally, there was a notable sex imbalance in our sample (17 men and 1 woman). This disproportion reflects the higher prevalence of aortic arch disease among men, as reported in previous studies.[21] However, it may limit the applicability of our findings to female patients, who may have different outcomes or complication rates. Future research should aim for a more balanced sample to improve the generalizability of the results across both sexes.

Lastly, the follow-up period was relatively short, averaging 7.78 months, which limits our ability to assess the long-term safety and durability of the Castor stent. Future studies should incorporate a follow-up period of at least 2 years to evaluate the long-term efficacy and potential late complications, including stent displacement, restenosis and other potential adverse events.

By addressing these limitations in future research, we can establish a stronger foundation for the clinical application of the Castor stent with direction-turnover and unwrapping techniques, particularly for high-risk patients who are unsuitable for open surgery.

Conclusions

The Castor single-branch stent represents an effective treatment option for patients with aortic arch disease who are unsuitable for thoracotomy and meet the specific criteria for endovascular repair. The application of the direction-turnover and the unwrapping techniques during stent placement demonstrated the ability to enhance surgical precision, improve the success rate and minimize guidewire entanglement. Short-term outcomes demonstrate safe and reliability, with promising potential for application. Further studies with larger sample sizes and extended follow-up are warranted to confirm these findings and evaluate long-term efficacy.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.15208395. The package includes the following files:

Supplementary Table 1 Analysis of normal distribution for continuous variables.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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An experimental rat model of non-alcoholic fatty liver disease: Ameliorative effect of green coffee and prediction of disease activity

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Abstract

Background. Non-alcoholic fatty liver disease (NAFLD) is currently a public health problem that affects many people worldwide. New-generation dietary agents can contribute to disease control. Also, prediction of liver disease activity with serum markers is an important factor to reduce the need for liver biopsies.

Objectives. The aim of this study was to compare the effects of 20 mg/kg green coffee bean extract (GCBE, standardized to 10 mg/kg chlorogenic acid), 10 mg/kg chlorogenic acid (CA) and 50 mg/kg CA on NAFLD activity score (NAS). We also aimed to create a NAS prediction model as an alternative to liver biopsies.

Materials and methods. Male Wistar rats were fed either a high-fat high-cholesterol (HFHC) diet (NAFLD groups, n = 32) or a normal diet (control groups, n = 24). Green coffee bean extract (20 mg/kg) and CA (10 mg/kg and 50 mg/kg) were given by daily oral gavage. At the end of the study, blood samples and liver tissues were collected. Biochemical parameters, glyoxalase 1 (GLO1) and oxidative stress parameters were analyzed. An experienced histopathologist graded NAS.

Results. Green coffee bean extract and 50 mg/kg CA significantly reduced NAS ($p < 0.01$ and $p < 0.001$, respectively). HFHC-diet induced NAFLD was associated with higher serum GLO1 levels ($p < 0.001$). Serum malondialdehyde (MDA) levels were positively correlated with NAS while GLO1 was negatively correlated with NAS ($p < 0.001$, $r = 0.698$; $p < 0.05$, $r = -0.367$, respectively). Serum MDA and triglyceride were found to be statistically significant in predicting NAS ($p < 0.001$).

Conclusions. Our study suggests that GCBE and CA can both ameliorate the development of NAFLD. Also, low-dose GCBE and high-dose CA showed similar hepatoprotective effects. Increased GLO1 in NAFLD may be a defense mechanism which is enhanced by GCBE and CA. Moreover, serum MDA and triglyceride levels are promising in NAS prediction.

Key words: malondialdehyde, oxidative stress, non-alcoholic fatty liver disease, chlorogenic acid

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Highlights

- The effects of green coffee bean extract (GCBE) and chlorogenic acid (CA) on the non-alcoholic fatty liver disease (NAFLD) population and on healthy individuals are different.
- GCBE and CA increase glyoxalase 1 (GLO1) in NAFLD.
- Increased GLO1 in NAFLD may be a defense mechanism.
- Both 20 mg/kg GCBE and 50 mg/kg CA can ameliorate NAFLD.
- Serum malondialdehyde (MDA) and triglyceride levels are promising in NAFLD activity score (NAS) prediction.

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease with a global prevalence of 25%.[1] This high prevalence rate parallels the increased prevalence of obesity, type 2 diabetes and metabolic syndrome.[2] Thus, current alarming state of NAFLD has raised the interest in hepatoprotective dietary agents for disease prevention.

In order to emphasize the importance of nutrition and metabolic risk factors in NAFLD, the terms "metabolic associated fatty liver disease" or "nutrition associated fatty liver disease" are also recommended instead of the term "non-alcoholic fatty liver disease".[3,4] Nutrition modulates liver lipid metabolism through different epigenetic pathways. A high-fat diet is found to downregulate the expression of silent information regulator 2 homologue 1 protein (SIRT1).[5] This downregulation increases the release of free fatty acids from mesenteric adipose tissue, which results in increased uptake of free fatty acids into the liver.[5]

Dicarbonyl stress is characterized by the excessive accumulation of reactive dicarbonyl metabolites.[6,7] These metabolites can easily interact with cellular proteins and DNA. Methylglyoxal is the most abundant dicarbonyl metabolite in humans.[8] Glyoxalase 1 (GLO1) is the primary enzyme of the dicarbonyl stress pathway and plays an essential role in methylglyoxal detoxification.[8] However, little is known about the role of GLO1 in NAFLD.

Screening patients with diabetes and metabolic syndrome who are at high risk of non-alcoholic steatohepatitis (NASH) is a key factor in NAFLD management. Predicting histological disease activity with serum parameters can decrease the number of liver biopsies. However, reliable serum markers are needed to detect early NASH.

Chlorogenic acid (CA) is the most abundant polyphenolic compound in green coffee beans.[9] The roasting process, which is necessary to produce brown coffee beans, was found to reduce chlorogenic acid content of green coffee beans by up to 95%.[9] Both chlorogenic acid and green coffee bean extract (GCBE) are well documented for their antidiabetic and hypolipidemic properties.[10,11]

However, comparison of the effects of GCBE and CA on NAFLD is crucial to determine which one should be preferred primarily for disease prevention. Due to the lower cost and easier production methods of GCBE, the use of GCBE is more cost-effective than of CA.

Objectives

The aim of our study was to compare the effects of 20 mg/kg GCBE (standardized to 10 mg/kg chlorogenic acid) and 10 mg/kg CA on NAFLD activity score (NAS). In the literature, different doses of CA (ranging from 10 mg/kg to 50 mg/kg) are used in different rat models.[12,13] We also aimed to compare the effects of low-dose (10 mg/kg) and high-dose (50 mg/kg) CA on NAS in NAFLD. Additionally, we developed a regression model for the prediction of NAS with serum parameters to address the need for reliable serum markers to detect early NASH.

Materials and methods

Animals

All animal experiments were performed in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Kobay Technology Co. Ltd. (Ankara, Turkey) provided 56 healthy male Wistar rats (13-week-old, 300–380 g).

Animal maintenance with 4 animals per cage was conducted in Experimental Animals Research Laboratory of Ankara University. Animals were maintained on a 12-h light–dark cycle at room temperature (22–24°C) with free access to food and water. No animals died during experimental protocols. At the end of the study, rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Intracardiac blood was collected under anesthesia prior to the removal of organs and tissues. Animals were sacrificed by cervical dislocation. Blood samples and liver tissues were collected after 12 h of fasting.

The study was approved by the Animal Ethics Committee of Ankara University to ensure ethical and humane treatment of the animals (ethical approval ID: 2019-19-77, issued on October 16, 2019).

Grouping and modeling

Animals were divided into 7 groups (n = 8). Sample size was decided according to previous studies.[14] Simple randomization (table of random numbers) was used for group allocation. Groups 1, 2 and 3 were fed a normal diet

Table 1. Typical sources of nutrients in rodent purified control diet

Nutrients	Typical sources
Protein (23 g per 100 g diet; 20% of total calories)	casein
Fat (4 g per 100 g diet; 10% of total calories)	soybean oil, cocoa butter
Carbohydrate (64 g per 100 g diet; 70% of total calories)	corn starch, maltodextrin, sucrose
Fiber (6 g per 100 g diet)	cellulose
Micronutrients (3 g per 100 g diet)	vitamin and mineral premixes

for 10 weeks. Normal diet was composed of 64 g carbohydrates (70% of total calories), 4 g fat (10 % of total calories) and 23 g protein (20 % of total calories) per 100 g of diet (Table 1). Group 1 was given no dietary agent. Groups 2 and 3 were given 20 mg/kg GCBE and 10 mg/kg CA, respectively.

Groups 4, 5, 6, and 7 were fed a high-fat high cholesterol (HFHC) diet (cat. No. D09052204; Research Diets, New Brunswick, USA) to induce NASH.[12] The HFHC diet was composed of 19 g carbohydrates (15% of total calories), 39 g fat (65% of total calories), 27 g protein (20% of total calories), 2 g cholesterol, and 0.5 g cholic acid per 100 g of diet. Group 4 was given no dietary agent. Groups 5, 6 and 7 were given 20 mg/kg GCBE, 10 mg/kg CA and 50 mg/kg CA, respectively. All dietary agents were given by daily oral gavage.

Reagents

Green coffee bean extract was obtained from Sepe Natural Organic Products Co. Ltd (İzmir, Turkey). It was extracted from *Coffea arabica* L. beans. Ethanol and water were used as solvents in the extraction procedure. The GCBE in our study was standardized to 50% chlorogenic acid using high performance liquid chromatography method (a dose of 20 mg/kg GCBE contained 10 mg/kg CA). Chlorogenic acid (purity ≥97.5%) was obtained from Acros Organics (Geel, Belgium). Other chemicals were obtained from Sigma–Aldrich (St. Louis, USA).

Serum biochemistry

Serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), amylase, lipase, total cholesterol, triglyceride, and high-density lipoprotein cholesterol (HDL-C) were analyzed with Beckman Coulter AU5800 autoanalyzer (Beckman Coulter, Inc., Hialeah, USA).

Serum superoxide dismutase (SOD) activity assay was based on the principle of inhibition of nitroblue tetrazolium reduction.[15] Malondialdehyde (MDA) levels were analyzed via thiobarbituric acid reaction.[16] The method developed by Aebi et al. was used for the measurement of catalase activity.[17] Glutathione peroxidase (GSH-PX) activity was measured using the method of Paglia et al.[18]

Histopathological analysis

Liver tissue samples were fixed and embedded in paraffin blocks. Four-micrometer sections were prepared using a Leica RM 2125 RT microtome (Leica Biosystems, Richmond, USA). After deparaffinization, samples were assessed using both hematoxylin & eosin (H&E) and Masson trichrome staining. An experienced histopathologist graded the NAS using the scoring system designed and validated by Kleiner et al.[19]

For electron microscopic analysis, liver tissues of rats from each group were fixed in phosphate-buffered glutaraldehyde (4%) (pH 7.4) at 4°C for 24 h and post-fixed in phosphate-buffered osmium tetroxide (1%) for 4 h, dehydrated through an ethanol series, and embedded in araldite-epoxy resin. Ultrathin sections were cut using diamond knives and a Leica Reichert Ultracut S ultramicrotome (Leica Biosystems). An 800-nm semithin section was prepared through the liver and stained with toluidine blue dye. Then, 80-nm thin sections were obtained from a selected area of the tissue samples defined as the semithin section, and these sections were stained with uranyl acetate and lead citrate. The ultrastructure of the liver was examined under a Hitachi HT7800 transmission electron microscope (Hitachi Corp., Tokyo, Japan).

Statistical analyses

All experiments were performed in duplicate, and statistical analysis was performed using IBM SPSS v. 21.0 software (IBM Corp., Armonk, USA). Since n = 8 per group nonparametric tests were used. Mann–Whitney (M–W) U test was used to analyze NASH model characteristics (group 1 vs group 4). Kruskal–Wallis (K–W) test followed by Dunn's multiple comparison test was used to compare animal characteristics, serum parameters and NAS within normal rat chow groups and within NAFLD groups. Spearman's test was used for correlation analysis between serum parameters and NAS. Also, a linear regression analysis was performed for NAS prediction in NAFLD groups (n = 32). Statistical significance was assumed at $p < 0.05$.

Results

Animal characteristics and serum biochemistry

Initial and final body weights were similar in normal rat chow and NAFLD groups. Both terminal body weight and weight gain during the study were higher in group 4 compared to group 1 (Table 2; M–W U test; $p = 0.038$, $U = 52$; $p = 0.050$, $U = 51$, respectively). Group 4 had higher terminal liver weight than group 1 (Table 2; M–W U test; $p < 0.001$, $U = 64$). Also, both group 2 and group 4 had a higher final liver weight/final body weight ratio than

Table 2. Animal characteristics

Weight	Statistics	Normal rat chow groups (n = 24)			K–W test		NAFLD groups (n = 32)				K–W test		M–W test group 1 vs group 4	
		group 1 control (n = 8)	group 2 GCBE (n = 8)	group 3 CA (n = 8)	H	p-value	group 4 control (n = 8)	group 5 GCBE (n = 8)	group 6 CA 10 (n = 8)	group 7 CA 50 (n = 8)	H	p-value	U	p-value
Initial body weight [g]	min Me max	287.00 346.00 377.00	335.00 367.50 379.00	307.00 359.00 374.00	3.27	0.195	300.00 361.50 386.00	342.00 369.50 399.00	307.00 368.50 382.00	323.00 366.50 401.00	1.01	0.798	41	0.328
Terminal body weight [g]	min Me max	395.00 450.00 514.00	403.00 442.50 513.00	392.00 458.00 493.00	0.02	0.990	352.00 562.50 626.00	439.00 489.50 583.00	442.00 498.00 559.00	440.00 503.00 601.00	3.40	0.334	**52**	**0.038**
Weight gain [g]	min Me max	72.00 124.50 156.00	43.00 85.00 145.00	68.00 93.50 119.00	5.26	0.072	58.00 183.00 208.00	101.00 135.50 204.00	129.00 140.00 192.00	107.00 151.00 200.00	3.57	0.311	51	0.050
Terminal liver weight [g]	min Me max	9.42 10.92 14.30	10.61 13.10 16.23	10.49 11.51 13.59	5.46	0.065	16.70 32.80 38.76	19.80 23.00 33.05	21.90 25.00 30.70	21.80 27.35 33.60	5.78	0.123	**64**	**<0.001**
Terminal liver/body weight [g]	min Me max	0.020 0.025 0.030	0.024 0.028 0.030	0.020 0.026 0.030	**8.07**[a]	**0.018**	0.050 0.057 0.070	0.040 0.048 0.060	0.040 0.052 0.060	0.050 0.053 0.060	6.59	0.086	**64**	**<0.001**

The Kruskal–Wallis (K–W) test was performed, followed by Dunn's multiple comparison test (statistically significant difference a: group 1–2) to compare normal rat chow groups and NAFLD groups. Dunn's test results are provided in Supplementary Table 1. Mann–Whitney (M–W) U test was used to compare 2 controls (group 1 vs group 4); min – minimum; Me – median; max – maximum; NAFLD – non-alcoholic fatty liver disease; GCBE – green coffee bean extract; CA – chlorogenic acid. Values in bold indicate statistical significance.

group 1 (Table 2; K–W test followed by Dunn's multiple comparison, H (2.00) = 8.07, p = 0.016; M–W U test; p < 0.001, U = 64, respectively).

As seen in Table 3, a comparison between group 1 and group 4 showed that HFHC diet-induced NASH caused an increase in serum glucose, ALT, AST, amylase, lipase, triglyceride, total cholesterol, and HDL-C (M–W U test; p = 0.002, U = 60 for triglyceride and p < 0.001, U = 64 for others). In the NAFLD groups, GCBE was linked to a substantial decrease in serum glucose, triglyceride and amylase levels (Table 3; K–W test followed by Dunn's multiple comparison, H (3.00) = 14.76, p = 0.003, H (3.00) = 16.87, p = 0.001, H (3.00) = 18.30, p < 0.001, respectively). Furthermore, the administration of 10 mg/kg of CA reduced serum glucose, triglyceride and amylase levels (Table 3; K–W test followed by Dunn's multiple comparison, H (3.00) = 14.76, p = 0.009, H (3.00) = 16.87, p = 0.009, H (3.00) = 18.30, p < 0.001, respectively). Also, 50 mg/kg chlorogenic acid reduced serum triglyceride levels in NAFLD (Table 3; K–W test followed by Dunn's multiple comparison, H (3.00) = 16.87, p = 0.037).

Due to more advanced cirrhosis, group 4 had lower ALT levels compared to groups 6 and 7 (Table 3; K–W test followed by Dunn's multiple comparison, H (3.00) = 13.17, p = 0.007, p = 0.016, respectively) and lower AST levels compared to groups 6 and 7 (Table 3; K–W test followed by Dunn's multiple comparison, H (3.00) = 11.57, p = 0.010, p = 0.043, respectively).

In contrast to NAFLD groups, GCBE and CA administrations resulted in significantly higher serum glucose in normal diet-fed groups (Table 3; K–W test followed by Dunn's multiple comparison, H (2.00) = 13.69, p = 0.002, p = 0.009, respectively). Chlorogenic acid also caused higher serum total cholesterol levels (Table 3; K–W test followed by Dunn's multiple comparison, H (2.00) = 9.48, p = 0.012).

Furthermore, GCBE administration increased ALT and amylase levels in normal rat chow groups (Table 3; K–W test followed by Dunn's multiple comparison, H (2.00) = 8.14, p = 0.013, H (2.00) = 12.06, p = 0.002, respectively).

Glyoxalase 1 and oxidative stress

As shown in Table 4; NAFLD was associated with higher serum GLO1 levels (group 4 vs group 1, M–W U test; p < 0.001, U = 64). In normal rat chow groups, GCBE administration reduced GLO1 levels (Table 4; K–W test followed by Dunn's multiple comparison, H (2.00) = 6.48, p = 0.036). In NAFLD groups, 10 mg/kg CA increased GLO1 levels (Table 4; K–W test followed by Dunn's multiple comparison, H (3.00) = 8.30, p = 0.036).

Serum catalase activity was suppressed in group 4 compared to group 1 (Table 4; M–W U test; p = 0.022, U = 11) Also, NAFLD was associated with a reduction in serum MDA levels (group 4 vs group 1, M–W U test; p < 0.001, U = 64).

Administrations of both GCBE and 50 mg/kg CA caused a reduction in MDA levels in NAFLD groups (Table 4; K–W test followed by Dunn's multiple comparison, H (3.00) = 19.58, p = 0.017, p < 0.001, respectively). In addition, GCBE administration significantly reduced MDA level in normal rat chow groups (Table 4; K–W test followed by Dunn's multiple comparison, H (2.00) = 20.48, p < 0.001).

Table 3. Biochemical characteristics

Serum biochemistry	Statistics	Normal rat chow groups (n = 24)			K–W test		NAFLD groups (n = 32)				K–W test		M–W test group 1 vs group 4	
		group 1 control (n = 8)	group 2 GCBE (n = 8)	group 3 CA (n = 8)	H	p-value	group 4 control (n = 8)	group 5 GCBE (n = 8)	group 6 CA 10 (n = 8)	group 7 CA 50 (n= 8)	H	p-value	U	p-value
Glucose [mg/dL]	min Me max	87.00 101.00 130.00	119.00 164.50 244.00	105.00 154.50 209.00	**13.69**[a,b]	**0.001**	151.00 160.00 200.00	57.00 93.00 154.00	90.00 112.50 149.00	107.00 128.00 169.00	**14.76**[d,e]	**0.002**	**64**	**<0.001**
ALT [U/L]	min Me max	33.00 38.50 46.00	39.00 51.00 65.00	34.00 42.00 56.00	**8.14**[a]	**0.017**	149.00 240.50 661.00	243.00 509.50 2512.50	280.00 978.00 2748.00	312.00 838.00 1337.00	**13.17**[e,f]	**0.004**	**64**	**<0.001**
AST [U/L]	min Me max	100.00 142.00 202.00	127.00 165.00 217.00	129.00 174.50 220.00	1.77	0.412	221.00 303.00 769.00	338.00 492.50 2912.00	348.00 960.50 3502.00	341.00 640.00 1272.00	**11.57**[e,f]	**0.009**	**64**	**<0.001**
Total cholesterol [mg/dL]	min Me max	42.00 47.00 54.00	42.00 66.00 98.00	51.00 72.00 82.00	**9.48**[b]	**0.009**	134.00 158.00 250.00	56.00 118.50 286.00	118.00 193.00 222.00	129.00 183.50 213.00	2.86	0.413	**64**	**<0.001**
HDL-C [mg/dL]	min Me max	21.00 25.50 30.00	22.00 30.50 42.00	24.00 27.50 35.00	3.95	0.139	43.00 57.50 71.00	19.00 44.50 85.00	33.00 55.00 66.00	37.00 56.00 66.00	1.10	0.777	**64**	**<0.001**
Triglyceride [mg/dL]	min Me max	33.00 40.00 58.00	33.00 68.00 108.00	33.00 56.00 107.00	5.31	0.070	52.00 67.50 101.00	17.00 23.50 65.00	21.00 29.50 45.00	21.00 33.00 52.00	**16.87**[d,e,f]	**0.001**	**60**	**0.002**
Amylase [U/L]	min Me max	1190.0 1441.5 1797.0	1649.0 1962.0 2353.0	1275.0 1666.5 1964.0	**12.06**[a]	**0.002**	2346.0 3213.5 4251.0	743.0 1574.0 2386.0	1245.0 2027.0 2613.0	2078.0 2381.0 2861.0	**18.30**[d,e]	**<0.001**	**64**	**<0.001**
Lipase [U/L]	min med max	3.00 5.00 6.00	5.00 7.00 8.00	2.00 5.50 8.00	5.37	0.068	15.00 23.50 37.00	6.00 18.00 49.00	20.00 30.50 36.00	15.00 21.50 36.00	3.54	0.314	**64**	**<0.001**

The Kruskal–Wallis (K–W) test was performed followed by Dunn's multiple comparison test (statistically significant difference [a] group 1–2, [b] group 1–3, [c] group 2–3, [d] group 4–5, [e] goup 4–6, [f] group 4–7) to compare normal rat chow groups and NAFLD groups. Dunn's test results are provided in Supplementary Table 2. Mann–Whitney (M–W) U test was used to compare 2 controls (group 1 vs group 4); min – minimum; Me – median; max – maximum; NAFLD – non-alcoholic fatty liver disease; GCBE – green coffee bean extract; CA – chlorogenic acid; ALT – alanine aminotransferase; AST – aspartate aminotransferase; HDL-C – high-density lipoprotein cholesterol. Values in bold indicate statistical significance.

Table 4. Serum GLO1 and oxidative stress parameters

Enzymatic antioxidants	Statistics	Normal rat chow groups (n = 24)			K–W test		NAFLD groups (n = 32)				K–W test		M–W test G1 vs G4	
		group 1 control (n = 8)	group 2 GCBE (n = 8)	group 3 CA (n = 8)	H	p-value	group 4 control (n = 8)	group 5 GCBE (n = 8)	group 6 CA 10 (n = 8)	group 7 CA 50 (n = 8)	H	p-value	U	p-value
GLO1 [ng/mL]	min Me max	38.57 39.71 42.01	34.37 36.58 39.75	35.42 37.76 47.85	**6.48**[a]	**0.039**	78.67 110.43 145.58	64.51 141.97 557.83	70.74 203.32 590.68	92.81 180.84 288.92	**8.30**[e]	**0.040**	**64.00**	**<0.001**
GSHPX [mIU/mL]	min Me max	96.00 151.20 240.00	105.60 124.80 153.60	96.00 122.40 148.80	2.31	0.314	110.40 172.80 249.60	96.00 134.40 216.00	115.20 148.80 163.20	115.20 139.20 187.20	1.64	0.649	34.60	0.798
Catalase [IU/mL]	min Me max	0.73 2.93 3.66	0.01 1.46 2.93	1.46 1.83 3.66	5.08	0.079	0.73 1.46 2.20	0.73 1.46 2.20	0.01 1.46 3.66	0.01 2.20 3.66	3.23	0.357	**11.00**	**0.022**
MDA [pmol/mL]	min Me max	206.00 228.00 253.00	66.00 71.50 82.00	94.00 100.50 109.00	**20.48**[a]	**<0.001**	124.00 146.00 160.00	98.00 104.50 140.00	99.00 116.00 123.00	86.00 96.50 112.00	**19.58**[d,f]	**<0.001**	**64.00**	**<0.001**
SOD [U/mL]	min Me max	7.40 7.86 8.32	6.91 7.45 7.93	7.06 7.25 7.57	**9.35**[b]	**0.009**	5.58 6.08 7.69	4.35 5.94 8.10	6.90 8.06 8.60	6.95 7.57 8.00	**17.57**[e,g]	**0.001**	**3.00**	**0.001**

The Kruskal–Wallis test was performed followed by Dunn's multiple comparison test (statistically significant difference [a] group 1–2, [b] group 1–3, [c] group 2–3, [d] group 4–5, [e] group 4–6, [f] Group 4–7, [g] group 5–6) to compare normal rat chow groups and NAFLD groups. Dunn's test results are provided in Supplementary Table 3. Mann–Whitney U test was used to compare 2 controls (group 1 vs group 4); min – minimum; Me – median; max – maximum; NAFLD – non-alcoholic fatty liver disease; GCBE – green coffee bean extract; CA – chlorogenic acid; GLO1 – glyoxalase 1; GSH-PX – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase. Values in bold indicate statistical significance.

Non-alcoholic fatty liver disease resulted in a significant decrease in serum superoxide SOD levels, as demonstrated in Table 4 (group 4 vs group 1). In NAFLD groups 10 mg/kg CA significantly increased SOD (Table 4; K–W test followed by Dunn's multiple comparison, H (3.00) = 17.57, p = 0.004). In normal rat chow groups, both GCBE and CA decreased SOD (Table 4; K–W test followed by Dunn's multiple comparison, H (2.00) = 9.35, p = 0.007).

Liver histopathology

The NAFLD activity score groups histological features into 5 categories: steatosis, fibrosis, inflammation, hepatocellular injury, and other findings (Mallory's hyaline and glycogenated nuclei). The NAFLD activity score was 0 for all normal rat chow-fed groups and NAFLD was excluded for all. However, all animals fed the HFHC diet were diagnosed with NAFLD (total NAS ranged from 9 to 22; Table 5). Both GCBE and 50 mg/kg CA significantly reduced total NAS (Table 5; K–W test followed by Dunn's multiple comparison, H (3.00) = 20.49, p = 0.002, p < 0.001, respectively). We observed a significant reduction in inflammation score with 10 mg/kg CA and 50 mg/kg CA (Table 5; K–W test followed by Dunn's multiple comparison, H (3.00) = 9.65, p = 0.005, p = 0.038, respectively). The administration of GCBE, 10 mg/kg CA, and 50 mg/kg CA resulted in a decrease in liver cell injury scores (Table 5; K–W test followed by Dunn's multiple comparison, H (3.00) = 19.84, p = 0.001, p = 0.004, p = 0.001, respectively). It was determined that GCBE, 10 mg/kg CA and 50 mg/kg CA all led to a decrease in other findings scores (Table 5; K–W test followed by Dunn's multiple comparison, H (3.00) = 31.00, p < 0.001 for all).

As shown in Fig. 1, light microscopic analysis showed that GCBE and 50 mg/kg CA both reduced hepatocyte ballooning. Additionally, 50 mg/kg CA reduced steatosis and inflammation. The presence of structurally normal hepatocytes was also noteworthy in both groups.

As shown in Fig. 2, ultrastructural micrographs of liver tissue belonging to the control group demonstrated normal hepatic cells. Sections of NASH groups showed macrovesicular and microvesicular lipid droplets, electron-dense bodies and irregular cell morphology, indicating the disruption of the cellular structure. In addition, inflammatory cells, particularly polymorphonuclear granulocytes and erythrocytes, were present around large oil droplets. It was observed that hepatocyte degeneration was similar in GCBE and 10 mg/kg CA groups, but the diameters of lipid droplets were smaller in both groups than in the NASH group.

Administration of 50 mg/kg CA was associated with NASH amelioration in terms of cell morphology and cytoplasmic structure in electron microscopic examination. Moreover, the number of lipid droplets decreased in this group compared to group 4 (Fig. 2). Additionally, the cell morphology and mitochondrial cristae inner contours were more regular.

Predicting NAS

As shown in Table 6, glucose, triglyceride, amylase, and MDA were positively correlated while ALT, AST and GLO1

Table 5. NAFLD activity scores

Activity scores	Statistics	NAFLD groups (n = 32)				K–W test	
		group 4 control (n = 8)	group 5 GCBE (n = 8)	group 6 CA 10 (n = 8)	group 7 CA 50 (n = 8)	H	p-value
Steatosis score	min Me max	5.00 5.50 7.00	4.00 4.00 5.00	3.00 4.00 7.00	4.00 4.00 6.00	**9.53**	**0.023**
Fibrosis score	min Me max	1.00 2.00 3.00	1.00 1.00 3.00	1.00 3.00 3.00	1.00 1.00 2.00	7.79	p = 0.050
Inflammation score	min Me max	4.00 6.00 6.00	2.00 5.00 5.00	2.00 4.00 5.00	2.00 4.00 5.00	**9.65**[e,f]	**0.022**
Liver cell injury score	min Me max	3.00 4.00 4.00	1.00 1.50 2.00	1.00 2.00 2.00	1.00 1.50 2.00	**19.84**[d,e,f]	**<0.001**
Other findings score	min Me max	2.00 2.00 2.00	0.00 0.00 0.00	0.00 0.00 0.00	0.00 0.00 0.00	**31.00**[d,e,f]	**<0.001**
Total score	min Me max	17.00 19.00 22.00	10.00 12.00 14.00	11.00 13.00 14.00	9.00 11.00 13.00	**20.49**[d,f]	**<0.001**

The Kruskal–Wallis (K–W) test was performed followed by Dunn's multiple comparison test (statistically significant difference; [d] group 4–5, [e] group 4–6, [f] group 4–7) to compare the groups. Dunn's test results are provided in Supplementary Table 4; min – minimum; Me – median; max – maximum; NAFLD – non-alcoholic fatty liver disease; GCBE – green coffee bean extract; CA – chlorogenic acid. Values in bold indicate statistical significance.

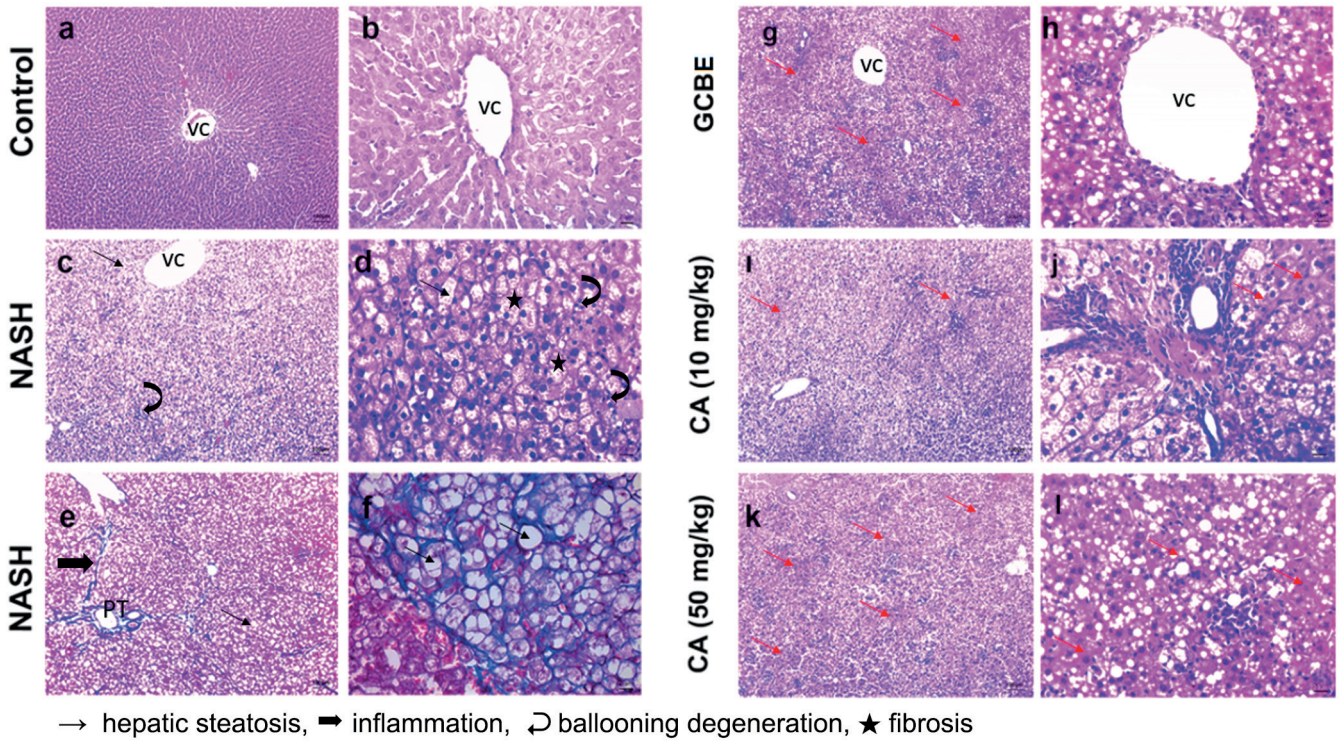

Fig. 1. Histological changes in the liver: control, NASH, GCBE (20 mg/kg), CA (10 mg/kg), and CA (50 mg/kg) groups (n = 8). Hepatoxylin & eosin (H&E) stain (A,B,C,D,G,H,I,J,K,L) and Masson trichrome stain (E,F) (×100 and ×400 magnification). A,B. In control group rats, liver tissue structure was histologically normal and hepatocytes were located around VC regularly. C,D,E,F. Significant hepatic steatosis, inflammation, ballooning degeneration, and fibrosis (see pictograms) were observed in NASH model group. G,H. In GCBE group, hepatocyte ballooning was found to decrease but macrovesicular and microvesicular steatosis still existed. Hepatocytes with normal histological appearance were observed around the vessel walls (→). Also, lymphocyte infiltration was significantly reduced compared to the NASH model group. I,J. Fibrosis localized to perisinusoidal and periportal areas was observed in 10 mg/kg CA group. K,L. In 50 mg/kg CA group, hepatic steatosis, inflammation and ballooning degeneration were found to decrease while number of hepatocytes with normal histological appearance increased

NASH – non-alcoholic steatohepatitis; GCBE – green coffee bean extract; CA – chlorogenic acid; VC – vena centralis; PT – portal triad.

were negatively correlated with NAS. According to linear regression analysis, serum MDA and serum triglyceride were found to be statistically significant in predicting NAS (R^2: 0.758, $p < 0.001$) (linear regression curves are shown in Fig. 3,4)

Discussion

In our study, the administration of GCBE and CA was associated with reductions in serum glucose, triglyceride and amylase levels in the NAFLD groups. The hypoglycemic and lipid-lowering effects of GCBE and CA have been shown in several studies.[10,11] However, the decrease in amylase levels is surprising because little is known about the protective effect of these dietary agents on the pancreas.

In the normal rat chow groups administrations of GCBE and CA (10 mg/kg) increased serum glucose levels compared to the control. Furthermore, GCBE administration increased ALT and amylase levels. These observations raise intriguing questions about the risks associated with the administration of GCBE and CA by healthy individuals without NAFLD.

Table 6. Correlation between serum parameters and NAS

Parameter	NAS	
	r	p-value
Glucose	**0.476**	**0.006**
ALT	**–0.567**	**0.001**
AST	**–0.532**	**0.002**
Total cholesterol	0.017	0.930
HDL-C	–0.260	0.890
Triglyceride	**0.487**	**0.005**
Amylase	**0.407**	**0.021**
Lipase	0.112	0.540
GLO1	**–0.367**	**0.040**
GSH-PX	0.250	0.170
Catalase	–0. 311	0.080
MDA	**0.698**	**<0.001**
SOD	–0.268	0.140

Spearman correlation was used; r – correlation coefficient; NAS – nonalcoholic fatty liver disease (NAFLD) activity score; ALT – alanine aminotransferase; AST – aspartate aminotransferase; HDL-C – high-density lipoprotein cholesterol; GLO1 – glyoxalase 1; GSH-PX – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase. Values in bold indicate statistical significance.

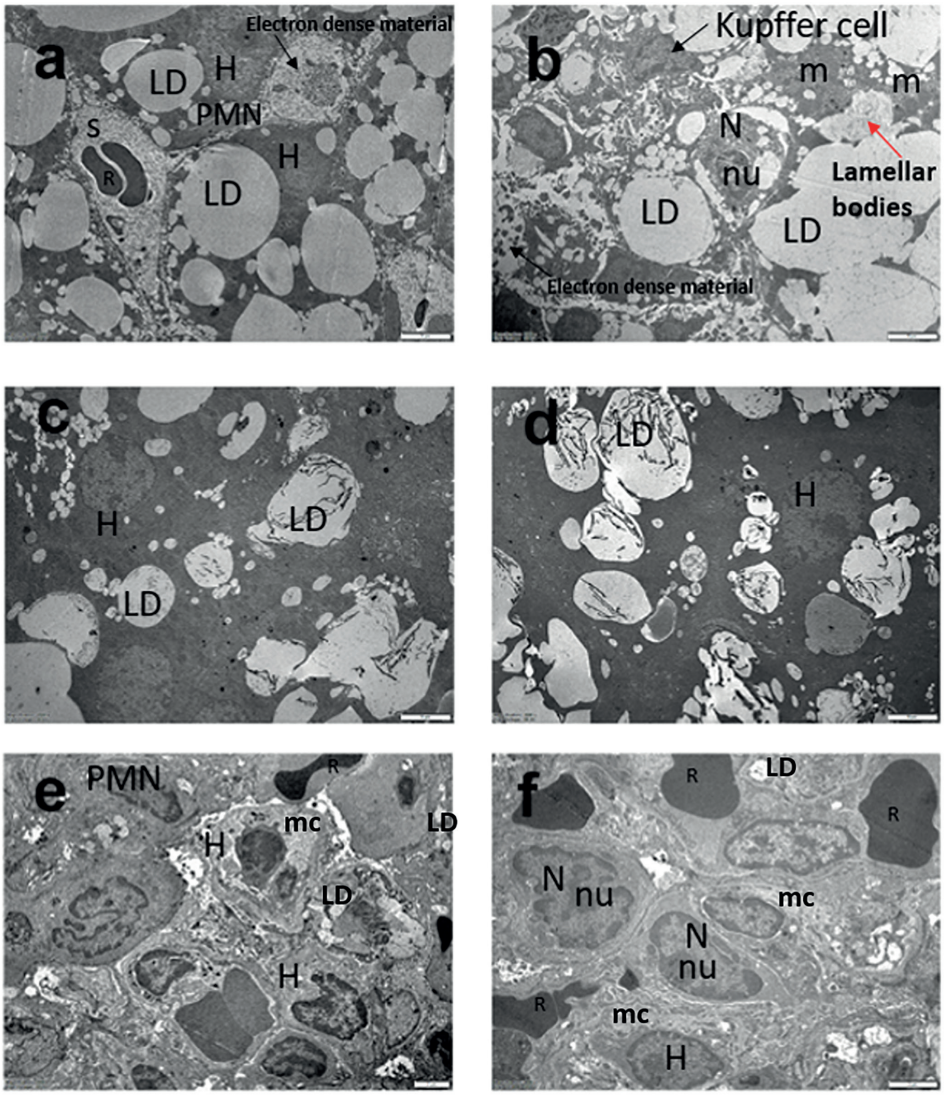

Fig. 2. Transmission electron microscopic images of NASH model, GCBE and 50 mg/kg CA groups: Hepatocyte (H), nucleus (N), lipid droplets (LD), sinusoids (S), erythrocyte (R), nucleolus (nu), polymorphonuclear neutrophils (PMN), mitochondria (m), and mitochondrial cristae (mc). A,B. Hepatic steatosis and structural damage in hepatocytes in NASH model group (×2,000 magnification); C,D. Macrovesicular and microvesicular steatosis exists in GCBE group (×2,000 magnification); E,F. A significant decrease in both number and diameter of lipid droplets was observed in 50 mg/kg CA group compared to NASH model group (×3,000 magnification) and (×4,000 magnification), respectively

NASH – non-alcoholic steatohepatitis; GCBE – green coffee bean extract; CA – chlorogenic acid.

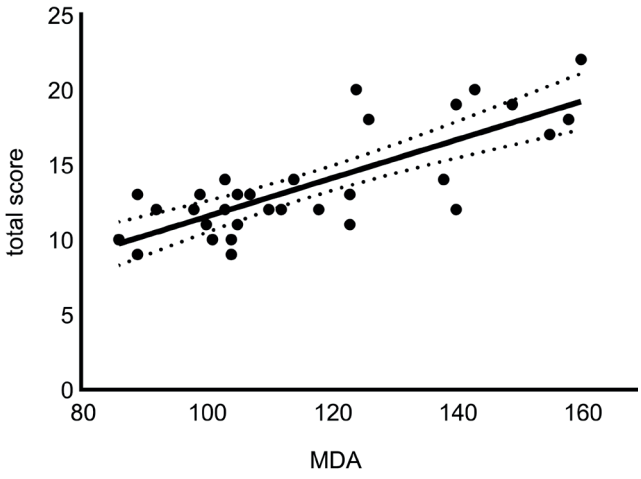

Fig. 3. Relationship between serum malondialdehyde (MDA) and total liver non-alcoholic fatty liver disease (NAFLD) activity score for NAFLD groups

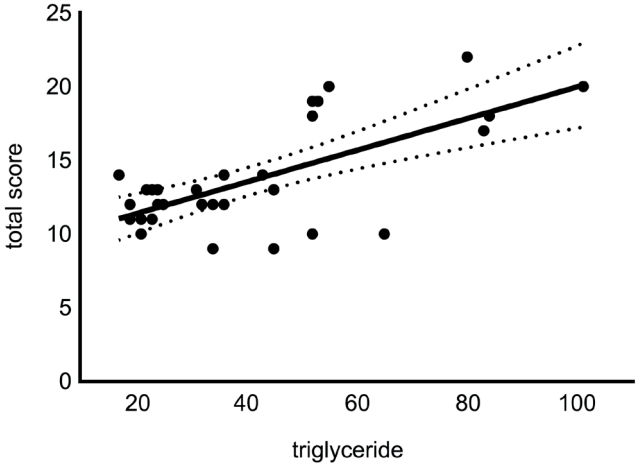

Fig. 4. Relationship between serum triglyceride and total liver non-alcoholic fatty liver disease (NAFLD) activity score for NAFLD groups

In HFHC diet-fed groups, intestinal absorption of polyphenols is expected to be more than in normal diet-fed groups, as the absorption of polyphenols increases in the lipid medium of the intestine.[20] Also, polyphenols can bind metal ions, which causes a risk of micromineral deficiency.[21] Micromineral deficiency was found to be

related to a decrease in antioxidant enzyme activity.[22] The increased amount of unabsorbed intestinal polyphenols and increased oxidative stress can help understand the adverse effects of GCBE and CA in the normal diet-fed groups.

In our study, HFHC diet induced NASH was associated with higher serum GLO1 levels. In NAFLD groups, the administration of both GCBE and CA increased GLO1 but it was statistically significant only in 10 mg/kg chlorogenic acid administration. Earlier observations suggest that increased GLO1 in obese individuals may be a defense mechanism against increased dicarbonyl stress.[7] Therefore, GLO1 may be one of the possible mechanisms by which GCBE and CA can ameliorate NAFLD.

The present study demonstrated a decrease in serum catalase and SOD activities as a consequence of NAFLD. Our finding aligns with the study by Swiderska et al.,[23] who found that serum catalase activity was statistically lower in the NAFLD group compared to the control. In our study administration of 10 mg/kg CA in the NAFLD groups led to increased serum SOD levels. In a study by Wang et al., CA was found to increase plasma SOD activity in rats, too.[24]

It has been established that there is a correlation between NAFLD and a decrease in serum MDA levels. Anti-MDA antibodies in the serum of advanced NAFLD patients were reported in earlier studies.[25] In our study, lower serum MDA levels in NAFLD groups may be caused by anti-MDA antibodies, which can bind to serum MDA and interfere with our MDA measurement. The administrations of both GCBE and CA decreased serum MDA in NAFLD groups. The suppression of serum MDA with CA and GCBE can refer to a peroxidation inhibitory effect of these dietary agents.

In our study, GCBE and 2 different doses of chlorogenic acid all reduced NAS. However, only GCBE and 50 mg/kg CA administrations caused a significant reduction. Hence, the hepatoprotective effect of 20 mg/kg GCBE containing 50% chlorogenic acid was more significant than 10 mg/kg purified chlorogenic acid. Green coffee also contains different polyphenols such as feruloylquinic acids, dicaffeoylquinic acids and cinnamoylquinic acids.[9] These different polyphenols may play a role in making GCBE more effective than pure chlorogenic acid.

Additionally, serum MDA and triglyceride were found to be statistically significant for NAS prediction. In an earlier study it was shown that the majority of fatty acids used for triacylglycerol synthesis in the liver of NAFLD patients originated from the blood circulation.[26] Hence, serum dyslipidemia markers are promising in NAS prediction.

Limitations

Our study had some limitations. Several differences between humans and animals, in terms of physiology, anatomy and metabolism, make it difficult to apply data derived from animal studies to human conditions. However, the findings of our study provide important insight into human studies. Another limitation is that, as a result of our study design, we were only able to evaluate the disease-preventive effects of CA and GCBE. Additional studies investigating the therapeutic effect can also contribute to our knowledge.

Conclusions

The effects of GCBE and CA on the NAFLD population and on healthy individuals are different; especially their effect on healthy individuals is not clearly understood, necessitating further studies. Green coffee bean extract and CA can reduce amylase levels in NAFLD. This finding may indicate their ameliorative effect on pancreatic damage in NAFLD. Green coffee bean extract and CA increase GLO1 in NAFLD. Increased GLO1 may be one of the possible mechanisms to explain their hepatoprotective effect. Both 20 mg/kg GCBE and 50 mg/kg CA can be used in the prevention of NAFLD. Since they have similar hepatoprotective effects, GCBE can be preferred primarily as a more cost-effective dietary agent. Serum MDA and triglyceride are promising in NAS prediction. The use of dyslipidemia markers can reduce the need for liver biopsies.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.13954275. The package includes the following files:

Supplementary Table 1. Dunn's test results for Table 2.
Supplementary Table 2. Dunn's test results for Table 3.
Supplementary Table 3. Dunn's test results for Table 4.
Supplementary Table 4. Dunn's test results for Table 5.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Emergency medical team interventions in Poland during out-of-hospital deliveries: A retrospective analysis

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Abstract

Background. Emergency medical teams are a crucial component of healthcare systems, routinely providing essential care to pregnant patients in various situations.

Objectives. To evaluate the rate and outcomes of out-of-hospital deliveries attended by Emergency Medical Services (EMS) in Poland and identify areas for improvement in the care provided by emergency medical teams.

Materials and methods. This retrospective study was based on 41,335 EMS emergency calls to women in advanced pregnancy, of which 879 births were delivered directly by medical teams between January 2018 and December 2022. Data were obtained from the Polish National Monitoring Center for Emergency Medical Services, encompassing all EMS interventions in Poland.

Results. The study involved 879 EMS team interventions for pregnant women, with an average patient age of 29.87 years. Most patients were in their 2nd pregnancy (28.26%) and delivering for the 2nd time (25.77%). The postnatal condition of newborns, assessed using the Apgar score, was missing in 408 cases (46.52%) due to incorrect completion of documentation. Emergency Medical Services teams, predominantly P-type (basic) teams, handled 69.78% of deliveries, while S-type (specialist) teams were involved in 30.22% of cases. Medical procedures often performed during childbirth included manual assistance in spontaneous delivery, pulse oximetry, physical examination, examination of systemic blood pressure, obtaining peripheral intravenous access, and gynecological examination.

Conclusions. Given the rate of encountered cases and the gaps identified in medical documentation, there is merit in potentially implementing a dedicated form to be completed by medical teams when caring for a pregnant patient. Ongoing training and enhancements in the range of assistance provided to the mother and newborn are imperative for ensuring appropriate care.

Key words: pregnant women, health care, Emergency Medical Team, out-of-hospital birth

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Background

As in many other countries, the number of births in Poland is progressively decreasing.[1] In the first half of 2023, 139,000 live births were registered in Poland, reflecting a decrease of 14,700 compared to the corresponding period during the previous year. The natural increase, calculated as the difference between live births and deaths, was negative, reaching about 70,400. Such a reduction in the number of deliveries necessitates further measures to optimize the care of pregnant women and make every possible effort to reduce the risk to the newborn, which is particularly important in the case of out-of-hospital (OOH) deliveries.

In Poland, the primary location for childbirth is centralized in hospitals, with very limited alternatives. Despite the option for patients to choose the delivery location based on perinatal care standards, no public national maternity program is available. Home births are exclusively facilitated by midwives operating within their private practices.[2]

Examining the situation in other European countries, such as Finland, reveals that when the number of deliveries declines, maternity wards with fewer than 1,000 deliveries per year are often closed. This trend leads to a centralization of births in larger units, aiming to guarantee the highest standard of care for the mother and the newborn. Consequently, the risk of intervention by emergency medical teams in pre-hospital deliveries increases.[3]

In 2019, the total number of Emergency Medical Services (EMS) teams in Poland was 1,585, including those operating on a seasonal basis. The current organized EMS system is referred to as the National Monitoring Center for Emergency Medical Services (NMC-EMS). Modern EMS also incorporate specialized medical staff and are integral to the system. In addition to established professions like physicians and nurses, there is the (relatively new in Poland) profession of paramedics.[4]

Polish EMS constitutes a critical pillar of the healthcare system, designed to deliver timely and specialized care to individuals confronted with life or health-threatening emergencies. This all-encompassing system comprises essential elements, ranging from hospital emergency departments and EMS teams to advanced units like Helicopter Emergency Medical Services (HEMS) teams. Within this structure, both EMS and HEMS teams share the unified goal of providing on-site emergency medical care and ensuring the secure transport of patients to hospitals. Emergency Medical Services teams consist of diverse healthcare professionals, including physicians, emergency medical technicians and nurses. Notably, Polish EMS teams are classified as basic (non-physician-staffed) or specialist (physician-staffed), with team size of 2 or 3 responders.

The foundation of EMS in Poland rests on paramedic emergency medical teams, comprising paramedics trained to meet legal requirements. Notably, an increasing number of paramedics in Poland hold bachelor's degrees in medical rescue. Their education includes courses in obstetrics and the practical and theoretical aspects of providing emergency care to women in pregnancy-related emergencies, encompassing pre-hospital deliveries. As the landscape of EMS in Poland undergoes evolution, there is a decreasing inclusion of physicians within these teams. While a minority specialize in emergency medicine, their expertise significantly enhances the overall capabilities of the teams. The integration of diverse healthcare professionals and the continuous development of skill sets underscore the adaptability and effectiveness of the Polish EMS in addressing a wide array of medical emergencies.[5] Current provisions in the law on state EMS stipulate the existence of 1 specialist team for every 10 basic teams.

The current landscape is changing, with a noticeable decrease in ambulances with on-board physicians. This trend implies an increasing need for emergency medical responders to take proactive measures and decisions, especially in rare but perilous situations such as assisting a pregnant patient or performing neonatal resuscitation. Such transformation underscores medical professionals' need to be well-prepared to handle critical scenarios. It is crucial to reference established guidelines, such as those outlined by the European Resuscitation Council (ERC), to ensure the highest standards of care.[6,7]

Numerous scientific studies underscore the pivotal role of pre-hospital care as one of the pillars of care in managing emergencies. Proper optimization, thorough staff preparation and prompt transportation consistently and substantially influence the patient's subsequent outcomes.[8]

An important point is that pregnant women and childbirth represent a small percentage of all realized calls for emergency medical teams. In the period during which the calls were analyzed (2018–2022), 41,335 calls involved assisting a pregnant woman. According to data from the Statistics Poland, the EMS realized a total of 15,139,193 notifications during this period, with calls related to pregnancies accounting for only 0.273% of the total. Therefore, we can assume that working with pregnant women is rare for members of the emergency medical teams, which makes it challenging for them to maintain proper standards of care and current medical knowledge.

Objectives

The study aimed to determine the rate of births attended by EMS teams in Poland, identify the procedures performed by them, compare the procedures performed based on the presence of a physician in the team, and assess newborn condition following delivery by the EMS team.

Materials and methods

Study design

We conducted a cross-sectional study using data from the NMC-EMS from 2018–2022, encompassing all interventions conducted by EMS within the country. As no specific diagnostic code reliably identified OOH deliveries, we employed multiple search strategies to identify OOH deliveries that EMS took, defined as the 2nd stage of labor.

Before commencing the study, ethical approval was obtained from the Bioethics Committee of Wroclaw Medical University, Poland (approval No. KB-975/2022).

Participants

In the International Classification of Diseases, 10th Revision (ICD-10), the range designated by codes O30 to O92 encompasses categories related to pregnancy, childbirth and puerperium complications. Specifically, O30–O48 addresses complications associated with pregnancy and fetal development, O60–O77 pertains to issues related to childbirth, O80–O84 involves complications during the puerperium, and O85–O92 focuses on complications related to maternal healthcare.

Variables

The original database was filtered within the range of ICD-10 codes from O30 to O92 to isolate those involving pregnant women, amounting to 41,335 cases. Subsequently, 2 independent researchers conducted a manual search within the descriptions of interventions prepared by emergency medical teams. The focus of the search was to gather information about direct EMS involvement during childbirth. The 2nd stage of labor, involving the actual birth of the newborn, was defined as the focal point of the analysis.

Relying on the descriptions provided by the EMS, we identified 879 cases of interventions involving childbirth. Instances in which the delivery occurred in a gynecological emergency room in the presence of a physician or midwife were excluded from the analysis. Adhering to the definition outlined by the World Health Organization (WHO), individuals who experienced labor after the 22nd week of pregnancy were considered to have given birth.

Statistical analyses

The relationships between qualitative variables were analyzed using Pearson's χ^2 independence test followed by Bonferroni correction to reduce the chances of obtaining false positive results (type I errors). The Kolmogorov–Smirnov (K–S) test was used to verify the normal distribution of the participant's age (K–S test: $D = 0.053$, $n = 818$; $p < 0.001$). A significance level of $p < 0.05$ was adopted to indicate the presence of statistically significant relationships or differences. Statistical analysis employed IBM SPSS v. 26 software (IBM Corp., Armonk, USA).

Results

The survey encompassed 879 interventions by EMS teams for pregnant patients. The median patient age was 30 years (1st quartile (Q1) = 25 and 3rd quartile (Q3) = 34), with the youngest being 15 and the oldest 45. Most patients were in their 2nd pregnancy (249, 28.33%), and 227 (25.82%) were experiencing their 2nd childbirth. Unfortunately, some reports from EMS teams were incomplete, leading to missing information about the specific pregnancy and childbirth in 138 cases (15.70%) and 268 cases (30.49%), respectively. Additionally, in 403 cases (45.85%), emergency medical teams omitted information about the week of pregnancy.

The cases considered included 360 instances of full-term pregnancy (after 37 weeks of pregnancy) (40.96%) and 116 cases (13.20%) involving premature deliveries (before 37 weeks of pregnancy). In the remaining cases, there was no information about the week of pregnancy (403, 45.85%). The postnatal condition of the newborn, assessed using the Apgar score, was evaluated in 471 cases. Among these, 428 (48.69%) were rated as having a good condition (8–10 points), 23 (2.62%) an average condition (4–7 points) and 20 (2.28%) a poor condition (0–3 points).

Table 1. Characteristics of the study population

Variable		Value
Age [years] median (Q1–Q3)		30 (25–34)
Number of pregnancies n (%)	1st	51 (5.80)
	2nd	249 (28.33)
	3rd	193 (21.96)
	4th	124 (14.11)
	5th or more	124 (14.11)
	no data	138 (15.70)
Number of labors n (%)	1st	52 (5.92)
	2nd	227 (25.82)
	3rd	158 (17.97)
	4th	96 (10.92)
	5th or more	78 (8.88)
	no data	268 (30.49)
Duration of pregnancy n (%)	preterm pregnancy	116 (13.20)
	term pregnancy	360 (40.96)
	no data	403 (45.85)
Apgar score n (%)	8–10 points	428 (48.69)
	4–7 points	23 (2.62)
	0–3 points	20 (2.28)
	no data	408 (46.42)

Q1 – 1st quartile; Q3 – 3rd quartile.

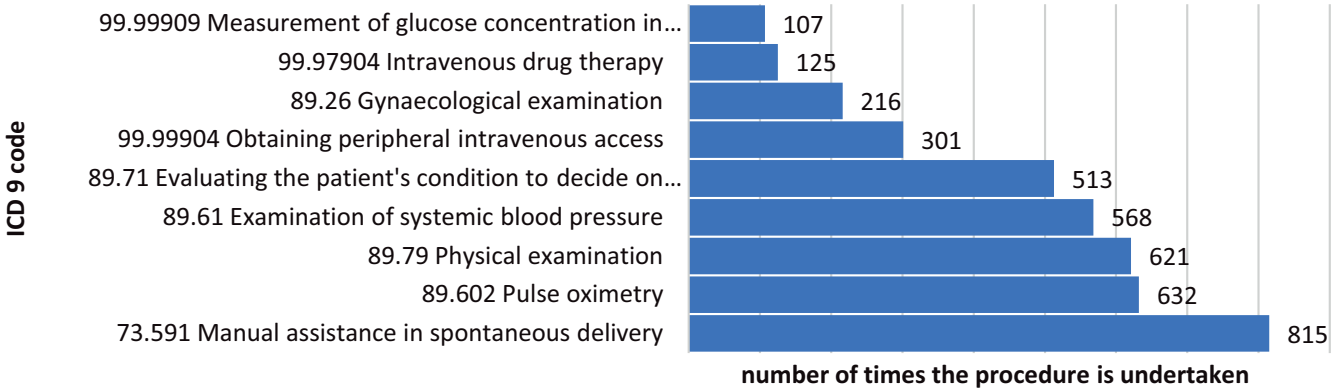

Fig. 1. Emergency medical procedures performed by Emergency Medical Services (EMS) teams attending a birthing patient

Table 2. Emergency Medical Services (EMS) group characteristics and intervention time

EMS team type, n (%)		Value, n (%)
Basic		612 (69.62)
Specialist		267 (30.38)
Intervention time, n (%)	6 AM–2 PM	273 (31.06)
	2 PM–10 PM	228 (25.94)
	10 PM–6 AM	378 (43.00)

However, in 408 cases (46.42%), no information was available regarding the Apgar score. Further details are presented in Table 1.

Most deliveries attended by emergency medical teams occurred under the care of P-type (basic) teams (612, 69.62%), with specialist teams handling deliveries in only 267 (30.38%) cases. The majority of deliveries occurred between 10 PM and 6 AM, totaling 378 (43.00%), followed by 273 (31.06%) interventions between 6 AM and 2 PM, and 228 (25.94%) between 2 PM and 10 PM (the data are presented in Table 2).

The medical procedures most frequently performed during the care of women giving birth included manual assistance in spontaneous delivery (815 cases, 92.72%), pulse oximetry (632 cases, 71.90%), physical examination (621 cases, 70.65%), examination of systemic blood pressure (568 cases, 64.62%), obtaining peripheral intravenous access (301 cases, 34.24%), and gynecological examination (216 cases, 24.57%). Detailed data are presented in Fig. 1. Emergency medical teams included oxytocin administration during labor in their reports in only 9 cases. However, there were no detailed descriptions of the reasons and purposes of its administration.

Table 3 contrasts the activities conducted based on the type of medical team, i.e., basic compared to specialized. Medical procedures, including physical examination, blood pressure measurements and pulse oximetry, were undertaken more frequently by primary emergency medical teams ($p < 0.05$). There were no significant differences in the frequency of gynecological examination, obtaining peripheral intravenous access and measurement of glucose concentration in arterialized capillary blood. However, it is worth emphasizing that these examinations were conducted less frequently than those mentioned above, being undertaken in 1 in 3 women at most.

Table 3. Comparison of the frequencies of procedures conducted by EMS P-type and EMS S-type medical team

Procedures conducted by EMS		EMS P-type	EMS S-type	χ^2 p-value*
Pulse oximetry, n (%)	yes no	487 (79.58) 125 (20.42)	152 (56.93) 115 (43.07)	48.03 <0.001
Physical examination, n (%)	yes no	463 (75.65) 149 (24.35)	165 (61.80) 102 (38.20)	17.49 <0.001
Examination of systemic blood pressure, n (%)	yes no	439 (71.73) 173 (28.27)	136 (50.94) 131 (49.06)	46.73 <0.001
Gynecological examination, n (%)	yes no	157 (25.65) 455 (74.35)	59 (22.10) 208 (77.90)	1.27 1.000
Obtaining peripheral intravenous access, n (%)	yes no	228 (37.25) 384 (62.75)	76 (28.46) 191 (71.54)	6.35 0.072
Measurement of glucose concentration in arterialized capillary blood, n (%)	yes no	89 (14.54) 523 (85.46)	22 (8.24) 245 (91.76)	6.69 0.060

χ^2 – χ^2 statistic value, degrees of freedom for all comparisons = 1; p-value considers the Bonferroni correction for multiple comparisons; EMS – Emergency Medical Services; P-type – basic team; S-type – specialized team.

Discussion

The current study illustrates the clinical support extended by EMS teams to women delivering OOH, shedding light on the most prevalent medical procedures and the postnatal condition of newborns. To the authors' knowledge, no prior studies in Poland, except for one describing the actual rate of deliveries attended by EMS, explored this aspect.[9]

The choice of birthplace is a subject of ongoing discussion among various organizations and associations in many countries, as is defining the necessary conditions and criteria for qualifying pregnant individuals for a planned home or hospital birth. The Polish Society of Gynecologists and Obstetricians (Polskie Towarzystwo Ginekologów i Położników (PTGiP)) has not formulated a clear position on home births.[10]

Hospital-based maternity care is frequently criticized for its medicalization of childbirth, and patients opting for community birth often intend to avoid, in their opinion, unnecessary interventions such as cardiotocography, episiotomy and epidural anesthesia. They consider an OOH birth to be safer than a hospital birth.[11]

The American College of Obstetricians and Gynaecologists (ACOG) recognizes that "many common obstetric practices are of limited or uncertain benefit for low-risk women in spontaneous labor."[12] However, it is important to note that childbirth is unpredictable and may sometimes occur in an unplanned setting, in which case medical assistance from EMS personnel may be required.[13,14]

Our study highlights deficiencies in the medical documentation maintained by emergency medical teams. It is crucial to underscore that deliveries are exceptional situations that do not occur daily, making them the most stressful emergency for EMS providers.[15] Therefore, it is understandable that documentation gaps may arise due to significant effort, exhaustion and lack of experience. However, the authors advocate for creating a dedicated card for OOH deliveries to enhance monitoring analysis and improve staff performance, ensuring comprehensive and appropriate information collection. Documentation deficiencies have also been acknowledged in other countries, indicating that the issue can also be expected in Poland.[13,16]

The additional documentation should be an integral part of the Command Support System for Polish EMS, in which the EMS team leader maintains each patient's medical records. The system should automatically run additional options for the assessment of a pregnant woman and possibly a newborn baby whenever a pregnant woman is assisted. Such a solution would not only guide the members of the EMS on the correct procedures but also provide better opportunities to monitor the quality of care.

Considering the declining birth rates in Poland and the consequent decrease in the number of gynecological and maternity hospitals, there is a significant likelihood that medical care will encounter new challenges. Many studies suggest a correlation between prolonged travel time for OOH births and an increased risk of neonatal mortality.[17,18] In light of the above, emergency medical team interventions could increase despite the declining number of deliveries. This would entail heightened expectations and encountering progressively complex clinical scenarios. It appears essential to proactively prepare to prevent dramatic situations in the future.

Mothers exhibiting abnormal vital signs during the intrapartum period may be experiencing conditions that pose potential complications for both the birthing process and the newborn's wellbeing.[19] Paramedics may overlook these conditions unless they are familiar with typical maternal vital signs during pregnancy and understand the physiological changes that occur in the mother. For example, hypertensive disorders are linked to elevated maternal and fetal morbidity and mortality levels.[20] Increased blood pressure during labor can contribute to placental insufficiency and fetal hypoxia.[21] Additionally, it has been demonstrated to elevate the incidence of postpartum preeclampsia.[22] Due to the above, it is essential not to forget basic activities such as measuring blood pressure, even in non-standard situations involving emergency medical teams.

In their 2021 study, Schultz et al. underscored the pivotal role of active management in the 3rd stage of labor, particularly the immediate administration of oxytocin postpartum.[23] Given the potential life-threatening implications of primary postpartum hemorrhage (PPH) for the mother, this practice is a standard preventive measure against it in many Polish hospitals. Analyzing Queensland Ambulance Service data, Schultz et al. reported a robust 63.4% administration rate of oxytocin postpartum. Contrastingly, our research, derived from the available data, indicates a notably lower frequency of oxytocin administration, with only 9 mission descriptions of its use. The reasons for the infrequent use of oxytocin by emergency medical services are not apparent from the mission descriptions alone.

Nevertheless, aligning with WHO guidelines and the recommendations of various scientific societies, e.g., the Royal College of Obstetricians and Gynecologists, it is prudent to consider oxytocin administration in every woman giving birth without risk factors.[24,25] In cases where the administration is declined, this refusal should be fully documented in adherence to best practices and WHO recommendations. Further investigation into the factors influencing the variance in oxytocin administration rate is warranted to ensure the optimal application of preventive measures for PPH in emergency obstetric care.

The Polish Ministry of Health regulations define EMS activities performed autonomously by a paramedic in a type P-team, and the medications listed therein to be administered by the paramedic do not include oxytocin – therefore, the paramedic can only administer it on a physician's orders.[26] The Polish National Health Service's guidelines for the minimum equipment of a P-team do not specify what medications the P-team should be equipped

with. As a result, dispatchers do not equip the EMS teams with oxytocin. Therefore, it is worth considering expanding the table of pharmacological agents administered by the paramedic autonomously, which will give the possibility of introducing it to the EMS. It can be considered that the introduction of its administration is similar to drugs such as clopidogrel and ticagrelor, which are administered after consultation with the physician on duty at the facility to which the patient is transported.

An essential aspect of our study was the exploration of rarely addressed, real challenges encountered by EMS teams involved in delivering babies in Poland. Further research appears imperative to enhance education and support for emergency medical teams, ultimately ensuring the highest level of safety for both the delivering patient and her newborn. The authors believe a discourse on developing a new card tailored for EMS teams during visits to pregnant women is necessary to ensure the comprehensive inclusion of essential data. Our working group is poised to submit its proposal for these changes to decision-makers shortly.

Paramedics in emergency medical teams proceed mainly based on guidelines from recognized organizations such as the ERC. Therefore, instead of the Apgar scale, one could consider introducing a simplified assessment following the Newborn Life Support (NLS) guidelines for the resuscitation of a newborn based on the evaluation of 4 parameters: skin color, muscle tone, respiratory rate, and heart rate.[7] The compatibility of such an assessment with current guidelines will provide consistency in the algorithm and completed documentation.

Within medical records, it is worth considering introducing a separate section for evaluating the newborn and the procedures performed, activated when the field "childbirth" is marked in the existing documentation. The study showed that intravenous access was performed in 301 cases. However, the design of the current documentation does not allow for an unambiguous statement of whether the access was performed on the woman giving birth or the child. There is no clear place in the current documentation to record whether the child had an assessed level of saturation, required oxygen support or lung ventilation, given that the teams assessed 23 cases (2.62%) as medium (3–7 points) and 19 cases (2.16 %) as poor (0–2 points).

Considering some specific aspersions of the assessment and management of a woman in labor, it is worth considering the development of "Good Practices for the Management of a Woman in Labor," employing the necessary elements of assessment, management and documentation in such cases. Similar documents already exist, approved and published by the Polish Ministry of Health, including "Good Practices for the Conduct of Medical Dispatchers, Emergency Medical Teams and Emergency Department to a Patient with Hemophilia or Related Hemorrhagic Diathesis" and "Good Practices for the Management of a Patient with Suspected Stroke."[27]

Limitations

Despite the valuable insights gained from this study, several limitations merit consideration. The retrospective nature of the study is one of the most significant limitations. The reliance on EMS team reports, some of which needed to be completed, poses a challenge in constructing a comprehensive understanding of each obstetric intervention. Additionally, we are concerned that some paramedics may misunderstand the ICD-9 procedures associated with childbirth and birth assistance. Missing Apgar scores further limit the depth of the analysis and hinder a comprehensive assessment of neonatal outcomes.

The study's focus on EMS team reports might only capture some clinical context, potentially leading to underestimating or misrepresenting certain variables, though the focus on basic and specialized EMS teams offers valuable insights into their distinct roles. However, the specific criteria determining the assignment of teams to cases were not explored. This lack of clarity limits the depth of understanding regarding the decision-making process for team allocation. Despite these limitations, this study constitutes a foundational exploration of the challenges and complexities associated with emergency obstetric care. A noteworthy strength of this work is the meticulous manual examination of all descriptions of medical interventions, a process that demanded a considerable investment of time and thorough analytical scrutiny.

Conclusions

Childbirth is inherently unpredictable and can take place in unplanned pre-hospital settings, posing a significant risk of requiring the intervention of emergency medical teams. This is particularly important considering the decreasing number of deliveries and gynecological wards, and the distance from the patient's home to the nearest hospital. According to our study, significantly more medical procedures were conducted by teams lacking a doctor. The challenges posed by unique situations for emergency medical teams, such as childbirth, contribute to a need for sufficient diligence in maintaining documentation. Developing new forms for EMS teams to enhance documentation quality is crucial. Further research is warranted to enhance our understanding of the factors influencing emergency obstetric outcomes and to guide the development of targeted interventions for pregnant patients in emergency settings.

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COVID-19 health communication strategies for older adults: Chatbots and traditional media

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Conflict of interest
None declared

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Abstract

Background. The coronavirus disease 2019 (COVID-19) pandemic has significantly accelerated the development and use of new healthcare technologies. While younger individuals may have been able to quickly embrace virtual advancements, older adults may still have different needs in terms of health communication.

Objectives. To identify areas of interest and preferred sources of information related to the COVID-19 pandemic among older adults and to verify their eHealth competencies.

Materials and methods. The study was conducted between February 2022 and July 2022. It included listeners from the University of the Third Age (U3A) and younger students. Both groups received information about the HealthBuddy+ chatbot, a questionnaire that addressed respondents' interests about COVID-19, and the PL-eHEALS (eHealth Literacy Scale) questionnaire to measure their eHealth competencies.

Results. There were 573 participants in the study (U3A listeners – 303 participants, median age: 73 years (interquartile range (IQR): 69–77); young adult students – 270, median age: 24 years (IQR: 23–24). The primary source of information about COVID-19 for older adults was television (84.5%), and for younger adults, internet (84.4%). Among the older adults, only 17% ever interacted with a chatbot (younger adults – 78% respectively), and 19% considered it a trustworthy source of information on COVID-19 compared to 79% of younger respondents. Older adults and younger adults in our study were most interested in COVID-19 treatment methods (45.5% and 69.3%, respectively), symptoms of the disease (36.6% and 35.2%, respectively) and chronic diseases coexisting with COVID-19 (35.0% and 51.5%, respectively). However, their eHealth competencies were generally low (median (Me): 34; IQR: 30–39) compared to younger adults (Me: 42; IQR: 40–47).

Conclusions. Health education for older adults should be appropriately tailored to their current needs and differentiated. The level of eHealth competencies of older adults suggests that much work remains to narrow the gap between the eHealth competencies of the younger and older generations.

Key words: health education, older adults, information seeking, COVID-19, chatbot

Background

The COVID-19 pandemic has negatively affected many spheres of life, particularly health, limiting patients' access to diagnosis and treatment, including screening and participation in clinical trials. Faced with difficulties in accessing healthcare facilities, many people turned to the mass media or the internet for information on medical issues of interest. In the context of the ongoing pandemic, we have observed reduced quality of life among older adults, accompanied by an increase in depression and social isolation. These trends have become more pronounced as the situation has deteriorated.[1] Moreover, the digital divide between younger and older people has led to a bigger skills gap and more digital isolation among older adults.[2] The epidemiological situation has forced social and family life to move to the internet, leaving older adults behind. Lack of adequate digital competencies and access to electronic devices limits older adults' contact with family and friends, and prevents them from using the online health services and public services that were developed during the pandemic.[3] These factors make it significantly more difficult for older adults to receive reliable health information.

In response to these barriers and the recent epidemiological situation, several technological solutions for remote communication have been developed, including smartphone applications[4] and chatbots for disease monitoring, risk assessment, information dissemination, or vaccination schedules.[5] Montenegro et al. distinguished 6 goals in healthcare policy for using chatbots. One of these goals is to support older adults.[6] Although this group is often seen as digitally excluded, some studies indicate that chatbots are well accepted by older adults and effective in improving their overall wellbeing, including physical and mental health.[7,8] Wilczewski et al. showed that older adults reported chatbot-delivered health information to be accessible, practical and with low cognitive load.[9] On the other hand, older respondents who experienced long COVID (median age 63) in the study by Wu et al. indicated doubts about the chatbot's ability to provide relevant health information. Attitudes towards the use of chatbots depend on the subject matter, e.g., in terms of sleep and nutrition or collecting information on symptoms, individuals were positively inclined.[10] A study by Dennis et al. that investigated a telephone intervention (COVID-19 screening hotline) with a chatbot showed that participants rated the chatbot more positively than human agents because they felt more comfortable providing socially undesirable information without fear of judgment or stigma. Furthermore, perceptions of chatbot functionality are linked to the screening hotline provider and trust in that provider, suggesting how important the chatbot source is and whether it is trustworthy.[11]

HealthBuddy+ (Fig. 1), developed by the United Nations Children's Fund (UNICEF) Regional Office for Europe and Central Asia (ECARO) and the World Health Organization (WHO) Regional Office for Europe (WHO/Europe) in May 2020,[12] is one such conversational chatbot, supported by a trusted source. It was designed using natural language processing principles to address the societal need for credible and verified information on COVID-19, quarantine, testing, isolation, and protection, as well as debunking of misinformation. The UNICEF and WHO offices have been involved in adapting chatbot's functionality in 15 countries and 16 languages, including Poland, through the https://healthbuddy.plus/website and as an Android and iOS[13] smartphone application.

Data from December 2021 show that the chatbot had 450,000 users at that time, and 10,000 user questions were

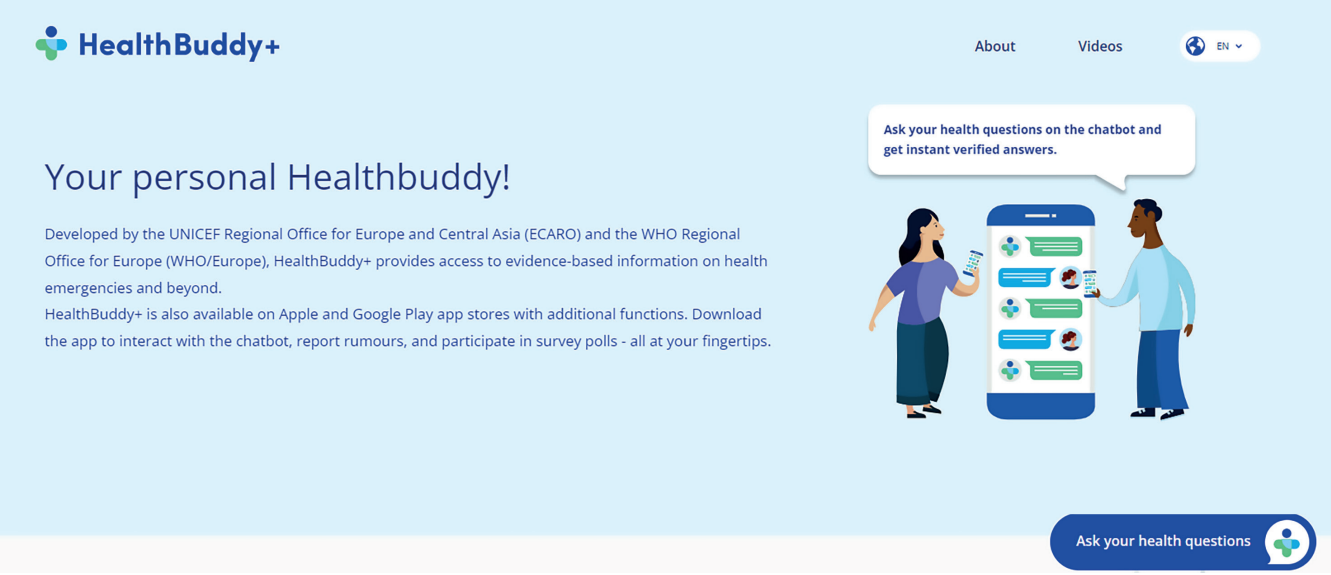

Fig. 1. Screenshot of the HealthBuddy+ chatbot website

analyzed, translated and contextualized by the HealthBuddy+ team at WHO and UNICEF to better understand user needs and improve the chatbot.[14] Our team was also indirectly involved in the improvement presented in this study.

Information about the pandemic, its associated restrictions, protective measures, symptoms, health consequences, and vaccinations had been disseminated through various media.[15] Media such as television, radio and the internet serve not only as essential sources of information but also have the ability to adapt content based on the audience's age or location to ensure effective health communication.[16] This requires an in-depth analysis of the needs of specific populations, their attitudes towards different sources of information, and the potential use of technological solutions such as chatbots for providing health information, which is crucial in such a rapidly evolving technological world.

Objectives

The launch of the HealthBuddy+ personal COVID-19 assistant encouraged us to explore the awareness of solutions such as chatbots among older and younger adults, the use of such solutions, and to assess whether it is considered a reliable source of health information on COVID-19.

The study aimed to compare the attitudes of older and younger adults towards HealthBuddy+ chatbot as a provider of information about COVID-19, to examine what information about COVID-19 both groups are looking for, and determine their eHealth competencies. We also wanted to find out where older and younger students obtain their information about COVID-19 and what other sources they use.

We hypothesized that older and younger adults differ regarding their experiences with chatbots and their attitudes toward them. We also expected that older and younger people will look for different information about COVID-19 and use different sources.

Materials and methods

The study was conducted between February 2022 and July 2022 among 573 consecutive participants: young adults, university students (270), and older adults, University of the Third Age (U3A) listeners (303). The median age of older adults was 73 years (interquartile range (IQR): 69–77), and the median age of young adults was 24 years (IQR: 23–24). The majority of participants in both study groups were female: 85% in the group of older adults and 61% in the group of younger adults. Respondents received the questionnaire in paper form. Before completing the questionnaire, respondents were provided with verbal information on the HealthBuddy+ chatbot in the form of a presentation, accompanied by an instructional video on how to use the chatbot. The questionnaires were distributed to both groups during a recess between classes. A total of 660 surveys were distributed, and 303 were returned from the senior group and 270 from the student group, yielding a 92% and 82% response rate, respectively. The study was conducted in Warsaw and Łódź, 2 cities located in central Poland.

Respondents received a self-administered questionnaire about their interests related to the pandemic and their sources of knowledge about COVID-19. They were also asked whether they had ever interacted with a chatbot and whether they thought it could be a reliable source of information about COVID-19. The questionnaire included additional questions about chronic diseases, vaccination, morbidity and mortality due to COVID-19 among relatives, self-assessment of COVID-19 knowledge, and education level of participants. The 2nd part of the study included an e-HEALS questionnaire in Polish to examine the eHealth competencies of both groups. This questionnaire was developed by Norman et al. in 2006.[17] In the same year, Norman et al. published the results of the level of these competencies in a group of 664 participants (370 boys, 294 girls) aged 13–21 years (mean = 14.95; standard deviation (SD) = 1.24) measured using eHEALS (eHealth Literacy Scale).[18] This scale quickly became a standard tool for assessing eHealth competencies by various internet participants. In 2019, the questionnaire was validated in a study by Duplaga et al.[19] consisting of 2 samples – sample 1 with 1,000 respondents (women and men) aged (mean ±SD) 64.16 ±9.55 years and sample 2 with 1,030 women aged 18–35 years. For sample 1, Cronbach's α coefficients were 0.90 and Guttman's distribution coefficients were 0.89, and for sample 2, Cronbach's α coefficients were 0.88 and Guttman's distribution coefficients were 0.81, confirming the scale's internal consistency. Moreover, Burzyńska et al. examined the Polish version of the eHEALS questionnaire in a representative sample of Polish social media users (n = 1,527, women = 89.8%, mean age 32 ±10.37 years, Cronbach's α = 0.84).[20] We decided to use the eHEALS questionnaire to support our study with a validated survey investigating reasons for the preferred sources of health information and provide a view of the eHealth competencies of participants.

Statistical analyses

The statistical analysis was carried out with Statistica v. 13.0 (StatSoft Inc., Tulsa, USA). The normality of the distribution of continuous variables was verified using the Shapiro–Wilk test. None of the continuous variables (age of participants and results from the eHealth questionnaire) were normally distributed. Continuous data are presented as median and IQR, and categorical variables as number and percentage. The Pearson's χ^2 test or Pearson's χ^2 test with Yates's correction (when at least 1

of the expected values was less than 5) was used to assess differences in categorical variables in both analyzed groups. Mann–Whitney U test was utilized to compare continuous variables. The presentation of test results also includes: χ^2 statistics Pearson's χ^2 test, z statistics Mann–Whitney U test and the degrees of freedom (df). A $p < 0.05$ was considered statistically significant.

Results

The questionnaire was completed by 573 participants: 303 seniors and 270 students. The median age of seniors was 73 years (IQR: 69–77), and the median age of students was 24 years (IQR: 23–24). Women were the majority in both studied groups: 85% in the senior group and 61% in the student group. The older adults attended U3A, while younger adults were university students. All college students had secondary education, while almost half of seniors (46%) had higher education. The experiences with COVID-19 among respondents and their relatives differed in both groups. Among the elderly, those ever diagnosed with COVID-19 represented 18%, hospitalization of a relative or friend accounted for 23%, and 20% reported that a relative or friend had died due to COVID-19. The respective ratios for younger participants were 34%, 24% and 12%. Details are presented in Table 1.

Most older adults in our study have never come across a chatbot (83%) and believe that a chatbot is not a reliable source of information about COVID-19 (33%) or have no opinion in this regard (48%). The young adults believe the opposite – a chatbot may be a reliable form of communicating information about COVID-19 (79%). In this group, 78% had previously encountered a chatbot of any kind (Table 2). Older adults and younger adults in our study were most interested in COVID-19 treatment methods (45.5% and 69.3%, respectively), symptoms of the disease (36.6% and 35.2%, respectively) and chronic diseases coexisting with COVID-19 (35.0% and 51.5%, respectively). Interest in COVID-19 vaccination was twice as high among students as among older adults (58.2% and 29.0%, respectively). These observations were statistically significant ($p < 0.001$) (Table 3). The preferred source of information on COVID-19 for older people was television (84.5%), while for younger people, it was the press and the internet (84.4%). Interestingly, it was younger people rather than older people who preferred information obtained from medical personnel (62.8% and 14.8%, respectively) (Table 4).

Table 1. Sociodemographic characteristics and experiences regarding COVID-19 of study participants

Variable		Older adults		Younger adults	
		n	n (%)	n	n (%)
Sex: women		303	258 (85%)	270	166 (61%)
Age [years], Me (IQR)		294	73 (69–77)	270	24 (23–24)
Educational status	Elementary	302	11 (4%)	270	–
	Secondary		151 (50%)		270 (100%)
	High		140 (46%)		–
Household	I live with other family members	–	157 (52%)	270	122 (45%)
	I live alone		145 (48%)		55 (20%)
	I live with flatmates		–		93 (34%)
COVID-19 experience	Have you ever been diagnosed with COVID-19?	302	55 (18%)	269	92 (34%)
	Has anyone in your surroundings – family, close friends – been hospitalized due to COVID-19?	302	68 (23%)	270	66 (24%)
	Has anyone in your family or close friends died from COVID-19?	301	60 (20%)	270	33 (12%)

Me – median; IQR – interquartile range.

Table 2. Contact with chatbot among older adults and younger adults

Survey questions on chatbot use		Older adults		Younger adults		χ^2 test
		n	n (%)	n	n (%)	
Have you ever come into contact with a chatbot?	Yes	276	46 (17%)	267	207 (78%)	$\chi^2 = 202{,}02$ df = 1 $p < 0.001$
	No		230 (83%)		60 (22%)	
Do you think that an online automated consultant could be a reliable source of information about COVID-19?	Yes	277	53 (19%)	265	209 (79%)	$\chi^2 = 226{,}49$ df = 2 $p < 0.001$
	No		92 (33%)		53 (20%)	
	I don't know		132 (48%)		3 (1%)	

df – degrees of freedom.

Table 3. Which of the following COVID-19 issues interest you most?

Interests regarding COVID-19	Older adults n = 303	Younger adults n = 270	χ^2 test
What is COVID-19	74 (24.4%)	20 (7.4%)	$\chi^2 = 30.141$ df = 1 p < 0.001
COVID-19 symptoms	111 (36.6%)	95 (35.2%)	$\chi^2 = 0.130$ df = 1 p = 0.718
How COVID-19 is spread	73 (24.1%)	42 (15.6%)	$\chi^2 = 6.486$ df = 1 p = 0.011
COVID-19 treatment methods	138 (45.5%)	187 (69.3%)	$\chi^2 = 32.708$ df = 1 p < 0.001
Contact with a person infected with COVID-19	44 (14.5%)	63 (23.3%)	$\chi^2 = 7.300$ df = 1 p = 0.007
Chronic diseases and COVID-19	106 (35.0%)	139 (51.5%)	$\chi^2 = 15.877$ df = 1 p < 0.001
Vaccinations against COVID-19	88 (29.0%)	157 (58.2%)	$\chi^2 = 49.415$ df = 1 p < 0.001
Populations at the highest risk of developing COVID-19	76 (25.1%)	58 (21.5%)	$\chi^2 = 1.033$ df = 1 p = 0.310
Side effects after vaccination	83 (27.4%)	61 (22.6%)	$\chi^2 = 1.748$ df = 1 p = 0.186
Tests for COVID-19	40 (13.2%)	79 (29.3%)	$\chi^2 = 22.374$ df = 1 p < 0.001
Personal protection methods	51 (16.8%)	55 (20.4%)	$\chi^2 = 1.186$ df = 1 p = 0.276

df – degrees of freedom.

To inform participants about the pandemic, we also inquired whether COVID-19 issues are addressed in university classes, including traditional universities and U3A. Among older adults, 17.2% indicated that these classes were a source of knowledge for them, compared to 61% of younger adults (Table 4).

Older adults' eHealth competencies were significantly lower than those of students. The median of the overall eHEALS score was 34 (IQR: 30–39) for older adults and 42 (IQR: 40–47) for students (z = –13.886, df = 422, p < 0.001) (Fig. 2). In particular, intergenerational differences emerged in questionnaire items such as: "I know what health resources are available on the internet", "I know where to find helpful health resources on the internet", "I have the skills I need to evaluate the health resources I find on the internet", "I can tell high-quality health resources from low-quality health resources on the internet", and "I feel confident in using information from the Internet to make health decisions". For all questions of the eHEALS questionnaire, the differences in responses were statistically significant

Table 4. Preferred source of information on COVID-19

Source of knowledge about COVID-19	Older adults n = 291	Younger adults n = 269	Pearson's χ^2 test
TV	246 (84.5%)	46 (17.1%)	$\chi^2 = 254.739$ df = 1 p < 0.001
Radio	83 (28.5%)	15 (5.6%)	$\chi^2 = 50.978$ df = 1 p < 0.001
Press, internet	164 (56.4%)	227 (84.4%)	$\chi^2 = 52.118$ df = 1 p < 0.001
University lectures	50 (17.2%)	164 (61.0%)	$\chi^2 = 113.496$ df = 1 p < 0.001
Medical staff	43 (14.8%)	169 (62.8%)	$\chi^2 = 137.177$ df = 1 p < 0.001
Family	69 (23.7%)	30 (11.2%)	$\chi^2 = 15.150$ df = 1 p < 0.001
Friends	55 (18.9%)	38 (14.1%)	$\chi^2 = 1.970$ df = 1 p = 0.160
Others	7 (2.4%)	18 (6.7%)	$\chi^2 = 5.060$ df = 1 p = 0.025

df – degrees of freedom.

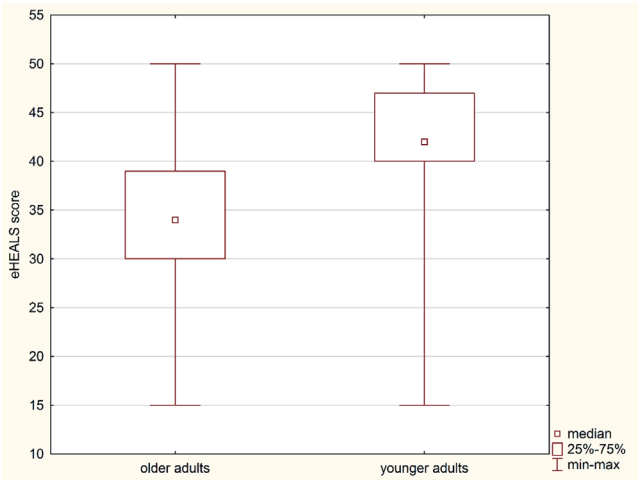

Fig. 2. eHEALS (eHealth Literacy Scale) Score in older adults and young adults

(p < 0.001). Detailed results of the eHealth competencies questionnaire are shown in Table 5.

Discussion

Our study showed that, unlike the younger group, most older respondents had never had contact with any chatbot. One of the reasons may be that older individuals prefer face-to-face interactions with another person, which is confirmed by some studies.[21,22] Others indicate older adults are

Table 5. eHealth competencies of older adults and students measured with the validated eHEALS (eHealth Literacy Scale) questionnaire in Polish

eHEALS	Older adults		Younger adults		Mann–Whitney test
	n	Me (IQR)	n	Me (IQR)	
How useful do you feel the internet is in helping you in making decisions about your health?	261	4 (3–4)	169	4 (4–5)	z = –7.5564 df = 428 p < 0.001
How important is it for you to be able to access health resources on the internet?	251	4 (3–4)	169	5 (4–5)	z = –11.4155 df = 418 p < 0.001
I know what health resources are available on the internet	252	3 (3–4)	167	4 (4–5)	z = –10.8363 df = 417 p < 0.001
I know where to find helpful health resources on the internet	245	3 (3–4)	169	4 (4–5)	z = –11.4367 df = 412 p < 0.001
I know how to find helpful health resources on the internet	244	4 (3–4)	168	4 (4–5)	z = –10.2334 df = 410 p < 0.001
I know how to use the Internet to answer my questions about health	244	4 (3–4)	168	4 (4–5)	z = –9.4799 df = 410 p < 0.001
I know how to use the health information I find on the internet to help me	233	4 (3–4)	169	4 (4–5)	z = –9.2977 df = 400 p < 0.001
I have the skills I need to evaluate the health resources I find on the internet	232	3 (3–4)	168	4 (4–5)	z = –9.1049 df = 398 p < 0.001
I can tell high-quality health resources from low-quality health resources on the internet	231	3 (3–4)	168	4 (4–5)	z = –9.7260 df = 397 p < 0.001
I feel confident in using information from the Internet to make health decisions	232	3 (3–4)	169	4 (3–4)	z = –6.9180 df = 399 p < 0.001

Me – median; IQR – interquartile range; df – degrees of freedom.

less willing to use technological solutions and learn to use them, which is linked to a combination of factors, including the lack of intergenerational activities within the family, difficulties in using devices, screens being too small to use comfortably, or anxiety about using technology.[23]

Moreover, in our study, some older respondents believed that a chatbot could not be a reliable source of information on COVID-19 or had no relevant opinion – which may be caused by the fact that most of them had never interacted with a chatbot before. In contrast, younger respondents had a predominant belief that it could be a reliable tool, while the proportion of younger respondents who have obtained information from a chatbot before was 78%. This may suggest that a lack of conviction results from not using such technological solutions. Interestingly, some other studies indicated that chatbots might be the most favored channel for sharing symptoms related to COVID-19 as they provide anonymity and reliable information.[11,24] Furthermore, it has been shown that chatbots can positively model health attitudes towards COVID-19 vaccination and influence health behavior.[25] Gudala et al. showed that, despite technological barriers, most older adults are sufficiently familiar with chatbot technology, especially those with higher socioeconomic status.[26] As mentioned in the Introduction, some studies indicate that chatbots are well-accepted by older adults and effective in improving their overall wellbeing.[9]

The primary source of information about COVID-19 among older respondents in our study was traditional media, particularly television. These findings are consistent with other studies,[27–29] but it is noteworthy that some respondents used the open question space to ask whether the provided information could be considered reliable. Consequently, they were not convinced that the information provided on television was reliable. Nevertheless, studies showed a positive correlation between the information presented on television regarding prevention, COVID-19 protection measures and health behavior in society, which may indicate that health information should reach older adults through this source.[30,31] The study by Wang et al. used this correlation to teach older adults to use the internet through TV sets connected to internet (Smart TV), which has proven to be an effective tool for the digital inclusion of older adults.[23] Only a small percentage of older respondents (17.2%) reported receiving information about COVID-19 from U3A classes. This is likely related to the fact that many of these institutions suspended their activities during the pandemic.

The technological advances we are witnessing cannot be stopped, so efforts to provide health information should be tailored to the population and vary according to the target group. Reaching out to older adults through traditional media is just one method, but older adults are not a homogeneous group; therefore, activating them in technology-oriented activities should be addressed. Given the vast technological advances between 2020 and 2024, including the development of artificial intelligence (AI), we can see changes in public attitudes toward chatbots and the potential for patient education explored in many studies.[32–34]

Furthermore, older participants indicated they were interested in COVID-19 treatment methods, symptoms of COVID-19 infection and its impact on chronic diseases. The categories selected by older adults suggest that even basic information about COVID-19 is not reaching them. Studies conducted at the beginning of the pandemic showed similar results – older adults were unsure about COVID-19 symptoms[35]; however, it is somewhat concerning as our research was conducted in 2022. There was a lot of information available from various sources, but this may lead to misinformation caused not only by misinformation from social media or the internet but also by the multiplication of misinformation by family members who pass it on to older relatives.[36] Topics related to vaccination were of more interest to younger respondents, which corresponds with the study by Elsner et al. conducted among high school students.[37] In another study conducted in Germany, students expressed the greatest interest in the spread of SARS-CoV-2 (89.6% of respondents), pandemic-related restrictions (85.9%) and personal protective measures (45.5%).[38] This may depend, in part, on the time in which the study was conducted, health policy changes occurring in the countries during the pandemic, or waves of infection.

One of the reasons for not utilizing technological solutions such as chatbots among older adults may be inadequate eHealth competencies. Thus, our study also examined the eHealth competencies of respondents. Results from the eHEALS questionnaire showed that the eHealth literacy of older adults was lower than that of the young adult population. Studies confirm that the eHealth competencies of older adults are low.[39–41] Although our older participants mainly had secondary or higher education, their eHealth competencies were still low, unlike in other studies[42–45] that found an association between low eHealth competencies and lower levels of education. Low levels of eHealth competencies may also be linked to poor digital competencies,[46] and both pose serious barriers to the use of technological solutions such as chatbots.

Limitations

This study has several limitations that could affect its results. First, our research was conducted on a non-randomized convenience sample. Therefore, the findings cannot be generalized to the entire population. Second, both analyzed groups were specific. Older respondents who were U3A listeners were assumed to be more open to acquiring knowledge and more educated than the average older adults. The students, in turn, were mainly medical school students, which may have an impact on their knowledge and information-seeking methods on medical topics. Third, the author's questionnaire used in the study was not validated, and no pilot study was conducted. Fourth, the questionnaires were self-administered, which may have led to self-report bias, e.g., false or inaccurate answers, although the researchers supervised the completion of the questionnaires and respondents answered questions about the questionnaires. Fifth, most of the respondents were women, but some studies suggest that gender is unlikely to affect willingness to use chatbots.[47]

Conclusions

Our study showed that despite the technological advances observed during the COVID-19 pandemic in disseminating information to different audiences, older adults still prefer to receive information through traditional media such as television. The categories of COVID-19-related areas of interest indicated by the older adults and the questions included in the questionnaire suggest that even basic information about the disease and the virus still needs to be improved. The level of eHealth competencies of the older adults and responses to the chatbot questions suggest that there is still a lot of work to be done to narrow the gap between the eHealth competencies of the younger and older generations. Digital health skills among older adults require attention and appropriate interventions. Given the positive impact of chatbots on the health behavior of older adults, workshops and exercises for seniors on the informed use of these applications should be considered, as well as greater involvement of older adults in activities on the use of technology – smartphones, computer, software – to ensure that they are not left behind in the process of technological progress that continues unabated. Intergenerational activities would also be a viable approach regarding health technology education and preventing social isolation. Future research should, therefore, focus on these issues, taking advantage of the new opportunities offered by AI.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Mapping the evolution of mitochondrial dynamics research: A bibliometric analysis of global trends and collaborations

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Abstract

Background. Mitochondrial dynamics is an important field in cell biology, encompassing mitochondrial fission and fusion. The balance between fission and fusion is responsible for the stability of the mitochondrial network and can be a regulator of mitochondrial function. Recent studies have emphasized that an imbalance in mitochondrial dynamics is the root cause of dysfunction and is involved in various stages, such as oxidative stress, inflammation and apoptosis. Reversing this imbalance can effectively alleviate disease conditions. Although the importance of mitochondrial dynamics has been widely recognized, there is still a lack of literature on the qualitative and quantitative description and analysis of advances in this field.

Objectives. This study is a bibliometric analysis of research trends, collaboration networks and thematic evolution in mitochondrial dynamics from 2000 to 2023.

Materials and methods. Using the Web of Science Core Collection (WoSCC) database, we performed a bibliometric review, applying VOSviewer and CiteSpace to visualize and analyze publications, citations, collaborations, and key word trends.

Results. We analyzed 332 publications, identifying China and the USA as leaders in research output and international collaborations. Significant contributions were made by institutions like Chiang Mai University and the California Institute of Technology (Caltech), with major research shifts from basic mitochondrial functions to roles in diseases like Alzheimer's and cardiovascular disease.

Conclusions. Mitochondrial dynamics research has expanded, with increasing attention to its role in disease mechanisms. Future research should further explore these connections, potentially leading to innovative treatments.

Key words: mitochondrial dynamics, bibliometrics, VOSviewer, CiteSpace, Science Citation Index

Introduction

Mitochondrial dynamics, a field within cell biology focused on the morphological variability of mitochondria, primarily encompasses mitochondrial fission and fusion. In 1898, Benda first observed the morphological diversity of mitochondria, which can be presented in either spherical or filamentous shapes.[1] Subsequently, in 1914, Lewis and Lewis discovered that mitochondria could change from one form to another following fission or fusion, thereby establishing the fundamental concept of mitochondrial dynamics.[2] Dynamin-related protein 1 (Drp1) is the principal protein mediating mitochondrial fission, recruited to the outer mitochondrial membrane (OMM) by mitochondrial dynamics proteins 59 (MiD59) and MiD50, where it aggregates to form interlocking structures.[3] In conjunction with fission 1 (Fis1) on the OMM, these aggregates constrict and sever the mitochondrion, producing 2 separate entities. Beyond transporting intramitochondrial components, fission is vital for isolating damaged mitochondrial segments to maintain a healthy mitochondrial network, which is crucial in the process of mitochondrial autophagy.[4] Mitochondrial fusion, divided into OMM fusion and inner mitochondrial membrane (IMM) fusion, is mediated by mitofusion 1/2 (Mfn1/2) and optic atrophy 1 (Opa1), respectively. Fusion allows mitochondria to share components, compensating for each other's deficiencies. Contrarily, the enhanced oxidative phosphorylation (OXPHOS) levels in a fused state aid in maintaining energy output under cellular stress. The balance between fission and fusion governs the stability of the mitochondrial network and dictates mitochondrial function.[5] Recently, mitochondrial dysfunction has been implicated in the pathogenesis of various diseases, making mitochondrial dynamics an emergent research focus. Imbalances in mitochondrial dynamics are at the root of dysfunction, contributing to oxidative stress, inflammation and apoptosis; rebalancing these dynamics may potentially alleviate these conditions.

For instance, in neurodegenerative diseases such as Alzheimer's disease (AD), the application of interventions that improve mitochondrial dynamics, such as antioxidants, has been shown to improve cognitive function in patients.[6] Mitochondrial dysfunction, such as oxidative stress, can stimulate the release of neurotransmitters from neurons, thus playing a protective role for neurons.[7] Mitochondrial dynamic imbalances often accompany disease states, which lead to mitochondrial structural disarray, contributing to disease onset.[8] Mitochondrial dynamic dysfunction causes abnormal oxidative stress in neurodegenerative diseases, increasing free radicals, which leads to neuronal changes, such as reduced membrane permeability and decreased neuronal excitability, making it an important therapeutic target.[9,10] Additionally, mitochondrial dynamics play a critical role in synaptic function, neurotransmitter release and axonal transport. For example, the activation of mitochondrial fission promotes mitochondrial fragmentation in the medial prefrontal cortex, leading to mitochondrial dysfunction, impairing excitatory synaptic transmission and contributing to stress-related depressive-like behaviors.[11] When quality control proteins like PINK1 in mitochondria are dysregulated, it leads to fewer axonal vesicles, abnormal synaptic connections and reduced neurotransmitter release, all of which are essential for neural circuit formation and synaptic efficacy.[12] Hence, mitochondrial dynamics is considered a promising research target.

Objectives

Bibliometric analysis is a method that qualitatively and quantitatively describes and analyzes the progress of a particular discipline or research area. With modern technology, results can be visualized using knowledge maps, making the outcomes more comprehensive, aiding in data interpretation and revealing inherent connections between pieces of information. To our knowledge, no bibliometric analysis of mitochondrial dynamics has been conducted so far. Therefore, we undertook a systematic analysis to explore the state and trends of mitochondrial dynamics research from 2000 to 2023.

Materials and methods

Data collection and analysis of mitochondrial dynamics research: A comprehensive search based on the WoSCC database

This study sourced data from the Web of Science Core Collection (WoSCC) database as of June 19, 2023. The WoSCC, widely regarded as the most authoritative database in bibliometrics, encompassing the Science Citation Index Expanded (SCIE) and the Social Sciences Citation Index (SSCI). To delve into the latest research trends in mitochondrial dynamics, we employed a precise search formula: [TS = (mitochondrial dynamics)] AND [Publication Type = (article)] AND [Language = (English)]. Considering that English literature published in English is more likely to be recognized and evaluated by the international academic community due to global usage of this language, we have added a language filter to the search strategy. This search spanned from 2000 to 2023 (Table 1), with the goal of comprehensively capturing scientific advances in mitochondrial dynamics during this period.

The retrieved data included various information such as titles, authors, keywords, abstracts, institutions, countries, languages, and cited references. All these data were downloaded as "full records and cited references" for subsequent in-depth analysis and research.[13] This study

Table 1. Summary of data source and selection

Category	Specific standard requirements
Research database	Web of Science Core Collection
Citation indexes	SSCI and SCIE
Searching period	January 2000 to June 2023
Language	"English"
Searching keywords	"Mitochondrial dynamic"
Document types	"Articles"
Data extraction	Export with full records and cited references in plain text format
Sample size	332

SCIE – Science Citation Index Expanded; SSCI – Social Science Citation Index.

aimed to identify research hotspots and trends in mitochondrial dynamics and analyze the contributions and collaboration patterns of different countries and institutions in this field.

Bibliometric analysis of mitochondrial dynamics research using VOSviewer and CiteSpace tools

In the data analysis phase, the filtered data were imported into 2 specialized bibliometric analysis and visualization tools, VOSviewer v. 1.6.19 (Centre for Science and Technology Studies, Leiden University, Leiden, the Netherlands) and CiteSpace v. 6.1.R3 (College of Computing & Informatics, Drexel University, Philadelphia, USA), which employ a probability-based data normalization method and offer a variety of display methods. These visualization methods, based on key word analysis and co-authorship networks, aid in gaining a deeper understanding of the research trends and critical nodes in mitochondrial dynamics.

CiteSpace 6.1.R3 is an application based on set theory data normalization methods. It provides a visual overview of the research progress in mitochondrial dynamics by analyzing the similarity of the most strongly cited burst keywords extracted from the research. Additionally, CiteSpace can identify current research frontiers in the field, offering valuable insights for future research directions.[13–15] To gain a deeper understanding of the global research landscape, we also employed the dual-map analysis method to examine the scientific citation network in mitochondrial dynamics worldwide. This approach visualizes the citation patterns and information flow between different academic disciplines, helping to reveal the interdisciplinary nature of mitochondrial dynamics research and the extent of international collaboration. This analysis provides a panoramic view of the core nodes in the academic network and their influence.

The combined use of these 2 tools reveals the core themes and trends in mitochondrial dynamics research and displays the dynamic changes in academic collaboration networks and research hotspots. Such analysis enables a deeper understanding of the research field's development trajectory, providing valuable references and guidance for future studies. These results are crucial in discussing how to promote academic exchange and collaboration in the field of mitochondrial dynamics and how to effectively share research findings globally.

Results

Trends and future prospects in mitochondrial dynamics research

After an extensive search of the WoSCC database, 359 papers were initially identified. Following further screening and evaluation, 332 articles were ultimately included in this study (Fig. 1). We found that the total number of articles related to mitochondrial dynamics has shown an upward trend (Fig. 2). It reflects the growing emphasis on scientific research in this field and indicates that there may be more breakthroughs and discoveries in the future. Moreover, this rising trend may also suggest that the research foundation in this area is being strengthened, the research community is expanding, and research methods and technologies are continuously advancing.

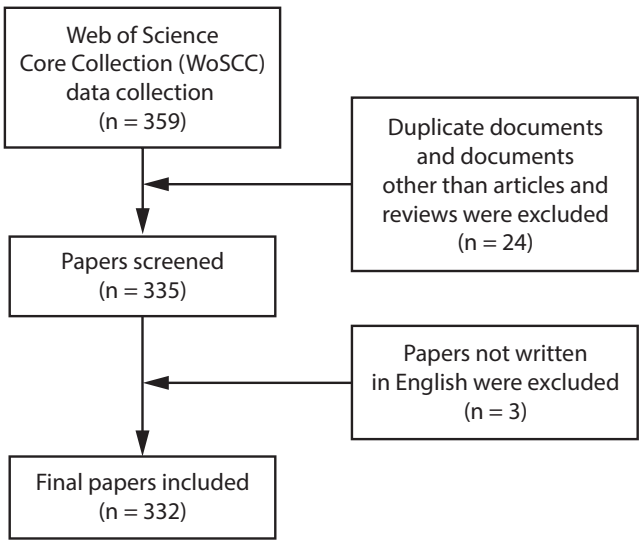

Fig. 1. The literature screening process from the initial 359 papers collected from the Web of Science Core Collection to the final inclusion of 332 papers for the study, after exclusions due to duplicates, document types and language criteria

We also observed a decline in the number of articles in 2018 and 2023 (Fig. 2), which could be attributed to various factors, such as fluctuations in research funding, changes in science policies or a shift in research interests. Notably, the final publication count for 2023 is expected to increase, as the data currently cover only the 1st half of the year. Therefore, research on mitochondrial dynamics will continue in the coming years, yielding more academic contributions and clinical applications.

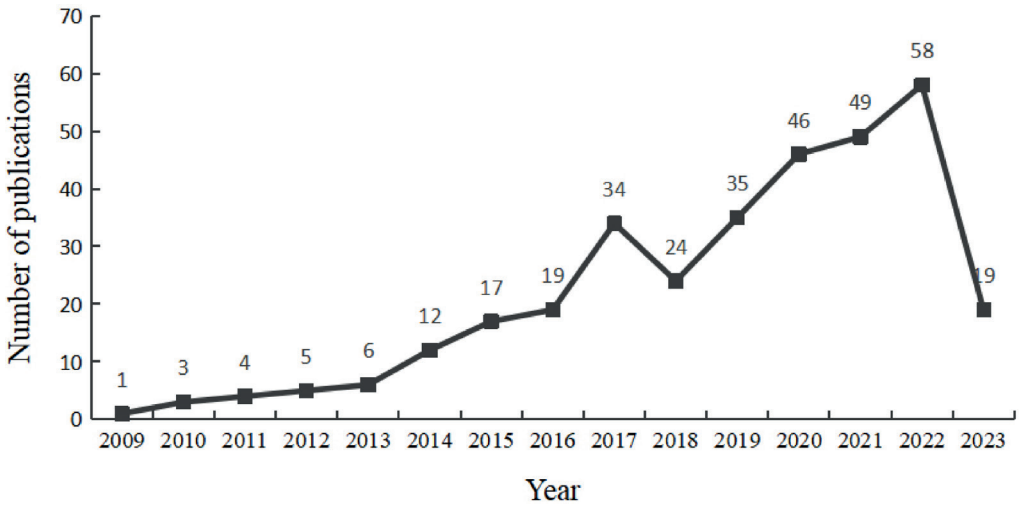

Fig. 2. Trends in mitochondrial dynamics research publications. The number of publications related to mitochondrial dynamics from 2009 to 2023

Table 2. The top 10 countries in terms of the number of publications

Rank	Country	Publications	Citations	Average citations/publications
1	China	196	4,508	23
2	USA	67	2,989	44.61
3	Italy	17	799	47
3	Thailand	17	230	13.53
5	Spain	11	250	22.73
6	France	10	177	17.7
7	South Korea	9	171	19
8	Brazil	8	146	18.25
8	Iran	8	90	11.25
10	UK	7	207	29.57

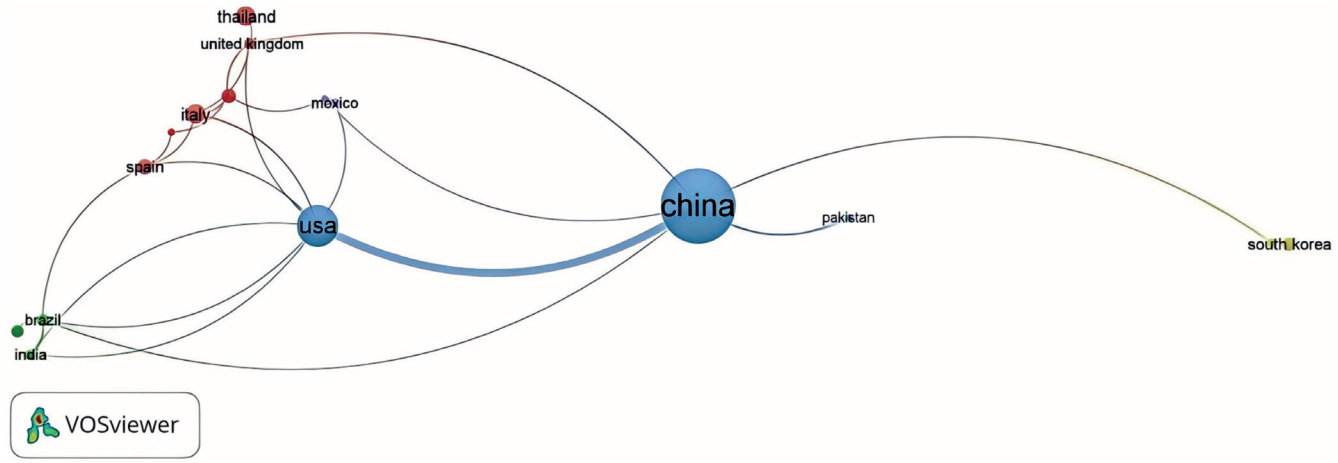

Fig. 3. Global collaboration in mitochondrial dynamics research. Country collaboration map. The circle size represents the number of publications, and lines denote their collaborations. The dots represent countries that have published relevant literature, with the size of the dots reflecting the level of contribution to the field and the number of publications. The lines connecting the dots represent collaborations between different countries

International collaboration and academic contributions in global mitochondrial dynamics research

In this study, 332 papers from 40 countries were analyzed. Table 2 lists the top 10 countries with the most published papers. In mitochondrial dynamics, China leads in publication quantity and citation count, followed by the USA and Italy, which also boast significant achievements (Fig. 3). However, the average citation count for the USA and Italy exceeds 40, approximately double that of China. The UK also achieved an average citation count

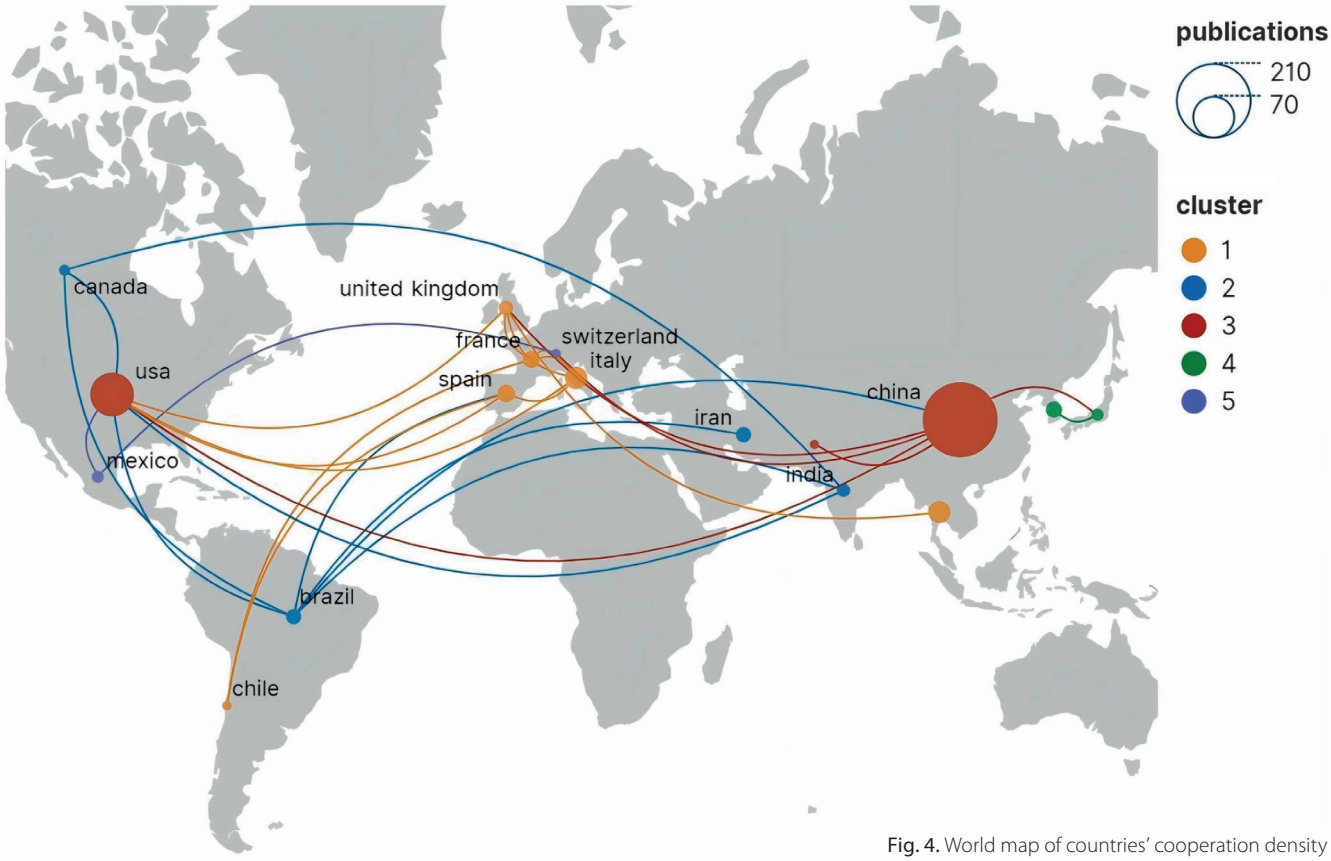

Fig. 4. World map of countries' cooperation density

of 29.57, indicating a higher quality of scientific research in these countries.

Further analysis involved 17 countries with more than three publications, showcasing their publication distribution (Fig. 4,5) and the strength of collaboration between countries (Fig. 6). Collaborations in mitochondrial dynamics are primarily between China and the USA, with other countries needing to strengthen their collaborative ties.

These data reveal the global development and scientific collaboration networks in mitochondrial dynamics research. While China dominates in publication volume, the high citation counts of the USA and Italy reflect their research quality and international influence. Despite fewer publications, other countries, such as the UK, also show significant impact through high average citation counts.

Overall, these findings suggest that while countries vary in their contributions to mitochondrial dynamics research, further strengthening international cooperation and exchange is vital to advancing the field. Enhanced collaboration among nations can foster knowledge sharing, technological exchanges and innovative ideas.

Global institutional distribution and collaboration trends in mitochondrial dynamics research

In this study, 482 institutions contributed to the publication of 332 articles. Table 3 lists the top 10 institutions by the number of published articles and citation counts. Chiang Mai University (Thailand) leads with 16 publications, followed by the Chinese Academy of Sciences (CAS). North Carolina Central University (USA) and several Chinese universities, such as Ningxia Medical University and Shanghai Jiao Tong University, have significantly contributed to mitochondrial dynamics research. The CAS has the highest average citation count, indicating its papers' exceptional scientific value. Since mitochondrial dynamics is a relatively new research area, we have yet to detect strong collaboration among institutions (Fig. 7). Closer collaboration is observed among Chinese universities, likely due to geographical proximity.

These findings reflect the expanding global reach of mitochondrial dynamics research, with varying contributions from institutions worldwide. The prominent roles of Chiang Mai University and CAS and other Chinese universities' active participation demonstrate Asia's significant impact and influence in this research area. In particular, the high citation rate of CAS highlights its leadership in scientific research and academic guidance.

Moreover, the study reveals a notable trend: despite being an emerging research field, tight collaboration among institutions has yet to be become prominent. It may be due to the development stage of this field, with institutions still exploring and establishing collaboration networks. Therefore, promoting international and cross-institutional collaboration, especially in knowledge sharing and resource

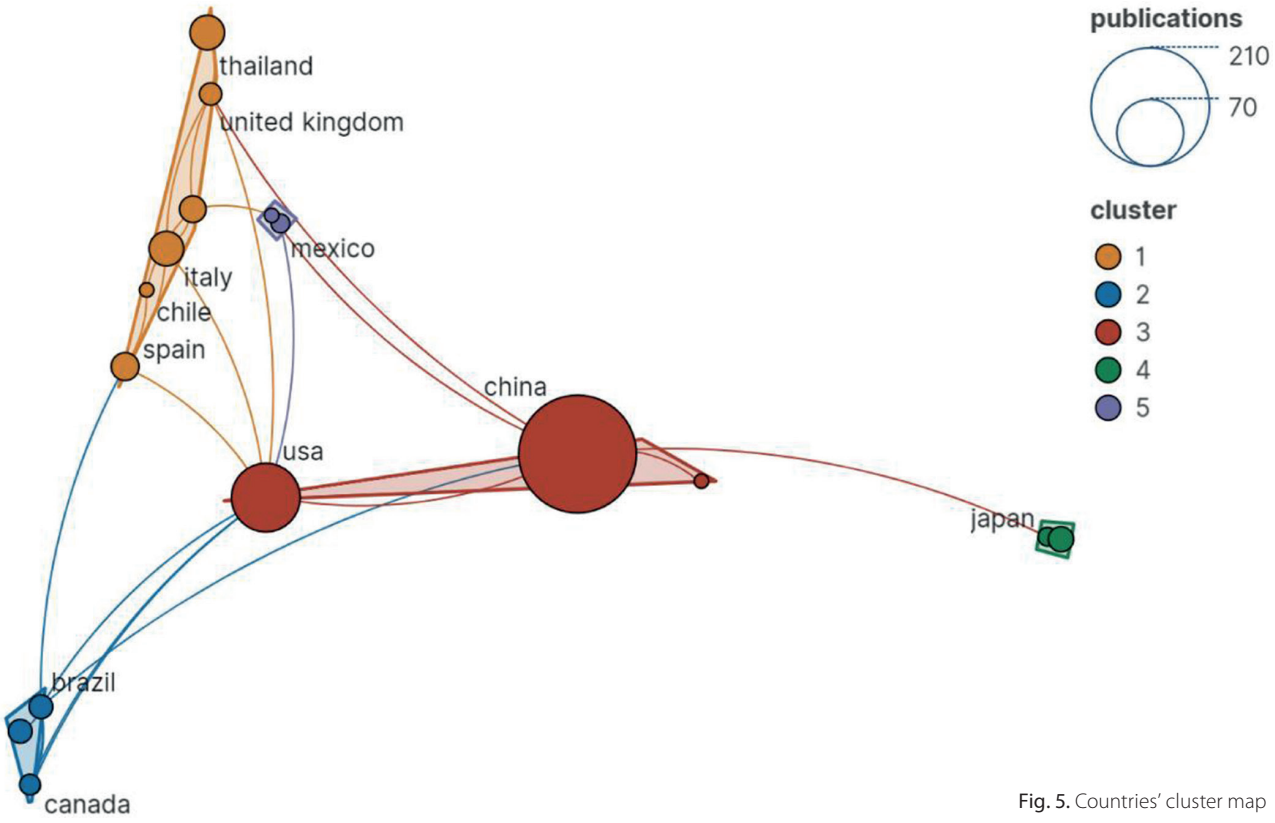

Fig. 5. Countries' cluster map

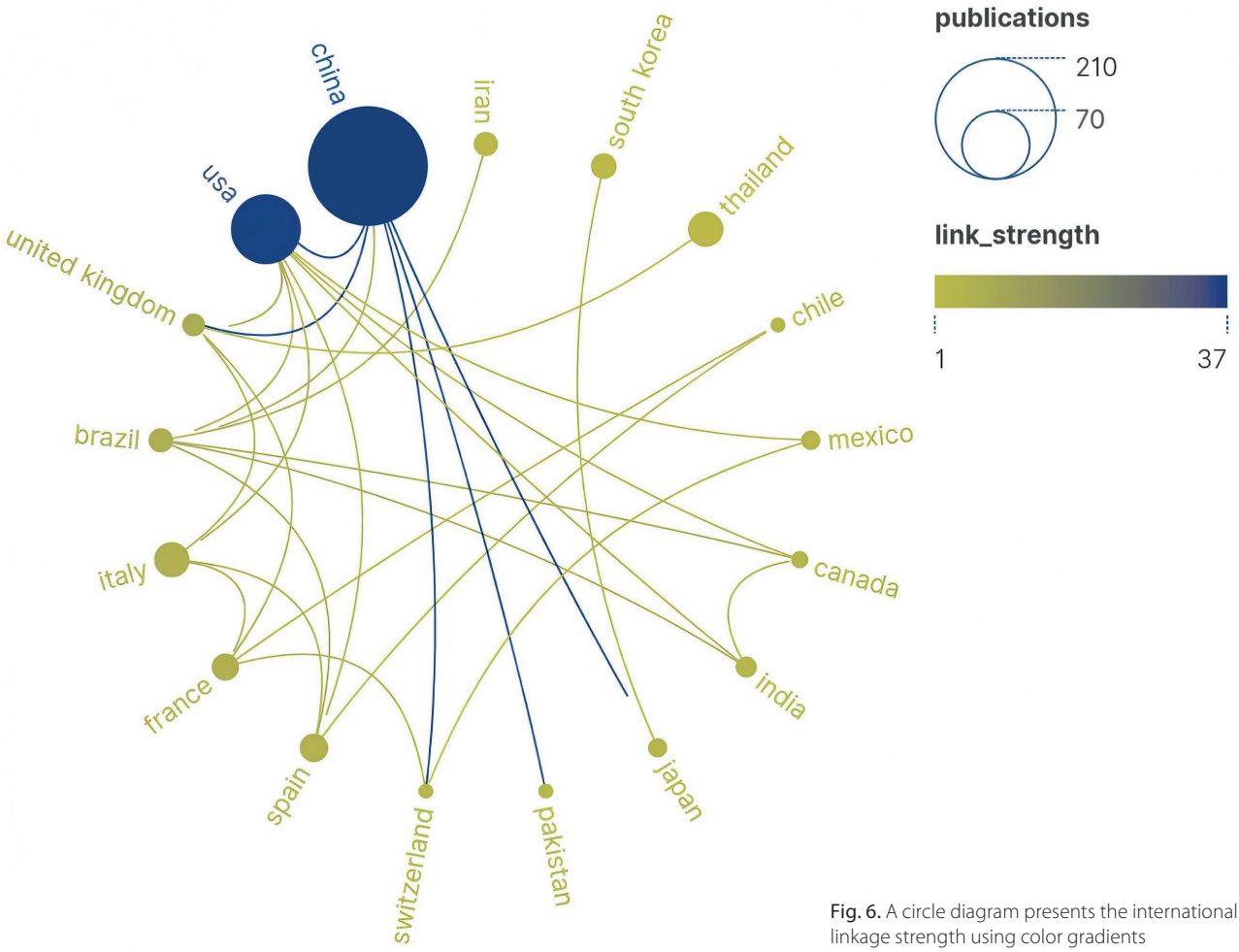

Fig. 6. A circle diagram presents the international linkage strength using color gradients

Table 3. Top 10 institutions conducting mitochondrial dynamics

Rank	Country	Publications	Citations	Average citations/publications
1	Chiang Mai University, Chiang Mai, Thailand	16	218	13.63
2	Chinese Academy of Sciences, Beijing, China	13	683	52.54
3	Ningxia Medical University, Yinchuan, China	10	304	30.4
4	Shanghai Jiao Tong University, Shanghai, China	10	290	29
5	Xi'an Jiao Tong University, Xi'an, China	8	378	47.25
6	Shandong University, Jinan, China	8	312	39
7	North Carolina Central University, Durham, USA	8	268	33.5
8	Fourth Military Medical University, Xi'an, China	8	224	28
9	Fudan University, Shanghai, China	8	192	24
10	Sun Yat-sen University, Guangzhou, China	8	187	23.38

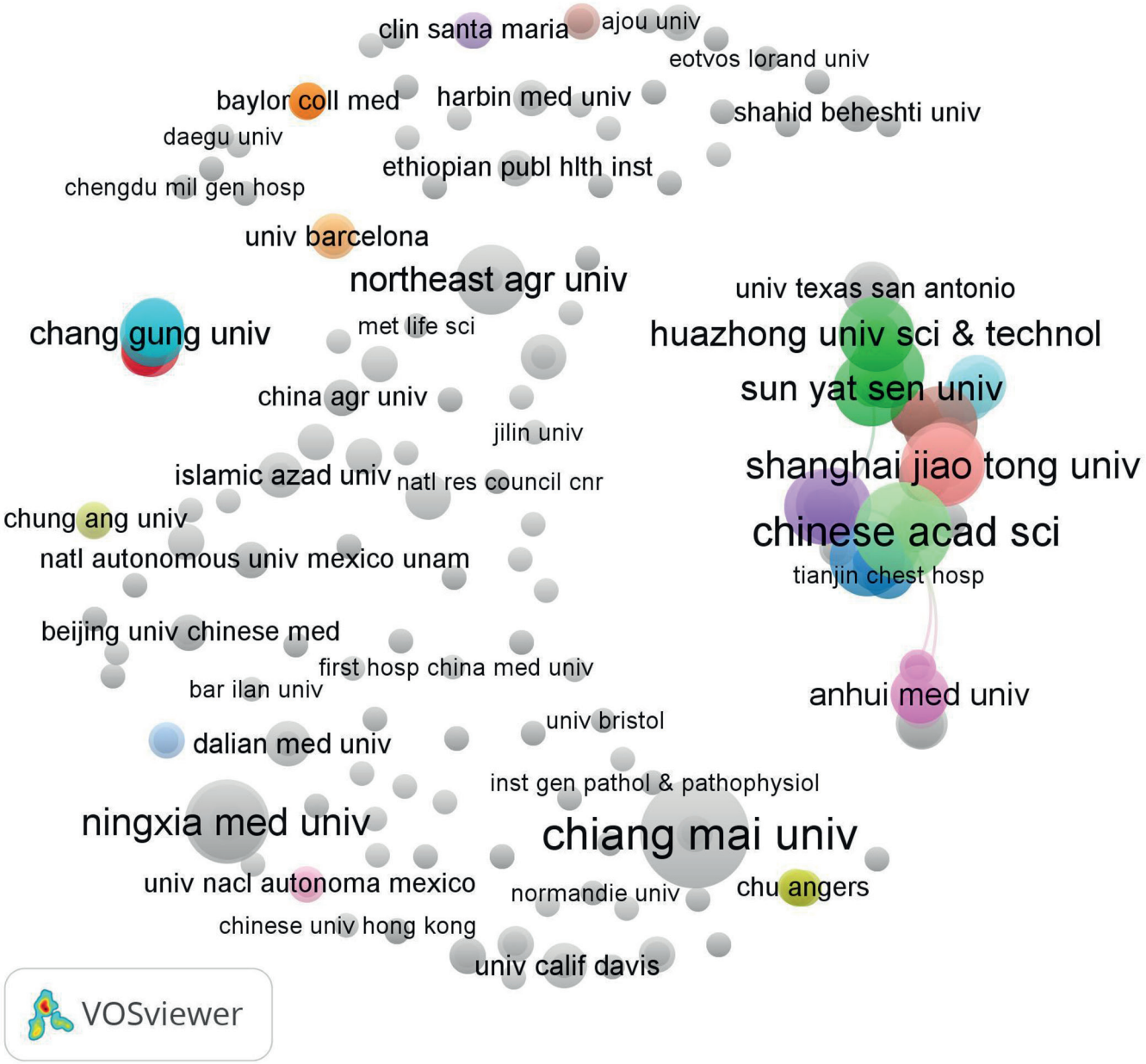

Fig. 7. Institutional contributions to mitochondrial dynamics research. The visual representation of the 482 institutions that have contributed to the publication of 332 articles in the field of mitochondrial dynamics

integration, is crucial for advancing the field of mitochondrial dynamics. As this area of research evolves, we anticipate more transnational and cross-institutional collaborations, leading to innovative outcomes and academic progress in this area.

Core authors and their academic impact on mitochondrial dynamics research

The 332 articles analyzed in this study encompass 2,227 authors (Fig. 8). Following Lotka's law,[16] authors with more than 2.8 publications were defined as core authors (Fig. 9,10), with the top 10 listed in Table 4. Notably, Nipon Chattipakorn from Chiang Mai University top the list with 16 publications, followed by Siriporn Chattipakorn (15 papers) and Andy P. Li (9 papers). It aligns with the results in Table 3, underscoring the significant influence of Nipon Chattipakorn and his team in the field of mitochondrial dynamics (Fig. 9).

Co-cited authors are those cited by at least 2 authors simultaneously. Among the 12,988 co-cited authors, Hsiu-Chen Chen leads with 116 citations (Supplementary Fig. 1,2). Only this author had more than 100 citations in this emerging research area (Table 4).

These data indicate a group of core authors driving the research frontier in mitochondrial dynamics. The significant achievements of Nipon Chattipakorn and his team at Chiang Mai University highlight their vital role in the field. Meanwhile, Hsiu-Chen Chen, as the most co-cited author, has widespread influence and recognition in mitochondrial dynamics research (Fig. 10).

These findings not only showcase the core academic strengths in mitochondrial dynamics but also identify the primary sources of knowledge dissemination and academic impact. As research deepens, these core authors and widely cited studies are expected to continue playing a pivotal role in driving the development of mitochondrial dynamics research. It also emphasizes establishing broader academic exchanges and collaboration networks to foster knowledge sharing and innovation.

Extensive distribution and impact of mitochondrial dynamics research in academic journals

Our study identified 332 articles on mitochondrial dynamics published across 198 academic journals (Supplementary Fig. 3,4). Table 5 lists the top 10 journals by the number of articles in mitochondrial dynamics, with *Oxidative Medicine and Cellular Longevity* and *Frontiers in Pharmacology* leading with 10 articles, followed by *International Journal of Molecular Sciences* with 9 articles.

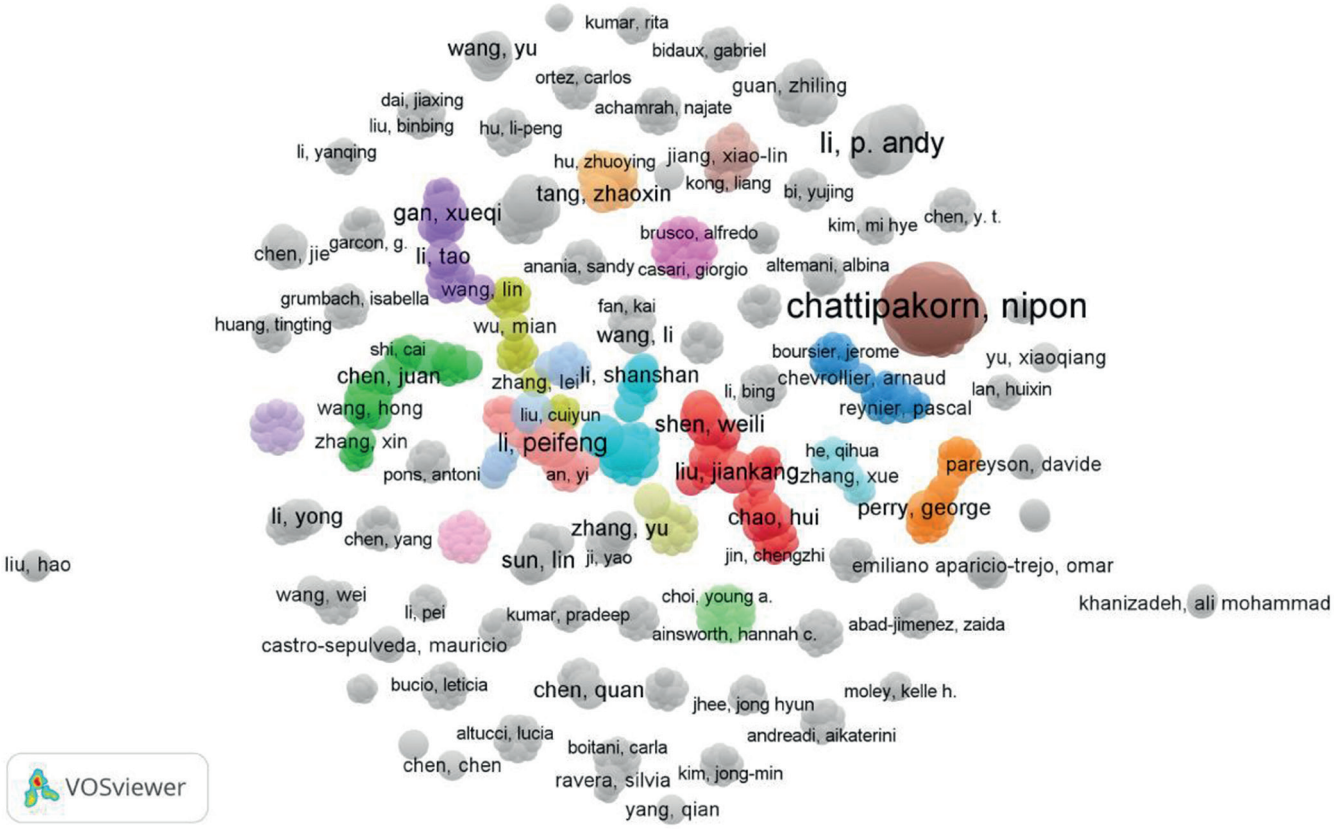

Fig. 8. Mapping the academic influence in mitochondrial dynamics research. A series of visualizations related to authorship in mitochondrial dynamics. The author co-occurrence network

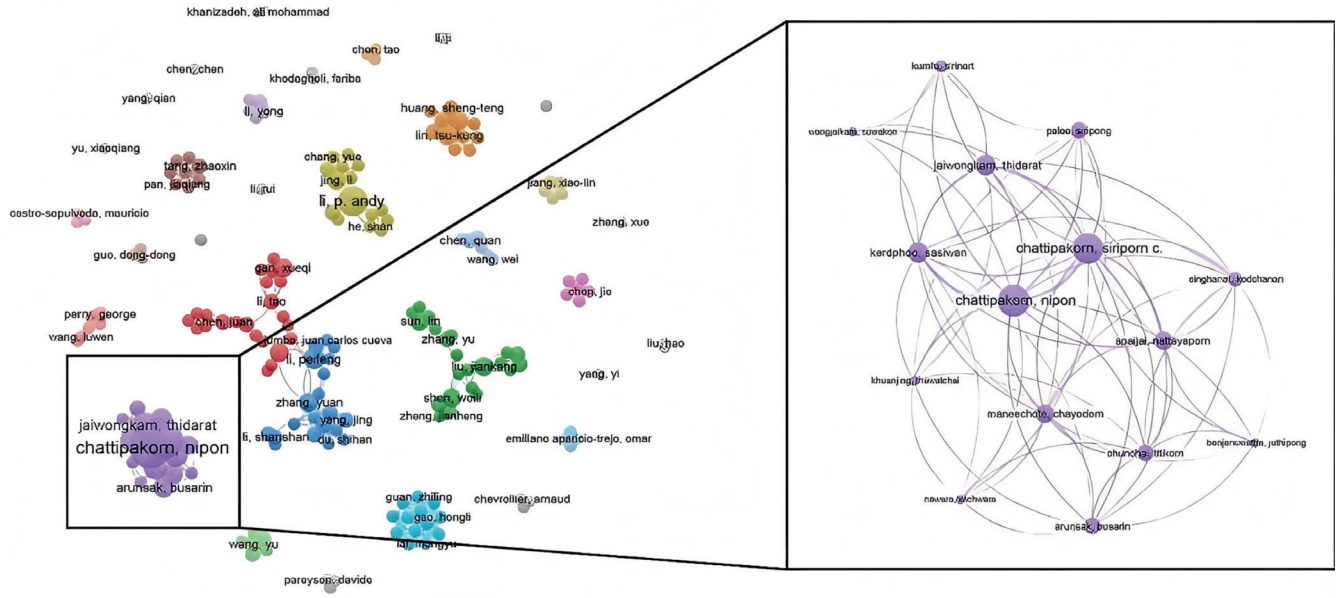

Fig. 9. Lotka's law defines the core authors as those with more than 2.8 publications

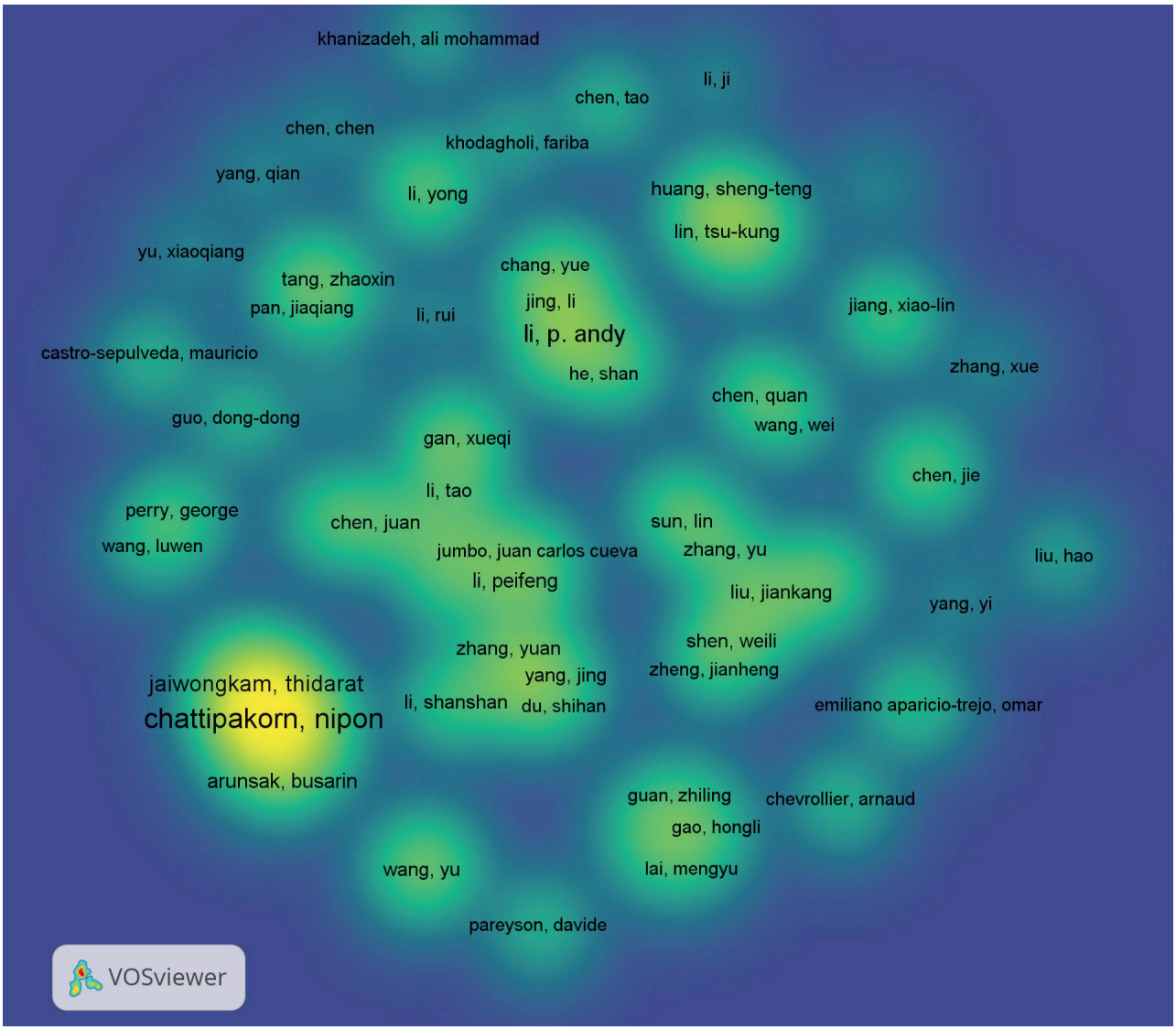

Fig. 10. Density visualization of core authors

Table 4. Top 10 authors and co-cited authors related to mitochondrial dynamics

Rank	Author	Publications	Citations	Co-cited author	Citations
1	Chattipakorn, Nipon	16	218	Chen, Hsiu-Chen	116
2	Chattipakorn, Siriporn C	15	197	Chan, David C	68
3	Li, Andy P.	9	253	Youle, Richard J	62
4	Jaiwongkam, Thidarat	7	119	Twig, Gilad	56
5	Kerdphoo, Sasiwan	7	99	Wang, Xiao Le	54
6	Apaijai, Nattayaporn	6	49	Ong, Sang-Bing	49
7	Maneechote, Chayodom	6	35	Westermann, Benedikt	46
8	Palee, Siripong	5	89	Manczak, Maria	45
9	Li, Peifeng	5	72	Otera, Hidenori	40
10	Chunchai, Titikorn	5	12	Smirnova, Ekaterina	40
10	Arunsak, Busarin	5	12		

Table 5. Top 10 journals related to mitochondrial dynamics

Rank	Journal	Publications	Citations	IF (2021)	JCR
1	*Oxidative Medicine and Cellular Longevity*	10	260	7.31	Q1
2	*Frontiers in Pharmacology*	10	33	5.988	Q1
3	*International Journal of Molecular Sciences*	9	113	2.598	Q2
4	*Biochemical and Biophysical Research Communications*	7	124	3.322	Q3
5	*Mitochondrion*	7	101	4.534	Q2
6	*Free Radical Biology and Medicine*	6	269	8.101	Q1
7	*Scientific Reports*	6	144	4.997	Q2
8	*Cells*	6	60	7.666	Q2
9	*Ecotoxicology and Environmental Safety*	5	84	7.129	Q1
10	*PLoS One*	4	199	3.752	Q2

IF – impact factor; JCR – Journal Citation Reports.

Table 6. Top 10 co-citation journals related to mitochondrial dynamics

Rank	Journal	Citations	IF (2021)	JCR
1	*Journal of Biological Chemistry*	637	5.485	Q1
2	*Proceedings of the National Academy of Sciences of the United States of America*	497	12.779	Q1
3	*Cell*	383	66.850	Q1
4	*Journal of Cell Biology*	368	8.077	Q1
5	*PLoS One*	354	3.752	Q2
6	*Nature*	326	69.504	Q1
7	*Science*	306	63.832	Q1
8	*Human Molecular Genetics*	243	5.121	Q1
9	*The EMBO Journal*	234	13.783	Q1
10	*Free Radical Biology and Medicine*	229	8.101	Q1

IF – impact factor; JCR – Journal Citation Reports.

Furthermore, these 332 papers collectively cited 2,377 journals, with the top 100 most-cited journals displayed in Supplementary Fig. 5,6. Table 6 lists the top 10 co-cited journals, including prestigious publications like *Cell*, *Nature* and *Science*. Notably, up to 90% of these top co-cited journals are ranked in the 1st quartile (Q1) of the Journal Citation Reports (JCR).

These data indicate that the academic outputs in mitochondrial dynamics are widely distributed across various scientific journals, encompassing multiple disciplines and research directions. Journals such as *Oxidative Medicine and Cellular Longevity*, *Frontiers in Pharmacology* and *International Journal of Molecular Sciences* significantly advance research, with numerous influential articles published.

The co-cited journals reveal the close association of mitochondrial dynamics research with other areas of life sciences, particularly in top-tier Q1 journals like *Cell*, *Nature* and *Science*. Their high citation counts underscore the importance and influence of mitochondrial dynamics research in life sciences, reflecting its wide recognition and esteem within the scientific community.

Mitochondrial dynamics research is published in multiple high-impact scientific journals, significantly contributing to knowledge dissemination and scientific communication within the field. As research progresses, this field will continue to showcase its findings in top-tier scientific journals, thereby advancing development and progress in the broader field of life sciences.

Analysis of key literature and future trends in mitochondrial dynamics research

Among the 332 articles on mitochondrial dynamics, the most cited is "Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling" (450 citations),[17] followed by "Sirtuin 3-dependent mitochondrial dynamic improvements protect against acute kidney injury" (282 citations)[18] and "Disruption of renal tubular mitochondrial quality control by myo-inositol oxygenase in diabetic kidney disease" (187 citations)[19] (Table 7[17–26]; Supplementary Fig. 7,8). These highly cited papers primarily focus on the vital roles of mitochondria in life activities such as energy production, metabolism, apoptosis, and intracellular signaling, as well as the impact of mitochondrial dynamics imbalance in diseases like acute kidney injury, neurodegenerative disorders, diabetes, and obesity.[20–26]

The relationships between co-cited references represent connections formed when an article published later cites 2 papers. VOSviewer visualizes co-cited references related to mitochondrial dynamics (Supplementary Fig. 9,10).[26] "Mitochondrial fission, fusion, and stress" by Youle and Van Der Bliek, published in *Science*, is among the top 10 most cited references (Table 8[3,4,27–34]). As a critical review article, it elaborates on the definitions of the 2 main aspects

Table 7. The top 10 cited references related to mitochondrial dynamics

Rank	Cited references	Authors	Year	Citations
1	Sirtuin regulation of mitochondria: Energy production, apoptosis, and signaling[17]	Verdin et al.	2010	450
2	Sirtuin 3-dependent mitochondrial dynamic improvements protect against acute kidney injury[18]	Morigi et al.	2015	282
3	Disruption of renal tubular mitochondrial quality control by myo-inositol oxygenase in diabetic kidney disease[19]	Zhan et al.	2015	187
4	Mitochondrial biogenesis in neurodegeneration[20]	Li et al.	2017	179
5	Mitochondrial membrane potential is required for MAVS-mediated antiviral signaling[21]	Koshiba et al.	2011	172
6	Butyrate regulates liver mitochondrial function, efficiency, and dynamics in insulin-resistant obese mice[22]	Mollica et al.	2017	168
7	Abnormalities of mitochondrial dynamics in neurodegenerative diseases[23]	Gao et al.	2017	150
8	Central role of mitofusin 2 in autophagosome-lysosome fusion in cardiomyocytes[24]	Zhao et al.	2012	149
9	Maternal metabolic syndrome programs mitochondrial dysfunction via germline changes across three generations[25]	Saben et al.	2016	138
10	Diet impact on mitochondrial bioenergetics and dynamics[26]	Putti et al.	2015	112

Table 8. The top 10 co-cited references related to mitochondrial dynamics

Rank	Co-cited references	Authors	Year	Citations
1	Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission[3]	Losón et al.	2013	25
2	Mitochondrial fusion and fission in cell life and death[4]	Westermann	2010	33
3	Fission and selective fusion govern mitochondrial segregation and elimination by autophagy[27]	Twig et al.	2008	40
4	Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development[28]	Chen et al.	2003	36
5	Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells[29]	Smirnova et al.	2001	32
6	Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria[30]	Cereghetti et al.	2008	29
7	Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells[31]	Otera et al.	2010	27
8	Functions and dysfunctions of mitochondrial dynamics[32]	Detmer et al.	2007	26
9	Mitochondrial dynamics and apoptosis[33]	Suen et al.	2008	26
10	Mitochondrial fission, fusion, and stress[34]	Youle et al.	2012	42

Drp1 – dynamin-related protein 1; Mfn1/2 – mitochondrial fusion proteins mitofusion ½; Opa1 – optic atrophy 1; Fis1 – fission 1; MiD49/51 – mitochondrial dynamics proteins 49/51.

of mitochondrial dynamics – fission and fusion – and details the mechanisms of these phenomena. Additionally, that paper discusses dynamic balance as a critical factor in maintaining mitochondrial network homeostasis under cellular stress: Fusion is impaired to prevent contamination of other mitochondria at a certain level of mitochondrial damage, and asymmetric fission is caused by uneven distribution of mitochondrial protein aggregates under cellular stress.

These analyses of highly cited literature and co-cited references reveal key research themes and trends in mitochondrial dynamics.[3,4,27–34] They showcase the critical role of mitochondria in cellular functions and diseases and highlight the importance of understanding the mechanisms of mitochondrial dynamic balance for future therapeutic strategies. With continued research, we expect more innovative findings to emerge, further propelling the progress of mitochondrial dynamics research in life sciences and medicine.

Key word analysis and research trends in mitochondrial dynamics

In this study of mitochondrial dynamics, 1,223 keywords were extracted from 332 articles. Six keywords appeared more than 50 times, including "oxidative stress", "fission", "apoptosis", "fusion", "mitochondrial dynamics", and "mitochondria" (Supplementary Fig. 11,12; Table 9). The most frequently occurring key word was "oxidative stress" (121 times), followed by "fission" (100 times) and "apoptosis" (97 times) (Table 9). Table 10 lists the top 20 molecular-, pathological process- and disease-related keywords associated with mitochondrial dynamics.

Table 9. Top 20 keywords related to mitochondrial dynamic

Rank	Keywords	Counts	Rank	Key words	Counts
1	oxidative stress	121	11	cell-death	32
2	fission	100	12	Mfn 2	32
3	apoptosis	97	13	expression	31
4	fusion	82	14	inhibition	30
5	mitochondrial dynamics	75	15	activation	29
6	mitochondria	72	16	mechanisms	26
7	mitochondrial dysfunction	47	17	metabolism	26
8	autophagy	43	18	Opa1	21
9	Drp1	42	19	skeletal-muscle disease	20
10	mitophagy	41	20	biogenesis	19

Drp1 – dynamin-related protein 1; Mfn1/2 – mitochondrial fusion proteins mitofusion ½; Opa1 –optic atrophy 1.

Table 10. Top 15 molecules, pathological process and disease related to mitochondrial dynamic

Rank	Molecules	Counts	Pathological process	Counts	Diseases	Counts
1	Drp1	42	oxidative stress	121	skeletal-muscle disease	20
2	mitofusin 2	32	fission	100	Alzheimer's disease	14
3	Opa1	21	apoptosis	97	cardiovascular disease	13
4	ROS	15	fusion	82	Parkinson's disease	13
5	amyloid-beta	7	mitochondrial dysfunction	47	cancer	12
6	cadmium	7	autophagy	43	obesity	12
7	cytochrome-c	7	mitophagy	41	insulin-resistance	11
8	Mdivi-1	7	cell-death	32	neurodegenerative diseases	8
9	PGC-1-alpha	7	metabolism	26	stroke	8
10	selenium	7	biogenesis	19	dominant optic atrophy	7
11	AMPK	6	inflammation	18	ischemia-reperfusion injury	7
12	copper	6	phosphorylation	18	diabetes	6
13	nitric-oxide	6	toxicity	15	hyperglycemia	6
14	parkin	6	morphology	10	sepsis	6
15	PINK1	5	ER stress	9	acute kidney injury	5

PGC-1-alpha – peroxisome proliferator-activated receptor-gamma co-activator 1 alpha; AMPK – adenosine 5'-monophosphate (AMP)-activated protein kinase; ER stress – endoplasmic reticulum stress; ROS – reactive oxygen species; Drp1 – dynamin-related protein 1; Mfn1/2 – mitochondrial fusion proteins mitofusion ½; Opa1 – optic atrophy 1.

In terms of molecules, the top 3 were essential proteins regulating mitochondrial dynamics: Drp1 (42 mentions), Mfn2 (32 mentions) and Opa1 (21 mentions). The most commonly mentioned pathological processes included oxidative stress (121 mentions), fission (100 mentions), apoptosis (97 mentions), fusion (82 mentions), mitochondrial dysfunction (47 mentions), and autophagy (43 mentions). The most studied diseases in the field of mitochondrial dynamics included skeletal muscle disease (20 mentions), AD (14 mentions), cardiovascular diseases (13 mentions), Parkinson's disease (13 mentions), and cancer (12 mentions).

The color changes in the overlay visualization exported from VOSviewer represent the average publication year (APY) (Supplementary Fig. 13). CiteSpace's timeline view clusters evolving high-frequency keywords (Supplementary Fig. 14). The largest cluster is No. 0 (death), followed by No. 1 (apoptosis), No. 2 (reperfusion injury), and No. 3 (acute lung injury). Notably, key word clusters focused on detailed mechanisms, such as No. 0 (death) and No. 1 (apoptosis), have ceased in recent years, while others related to specific diseases, like No. 2 (reperfusion injury) and No. 3 (acute lung injury), continue to develop.

Additionally, we detected citation bursts to better understand the development of mitochondrial dynamics research (Supplementary Fig. 15). Citation bursts reflect the frequency of any key word over a specific time. Analysis of citation bursts not only focuses on the evolution of research hotspots but also summarizes recent research trends and suggests potential directions for future studies. The distribution of burst keywords is relatively even, with no year experiencing a sudden influx of burst terms. Opa1 exhibits the most robust burst (strength = 4.6), while axonal transport had the most extended burst duration from 2011 to 2017.

These analyses reveal the main foci and trends in mitochondrial dynamics research. The distribution of keywords indicates that pathological processes like oxidative stress, fission and apoptosis are current research focuses, while diseases related to mitochondrial dynamics, such as skeletal muscle disease and AD, are also receiving extensive attention.

Dual-map analysis of mitochondrial dynamics research

Supplementary Fig. 16 presents a dual-map overlay visualization analysis of research on cellular mitochondrial dynamics from 1956 to 2023. The dual-map analysis illustrates global academic collaboration and the knowledge flow patterns between disciplines. The research primarily focuses on molecular biology, medicine and chemistry. The close citation relationships between these fields reflect the active interdisciplinary collaboration network, with molecular biology and medicine playing a particularly dominant role in advancing mitochondrial dynamics research.

Based on the specific details of the dual-map analysis, the left and right sides, respectively, show the distribution patterns of citing journals and cited journals. The clustering of citing journals is mainly concentrated in the fields of "Medicine, Medical, Clinical," "Biology, Molecular, Immunology" and "Physics, Materials, Chemistry," indicating that articles on mitochondrial dynamics are primarily published in journals focusing on clinical medicine, biological sciences and chemistry. On the other hand, the cited journals show concentrations in "Molecular, Biology, Genetics," "Health, Nursing, Medicine" and "Physics, Materials, Chemistry," suggesting that research in this field relies on foundational discoveries in genetics, molecular biology and clinical studies. The citation paths between the left and right maps demonstrate the main citation flows from "Medicine, Medical, Clinical" to "Molecular, Biology, Genetics," and from "Biology, Molecular, Immunology" to "Health, Nursing, Medicine." This further reveals the close connection between clinical medicine and fundamental biological research in mitochondrial dynamics, underscoring the interdisciplinary nature of the field.

This dual-map analysis not only highlights the core research disciplines within the mitochondrial dynamics field but also reveals global trends in knowledge flow and interdisciplinary collaboration.

Discussion

The WoSCC database shows that between 2000 and 2023, 332 articles on mitochondrial dynamics were published in 198 academic journals, authored by 2,227 researchers from 482 institutions across 40 countries. This trend indicates the growing global interest and research in mitochondrial dynamics.

China's contribution to mitochondrial dynamics research is particularly notable, leading the world in published papers. The performance of 8 high-output institutions, including the CAS, underscores the increasing emphasis on mitochondrial dynamics research in Chinese institutions. While the number of publications from the USA, Italy and the UK lags behind China, these countries' articles garner more citations, indicating superior quality. However, the collaborative network among countries remains at a nascent stage. Aside from the close ties between China and the USA, connections among other countries are relatively sparse, suggesting a need for stronger international cooperation and more profound exchange to understand mitochondrial dynamics comprehensively.

Nipon Chattipakorn from Chaing Mai University is the most prolific author in this field, mainly researching the role of mitochondrial dynamics in myocardial ischemia/reperfusion (I/R) injury. His papers indicate that a single dose of melatonin, acute metformin, erythropoietin (EPO), and donepezil alleviate myocardial I/R injury, an effect achieved through the regulation of mitochondrial dynamics.[35–38] Hsiu-Chen Chen from the California Institute of Technology (Caltech) is the most cited author,

focusing on the intrinsic mechanisms of mitochondrial dynamics, including molecular expression related to Drp1 recruitment (Fis1, Mff, MiD49, and MiD51), AMPK-mediated mitochondrial fission and mitochondrial heterogeneity and dysfunction caused by mitochondrial fusion disorders.[3,39,40] Notably, a paper from 2003 by Chen et al. titled "Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development" is also among the top ten most-cited articles. This article highlights the crucial role of mitochondrial fusion in embryonic development, stating that fusion proteins MFN1 and MFN2 exist as homotypic or heterotypic oligomers, promoting mitochondrial fusion either cooperatively or independently, thus maintaining mitochondrial collaboration and protecting against respiratory dysfunction.[28]

Oxidative Medicine and Cellular Longevity published the most articles in the journal analysis. Additionally, co-cited journals include high-quality publications such as *Proceedings of the National Academy of Sciences of the United States of America*, *Journal of Cell Biology*, and the top-tier journals *Cell*, *Science* and *Nature*.

As previously mentioned, the cited references primarily discuss the impact of mitochondrial dynamics on specific disease types, while co-cited references focus more on analyzing molecular and cellular intrinsic mechanisms. Most cited references were published after 2015, while most co-cited references predate 2010, aligning with the research shift from mechanisms to disease. We observed a fluctuation in the publication volume of mitochondrial dynamics-related literature in 2018 and 2023. It may be due to the interdisciplinary integration of mitochondrial dynamics with other fields, such as bioinformatics and systems biology, leading researchers to publish their work in these emerging interdisciplinary areas, thus impacting the publication volume in the mitochondrial dynamics field. As research on mitochondrial dynamics progresses, the complexity and technical demands of the studies may increase, resulting in only laboratories equipped with specific technologies and resources being able to conduct relevant research, consequently affecting the overall publication volume.

Through key word iteration, research trends were mapped. Using overlay and timeline visualizations, we visualized the evolution of keywords. By analyzing key word co-occurrence (Tables 8,9), key word overlay and timeline (Supplementary Fig. 13,14), and key word bursts (Supplementary Fig. 15), we objectively assessed the hotspots and frontiers in mitochondrial dynamics research. These 3 aspects are summarized below.

Mitochondrial dynamics imbalance and disease: From oxidative stress to inflammation

An imbalance in mitochondrial dynamics is the fundamental cause of mitochondrial dysfunction, affecting various aspects such as oxidative levels, metabolic regulation, autophagy, and inflammatory responses. Our key word analysis revealed that early research predominantly focused on oxidative stress. Reactive oxygen species (ROS), representative components in oxidative stress, can be overproduced following an imbalance in dynamics. As typical byproducts of the electron transport chain (ETC), ROS are highly reactive superoxide anions with strong oxidizing properties that can damage proteins, lipids and DNA.[34] Mitochondrial DNA (mtDNA) is especially susceptible to ROS-induced mutations and damage. The progressive accumulation of mutated mtDNA can lead to the loss of functional respiratory chain complexes, resulting in bioenergetic decline and cell death.[41]

Mitophagy, a frequently mentioned key word, is a protective mechanism for quality control by eliminating outdated or damaged mitochondrial components (Table 9). It has been established that mitophagy is necessary for fission.[27] Dysfunctional components can be isolated and degraded through fission to compensate for nutrient scarcity. Furthermore, fusion proteins also participate in mitophagy. The PINK1/parkin pathway, known to mediate mitophagy, involves Mfn2, specifically colocalizing with PINK1 on the OMM during depolarization. Phosphorylated Mfn2 by PINK1 acts as a receptor to recruit activated parkin, prioritizing its ubiquitination.[42,43] Following Mfn2 ubiquitination, mitophagy initiators such as LC3 are recruited, initiating mitophagy.[44] Additionally, Opa1 can be cleaved into 2 isoforms, long Opa1 and short Opa1 (L-Opa1 and S-Opa1). Under mitochondrial stress conditions, S-Opa1, derived from the hydrolysis of membrane-anchored L-Opa1, can be selectively used for mitophagy.[45]

Mitochondria-induced inflammation is a recent research interest. Upon injury, mitochondrial contents are released into the cytoplasm. Among these, mtDNA, a primary endogenous damage-associated molecular pattern (DAMP), induces inflammation through 2 main pathways due to its high specificity.[46] In the 1st pathway, mtDNA is released into the cytoplasm through the permeability transition pore complex (PTPC) or pores formed by apoptotic regulators (Bax) and BCL2 antagonist/killer 1 (Bak1).[47,48] Cytosolic DNA sensor cGAS catalyzes the formation of cyclic dinucleotides (cGAMP) upon binding DNA, initiating inflammatory responses via cGAMP-interacting protein 1 (STING1) and subsequent synthesis of cytokines like interferon-β1 (IFN-β1), interleukin (IL)-6 and tumor necrosis factor (TNF). On the other hand, ROS-oxidized mtDNA can activate the NLRP3 inflammasome, promoting the secretion of downstream IL-1β and IL-18.[49,50] Additionally, mtDNA can endogenously bind toll-like receptor 9 (TLR9) in endosomes, activating the downstream classical nuclear factor kappa B (NF-κB) inflammatory pathway.[51] Rodríguez-Nuevo et al. found that specific deletion of OPA1 in muscles leads to mitochondrial dysfunction and increased mtDNA content. Conversely, the depletion of mtDNA reversed the activation of the TLR9 and NF-κB pathways and the inflammatory response.[52] Irazoki et al. also observed that acute downregulation

of dynamin proteins (Mfn1, Mfn2, Fis1, Drp1) in myoblasts leads to mtDNA release and differential inflammatory responses: Inhibition of fusion proteins Mfn1 or Mfn2 triggers mitochondrial fragmentation and TLR9-dependent NF-κB activation, while inhibiting Drp1 or Fis1 causes mitochondrial elongation, accompanied by NF-κB-dependent and type I interferon inflammatory responses.[53] Although dynamics imbalance leading to mtDNA extramitochondrial localization and triggering TLR9 or cGAS-dependent inflammation has been reflected in literature, more detailed mechanisms yet to be fully defined could be future research targets.

In addition to the mechanisms discussed above, aspects such as mitochondrial biogenesis, endoplasmic reticulum stress, calcium homeostasis, and protein phosphorylation have attracted widespread attention and are continually updated, warranting further exploration.

Mitochondrial dynamics and cell death: From apoptosis to the diversity of programmed cell death

In the field of mitochondrial dynamics, apoptosis is the most frequently mentioned form of cell death and has been extensively studied. The classic apoptotic pathway involves the complex of Bax and Drp1.[54] Under mitochondrial stress, increased fission is accompanied by enhanced synthesis of the Bax-Drp1 complex. It leads to increased OMM permeability and cytochrome c release, activating caspase cascades and ultimately inducing apoptosis. Evidence suggests that defective mitochondrial fusion also responds to apoptotic signaling. Degradation or knockdown of Mfn2 enhances apoptotic intensity, while apoptosis in human retinal endothelial cells under hyperglycemic conditions is reversed following Mfn2 overexpression.[55–57] Additionally, caspases activated during apoptosis are associated with organelle fragmentation and cristae remodeling. Opa1 maintains the integrity of cristae junctions, preventing cytochrome c release during apoptosis and thus exerting a protective function.[58]

Programmed cell death (PCD) has always been a widely studied field, encompassing necroptosis, pyroptosis and ferroptosis, in addition to apoptosis. Necroptosis mentioned in the keywords is an inflammatory PCD mediated by the activation of receptor-interacting protein kinase 1 (RIP1), receptor-interacting protein kinase 3 (RIP3) and mixed lineage kinase domain-like protein (MLKL), characterized by cell swelling, membrane rupture and release of cell contents. In a chronic kidney disease (CKD) rat model, increased expression of RIPK1, RIPK3 and MLKL along with Drp1 was observed, which was reversed after treatment with the RIPK1-targeting drug necrostatin-1 (Nec-1), suggesting excessive mitochondrial fission might be involved in necroptosis.[59]

Although pyroptosis and ferroptosis were not mentioned in the keywords, continuous research indicates potential links with mitochondrial dynamics. Pyroptosis is characterized by forming pores on the cell membrane and releasing cell contents, such as pro-inflammatory cytokines.[60] The classic pathway of pyroptosis involves the activated NLRP3 inflammasome, which assembles and activates caspase-1 to cleave gasdermin D, a member of the gasdermin family, mediating membrane pore formation and enabling maturation and release of IL-1β and IL-18. Existing studies reveal connections between mitochondrial dynamics and the NLRP3 inflammasome. RNA virus infection mediates the assembly of the RIP1-RIP3 complex, activates Drp1, and then guides its mitochondrial translocation, excessive fission leading to mitochondrial damage, and NLRP3 inflammasome activation.[61] CaMKII activation-induced Drp1 phosphorylation and mitochondrial fission produces excessive mitochondrial ROS (mtROS), driving NLRP3 inflammasome activation.[62] Additionally, divalent manganese (Mn^{2+}) inhibits Mfn2 expression, promoting mitochondrial superoxide generation and accumulation and triggering NLRP3 inflammasome activation in microglia.[63]

Ferroptosis is a PCD characterized by excessive iron accumulation and lipid peroxidation. Excessive mitochondrial fission, due to the overproduction of ROS and increased cellular oxidative levels, is considered a contributing factor to ferroptosis. Ferroptosis in damaged intestines induced by cisplatin was observed alongside increased protein and mRNA expression levels of fission-related proteins Drp1 and Fis1. In contrast, treatment with the antioxidant vitamin D_3 inhibited ferroptosis, concurrently reducing ROS accumulation and excessive mitochondrial fission.[64] The ferroptosis inducer erastin, in combination with celastrol, mediated cell death in non-small cell lung cancer (NSCLC) cells, accompanied by abundant ROS and enhanced mitochondrial fission.[65] Targeting mitochondrial fusion-mediated ferroptosis, increased Mfn1/2-dependent mitochondrial fusion guided by STING1 led to ferroptosis in human pancreatic cancer cells, while knocking down STING1 or Mfn1/2 genes reduced their sensitivity to ferroptosis.[66] In a mouse model of brain I/R injury, selenium enhanced Mfn1 expression to promote mitochondrial fusion, significantly reducing oxidative stress and iron accumulation and ultimately ensuring higher mouse survival.[67]

Regulation of mitochondrial dynamics and disease progression: From energy metabolism to cellular aging

Our findings (Supplementary Fig. 11–15; Tables 9,10) indicate that diseases related to mitochondrial dynamics are an important research area. In the case of neurodegenerative diseases, chronic progressive damage to neurons is critical. Given neurons' high energy metabolic demands and the inability of adenosine triphosphate (ATP) to be internally transported due to rapid hydrolysis, the proper distribution of mitochondria (the sole supplier of ATP) becomes crucial. This distribution depends on altered

mitochondrial dynamics and is particularly susceptible to mitochondrial dysfunction, thereby highlighting the importance of mitochondrial dynamics.[68–70]

Bibliometric analyses indicate that AD is the most frequently studied neurodegenerative condition in academic literature, with Parkinson's disease also receiving significant attention. Earlier studies found potential mitochondrial network fragmentation in neuronal brain biopsies from AD patients.[71] Subsequent research revealed that amyloid-beta (Aβ) and other AD-related damage directly activate calpains, leading to cleavage of dynamin-like protein 1 (DLP1, a homolog of Drp1) and Mfn2, skewing dynamics towards hyperfission.[72] However, it remains controversial that the expression of Drp1 is reduced in AD brains, similar to the fusion proteins (OPA1, Mfn1 and Mfn2), while the levels of the cleavage factor Fis1 are significantly increased.[73,74] Yet, the DLP1 inhibitor Midivi-1 consistently shows early rescue of mitochondrial morphology and motility, as well as alleviation of amyloid pathology and cognitive function improvement, possibly by reducing Aβ production.[75,76] The exact role and mechanism of Drp1 in hyperfission remain to be confirmed.

Parkinson's disease is the 2nd neurodegenerative disease discussed, also considered a mitochondrial disease, characterized by the gradual death of dopaminergic neurons due to intracellular accumulation of alpha-synuclein (α-syn). Existing evidence supports the localization of α-syn to mitochondria.[77,78] The α-syn influences mitochondrial size by directly acting on the fusion-fission process. In mice overexpressing the A53T α-syn mutant, reduced expression of Mfn1, Mfn2 and Drp1 was observed, with mitochondrial expression of α-syn enhancing the mitochondrial fragmentation phenotype.[79] Similarly, in SH-SY5Y cells, overexpression of A53T led to increased translocation and expression of DLP1 targeted to mitochondria.[80] Overall, the reliance of neurons on mitochondria determines the irreplaceable importance of mitochondrial dynamics in neurodegenerative diseases, with its application and regulatory roles continually explored.

Moreover, mitochondrial dynamics are involved in the pathogenesis of other diseases. Dysregulation of Opa1 decreased L-Opa1, increased S-Opa1-induced mitochondrial fragmentation and reduced OXPHOS, leading to left ventricular dysfunction and myocardial atrophy.[81] Increased mitochondrial fragmentation is a critical factor in reduced OXPHOS, diminishing the ability to oxidize lipids and accumulating undesired lipid species such as triglycerides and diacylglycerol. Notably, increased diacylglycerol promotes protein kinase C (PKC) activation, which disrupts the insulin signaling cascade by inhibiting insulin receptor substrate-1 (IRS-1) activity. This inhibition leads to a reduction in phosphatidylinositol 3-kinase (PI3K) activity, a key factor in insulin signaling. As a result, impaired PI3K signaling contributes to insulin resistance, which is associated with the development of type 2 diabetes.[82] In the loss of skeletal muscle mass and strength, exercise training represents a practical therapeutic approach to recovery. Studies confirm that increased expression of Mfn1 and Mfn2 enhances mitochondrial fusion, leading to an enlarged cristae surface area (CSA), which facilitates cross-mitochondrial network junctions. This structural adaptation supports the overall elevated OXPHOS levels by improving mitochondrial efficiency and inter-organelle communication.[81,83] Concurrent pDrp1 Ser616 and Fis1 activity increases are also observed.[83,84] Exercise training-induced increases in Mfn2 and Drp1 expression in skeletal muscles of aging rats salvaged muscle endurance and enhanced exercise performance.[85]

Limitations

This study also has some limitations. Data were collected using the WoSCC. Although WoSCC strives to minimize bias, this factor in any academic database is complex and multifaceted. The scientific impact of publications, determined by citations, is now commonly regarded as a measure of research quality. However, while citations may assess the scientific influence of a study, they do not necessarily reflect its impact outside the scientific community. Furthermore, numerous factors influence citations, and the meaning of citations can vary widely, including negative citations.[86] Much of the literature in WoSCC comes from English-language publishers, which introduces potential language bias.[87]

Moreover, we added an English-language filter to our search criteria, given its international recognition and evaluation, which may also introduce potential publication and language bias. In future research, we plan to expand the language scope to include a more comprehensive analysis of literature related to mitochondrial dynamics.

Currently, research in mitochondrial dynamics primarily focuses on changes in mitochondrial dynamics in diseases,[88] mitochondrial homeostasis and quality control.[89] However, significant gaps remain in the upstream regulatory mechanisms of mitochondrial dynamics and the development of drugs targeting mitochondrial dynamics, areas that warrant further investigation by researchers.

Conclusions

Research in mitochondrial dynamics has shown steady and consistent growth. Through bibliometric analysis, we have identified the leading countries contributing significantly to this field, with China and the USA at the forefront, as well as relevant research institutions, authors, journals, and references. Current studies indicate that mitochondrial dynamics are active in a diverse array of mitochondrial-related cellular events, interacting with various forms of PCD and demonstrating potential

applications across different diseases. Additionally, mitochondrial dynamics are involved in a wide range of pathophysiological processes and play a crucial role in various diseases such as AD, Parkinson's disease and diabetes. It highlights their potential as therapeutic targets for disease treatment and underscores the value of developing targeted drugs aimed at mitochondrial dynamics. Our findings open up new research avenues for their potential in disease therapy, which may guide future studies and offer new insights.

Ethics approval and consent to participate

Ethical approval was not applicable because this study is based exclusively on published literature.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.14963828. The package includes the following files:

Supplementary Fig. 1. Visualization of co-cited authors' network.

Supplementary Fig. 2. Density visualization of co-cited authors.

Supplementary Fig. 3. Journal landscape in mitochondrial dynamics research. Visualizations map the journal landscape within the mitochondrial dynamics research field. Network visualization maps out the 198 journals that have published 332 articles.

Supplementary Fig. 4. Density visualization reveals the concentration of publications across these journals.

Supplementary Fig. 5. Network visualization of co-citation journals.

Supplementary Fig. 6. Density visualization of co-citation journals.

Supplementary Fig. 7. Key references shaping mitochondrial dynamics research. The citation and co-citation networks within mitochondrial dynamics research. Network visualization pinpoints seminal papers with the highest citation counts.

Supplementary Fig. 8. Density visualization highlights the clusters of most frequently cited references.

Supplementary Fig. 9. Network visualization of co-cited references elucidates the interconnectivity between references.

Supplementary Fig. 10. Density visualization of co-cited references further emphasizes the impact and relevance of these works within the scientific community.

Supplementary Fig. 11. Mitochondrial dynamics: A key word analysis of research trends and foci. The critical thematic elements shaping mitochondrial dynamics research. The key word network identifies the most common terms used across 332 articles.

Supplementary Fig. 12. The key word density map provides a visual concentration of these terms, indicating areas of high research activity.

Supplementary Fig. 13. An overlay visualization based on the average publication year shows the evolution of research focus over time, with recent years gravitating toward specific molecular mechanisms.

Supplementary Fig. 14. The timeline view clusters evolving keywords, with the most significant clusters associated with fundamental processes like death and apoptosis. Each horizontal line represents a cluster; the smaller the number, the larger the cluster (No. 0 is the largest). The time is performed at the top, and keywords are located at their first co-occurrence time in the clusters.

Supplementary Fig. 15. Top 25 keywords with the most robust citation bursts (sorted by the starting year). The red bars mean citation burstiness.

Supplementary Fig. 16. Dual-map analysis of mitochondrial dynamics research. The left map shows the citing journals, while the right map represents the cited journals. The paths illustrate the citation flow between disciplines, highlighting the key knowledge sources and research directions in the field.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Novel therapies in SLE treatment: A literature review

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Abstract

Systemic lupus erythematosus (SLE) is a chronic, autoimmune inflammatory disease with a multisystem manifestation and a variety of clinical symptoms. Over the last decades, the prognosis and life expectancy of patients with SLE improved significantly due to the implementation of corticosteroids combined with immunosuppressive agents. Nevertheless, the use of these medications is often associated with the occurrence of serious side effects and additional deterioration of organ function. Therefore, developing and implementing novel therapies that are both safer and more effective in managing disease is crucial. For a long time, European Alliance of Associations for Rheumatology (EULAR) recommended only 2 biological agents in the treatment of SLE: belimumab and rituximab. However, in 2023, anifrolumab, an interferon (IFN) receptor inhibitor, and voclosporin, a novel calcineurin inhibitor, appeared in new SLE treatment guidelines. In addition, several biological agents are targeting different cells or cytokines that are being evaluated in phase II and III clinical trials. Apart from that, experimental therapies such as targeting of plasma cells, chimeric antigen receptor T-cell therapy (CAR-T) or stem cell transplantation appear promising in the treatment of the severe forms of SLE.

Key words: systemic lupus erythematosus, calcineurin inhibitors, biological therapy, monoclonal antibodies

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Introduction

Systemic lupus erythematosus (SLE) is a chronic, multifaceted autoimmune disease with a broad range of symptoms and a variable prognosis. Early diagnosis and initiation of the treatment are crucial to alleviate symptoms and reduce mortality.[1] The development of new medications is necessary to further extend the patients lifespan and improve their quality of life. Systemic lupus erythematosus affects primarily women of reproductive age, with female to male ratio of 9:1.[2,3] Its incidence for the global population is estimated to be 5.14 per 100,000 person/years.[4]

Common SLE manifestations often include fatigue, weight loss, arthritis, and skin rashes, especially butterfly-shaped rash, which spreads across cheeks and nose (malar rash).[5] Affected individuals commonly experience photosensitivity, provoking skin reactions upon sun exposure. Throughout the disease, lesions in internal organs can be seen. Lupus nephritis (LN) is one of the most frequent severe organ manifestations of SLE, carrying a substantial morbidity and mortality risk, with approx. 20% of patients advancing to end-stage renal disease (ESRD).[6,7] Although less common, neuropsychiatric SLE manifests across a spectrum of presentations, ranging from mild cognitive dysfunction to severe psychosis.[8] Furthermore, SLE may give a rise to manifestations in pulmonary, gastrointestinal and cardiovascular system, while also affecting hematopoiesis.

Although the short-term and median outcomes of SLE patients have improved over the past decade, the long-term prognosis is still unfavorable.[9,10] The course of the disease is burdened by comorbidities in addition to the side effects of the treatment used.[11,12] These include cardiovascular disease such as arteriosclerosis, hypertension, osteoporosis, and increased frequency of infections.[13] The prevalence of psychiatric disorders such as depression and anxiety is significantly higher compared to the general population.[14] Frequently occurring fatigue interferes with the ability to perform routine daily activities.[15] A substantial number of individuals are forced to reduce their work hours or even retire.[16]

Systemic lupus erythematosus is associated with the production of autoantibodies targeted mainly against autoantigens coming from cell nuclei and the formation of immune complexes that cause damage to various organs. However, the exact cause of the disease is still unknown. It is being suggested that genetic, environmental and hormonal factors play a role in self-immunization.[17,18] Its multifactorial pathogenesis together with its variable clinical phenotypes pose a challenge to the treatment. However, the rising knowledge of the pathological pathways taking part in the disease progression allows us to introduce new therapeutic solutions.

Objectives

Due to the increasing incidence of SLE and the growing number of patients who do not respond to previously used medications, this systematic review aims to present newly introduced SLE therapies as well as treatments with potential for broader use in the future (Fig. 1).

Materials and methods

The literature search used PubMed, Embase and Google Scholar databases, as well as references from relevant articles and internet sources. Search terms included “SLE novel therapies”, “SLE biological medications”, “SLE future therapies”, “SLE voclosporin”, “SLE anifrolumab”, “SLE obinutuzumab”, “SLE dapirolizumab”, “SLE deucravacitinib”, “SLE ustekinumab”, “SLE litifilimab”, “SLE plasma cells targeting”, “SLE CAR-T”, and “SLE stem cell transplant”. The authors screened the titles and abstracts to identify relevant articles, with the last literature search performed on May 5, 2024. Finally, we have included 116 studies eligible for our review.

Immunosuppressive medications

Voclosporin

Calcineurin inhibitors (CNIs) are immunosuppressive medications widely used in transplantology and the treatment of autoimmune diseases. Calcineurin inhibitors disrupt the intrinsic calcium signaling pathway, ultimately leading to decreased T-cells activation, proliferation and differentiation. What is more, CNIs act in nephron podocytes to stabilize the actin cytoskeleton, thereby exerting an antiproteinuric effect. This unique characteristic of CNI makes them ideal candidates for the treatment of autoimmune glomerulonephritis, including LN.

Voclosporin is a novel CNI indicated for the treatment of adult patients with active class III, IV and V LN, and in combination with mycophenolate mofetil (MMF). Compared to cyclosporin, low-dose voclosporin seems to have a lower nephrotoxicity, and compared to tacrolimus, a lower diabetogenic effect.[19] AURORA 1 study assessed the efficacy and safety of voclosporin compared to placebo in patients with biopsy-confirmed LN over the course of 2 years. Voclosporin in combination with MMF and low-dose steroids caused clinically significant complete renal responses compared to placebo (41% vs 23% of patients), with a comparable rate of serious adverse events (21% in both groups).[20] The continuation of this study, AURORA 2, further evaluated the long-term efficacy with adverse effects. Furthermore, it determined biochemical and hematological outcomes. Over a 3-year follow-up, the rate of adverse events was similar to that seen in the AURORA 1 study. Hypertension and decreased glomerular filtration rate (GFR) were observed more frequently with voclosporin. However, the mean corrected estimated GFR (eGFR) was within the normal range and

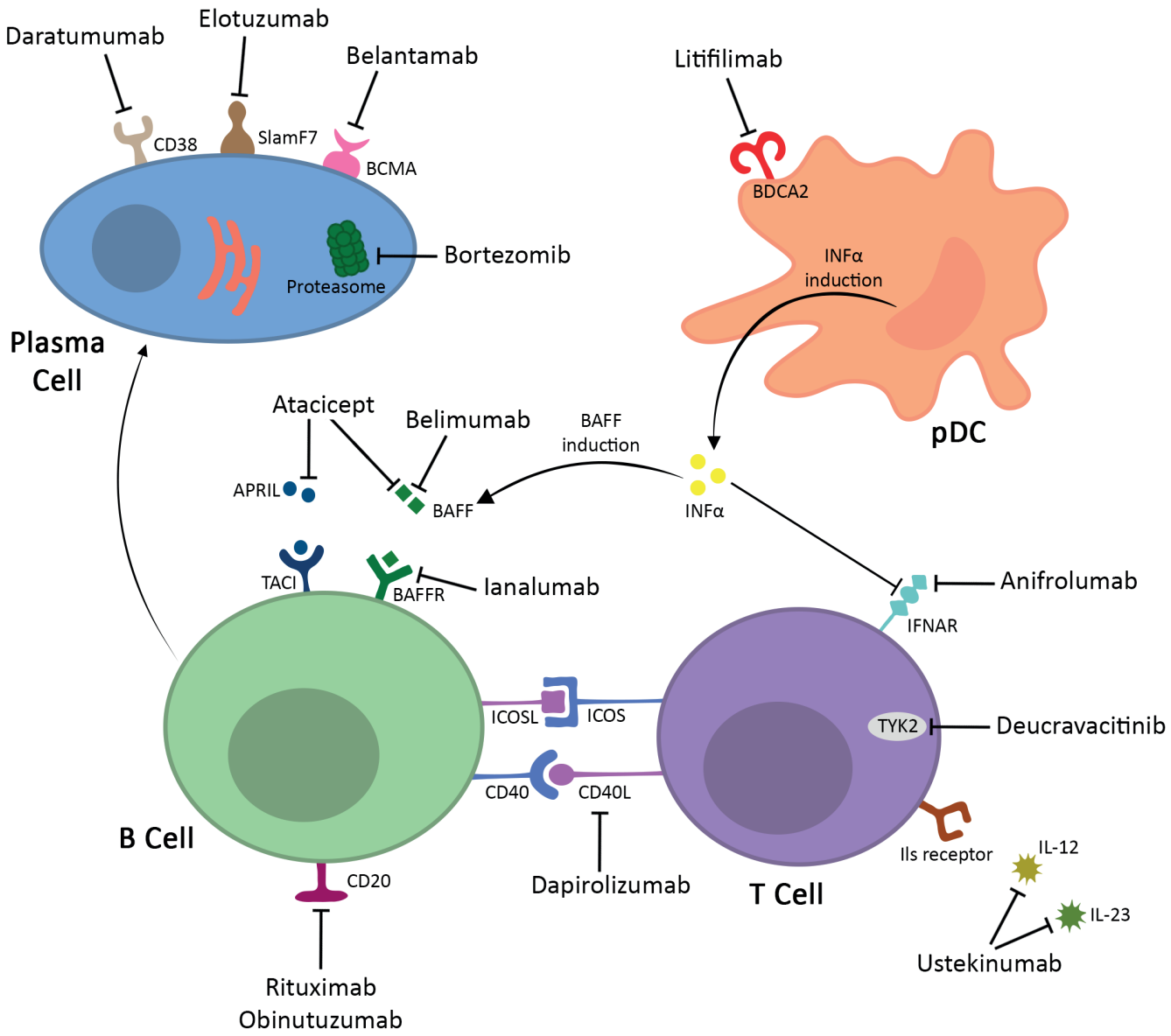

Fig. 1. The antibodies used and their therapeutic targets, as well as the cells that play a critical role in the control of the systemic lupus erythematosus (SLE) progression

APRIL – a proliferation-inducing ligand; BAFF – B-cell-activating factor; BAFFR – BAFF receptor; BCMA – B-cell maturation antigen; BDCA2 – blood dendritic cell antigen 2; CD40L – CD40 ligand; ICOS – inducible T-cell co-stimulator; ICOSL – ICOS ligand; IFN-α – interferon alpha; IFNAR – type 1 interferon receptor; ILs – interleukins; pDC – plasmacytoid dendritic cell; SlamF7 – signaling lymphocytic activation molecule F7; TACI – transmembrane activator and calcium modulator and cyclophilin ligand interactor; TYK2 – tyrosine kinase 2.

stable in both groups. A complete renal response occurred in 50.9% of patients in the treatment group compared to 39.0% in the placebo group.[21] It has been reported that the combination of voclosporin and MMF does not require a change in the dosage of MMF.[22]

Targeted therapies

Anifrolumab

The interferon (IFN) pathway has attracted a lot of attention as one of the key factors in pathogenesis of SLE and as a promising aim of novel treatment methods.[23] The "type I IFN signature", or overexpression of gene transcripts in the IFN pathway, is a hallmark of SLE patients.[24] This, in turn, results in the dysfunction of peripheral tolerance mechanisms. The interferon promotes activation of Th cells, improves plasmacytoid dendritic cells abilities to present antigens, and induces the production of various cytokines.[17] The IFN overproduction is probably mediated by exposure of plasmacytoid dendritic cells (pDC) to serum immune complexes and increased neutrophil extracellular trap (NET) formation, while simultaneously reducing the ability to degrade them. Other proposed triggers are bacterial and viral infections, dysregulation in gut microbiome and increased estrogen concentrations. Over

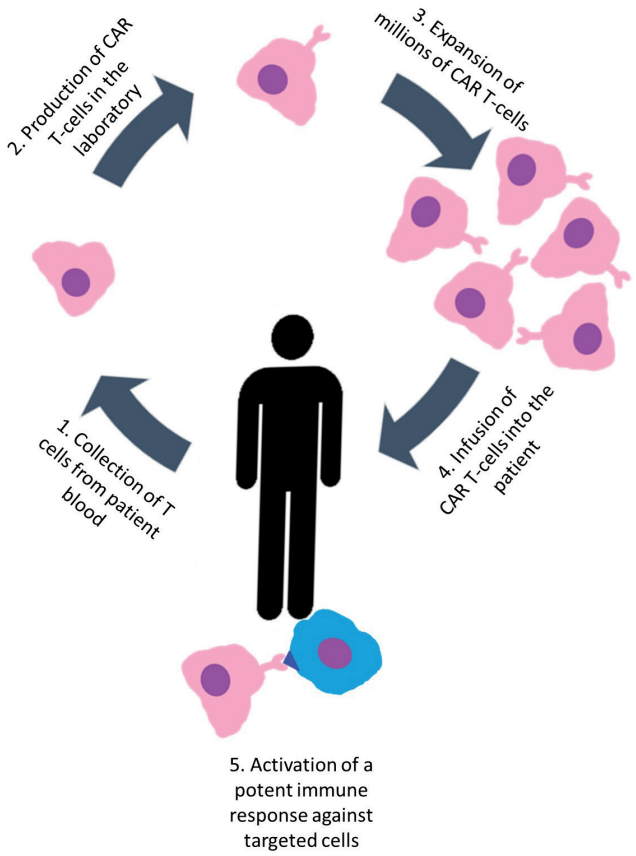

Fig. 2. Chimeric antigen receptor T-cell therapy (CAR-T). The process begins with the collection of the T cells from the patient's blood and ends with the activation of a potent immune response against the targeted cells

half the loci associated with SLE encode proteins related to cell IFN production or response.[25]

Anifrolumab is becoming a promising therapeutic option for patients with SLE and is currently used in the therapy of moderate-to-severe SLE.[26] Anifrolumab is a human monoclonal immunoglobulin 1 (IgG1) antibody to the type I IFN receptor subunit 1 (IFNAR1) that binds to type I interferon receptors blocking their activation and induction of interferon-related genes. Numerous studies have proven that type I IFN system plays a crucial role in the SLE etiopathogenesis.[27,28] Sandling et al. identified an increased expression of type I IFN-inducible genes in SLE patients, referred to as the 'IFN signature.' Additionally, Beachler et al. demonstrated that polymorphisms in genes such as *IKBKE* and *IL8* are associated with dysregulated type I IFN signaling, potentially increasing susceptibility to SLE by altering inflammatory pathways.[29,30] In addition, a causative role of the type I IFN system in the development of SLE has been demonstrated, as individuals treated with IFN-α developed this disease that was indistinguishable from the naturally occurring disease.[31] Type I IFNs are a family of cytokines that include 13 subtypes of IFN-α, as well as IFNβ, IFNε, IFNκ, and IFNω. All of these type I IFNs initiate a signaling cascade by binding to the receptor complex composed of IFNAR1 and IFNAR2 and thus inducing conformational changes in these receptors.[32–34] This leads to activation by the tyrosine kinases JAK1, which interacts with IFNAR2, and TYK2, which in turn interacts with IFNAR1. Janus kinases activate STAT1 and STAT2, which then initiate gene transcription, specifically IFN-stimulated response elements found in IFN-stimulated genes. Hence, any alternations in type I IFN signaling pathway might disturb homeostasis and prolong the biological effects of IFNs, which consequently might cause uncontrolled destructive effects observed in SLE.

Following the newest European Alliance of Associations for Rheumatology (EULAR) 2023 recommendations, the first-line treatment of SLE is hydroxychloroquine at a target dose of 5 mg/kg real body weight/day. Glucocorticoids are recommended a maintenance dose ≤5 mg/day (prednisone equivalent), and if possible, they should be withdrawn. In patients with moderate-to-severe disease, there might be considered pulses of intravenous methylprednisolone. In patients not responding to hydroxychloroquine alone or in combination with glucocorticoids or patients unable to reduce their dose below the levels acceptable for chronic use, the use of immunomodulating agents or mycophenolate and/or biological agents, such as belimumab or anifrolumab, should be considered.[35]

Biological agents are critical for some patients to better control their disease, and that is why it is so important to develop and research these drugs. Belimumab and anifrolumab have demonstrated efficacy in controlling disease activity and allowing GC dose reductions. In 2021, the U.S. Food and Drug Administration (FDA) approved the use of anifrolumab in the SLE treatment. In contrast, belimumab has been used in clinical practice for over a decade.[32,33] There is no given hierarchy between anifrolumab and belimumab in the EULAR 2023 recommendations as these 2 drugs have not been compared in the head-to-head trials. Apart from reducing the disease activity, anifrolumab has the potential to reduce the dose of corticosteroids used to treat SLE. In TULIP 1 and TULIP 2 trials, sustained corticosteroids tapering was achieved in 52% of patients in the anifrolumab group and in 32% of patients in the placebo group. In addition, it was possible to reduce the cumulative corticosteroid dose by 32% in taper responders in the anifrolumab group, the blood pressure was reduced and anifrolumab group experienced fewer side effects.[36] What is more, in patients with moderate-to-severe SLE, anifrolumab has been demonstrated to reduce the incidence of flares. In patients who achieved sustained corticosteroids reduction, 40.0% did not experience flares while on anifrolumab compared to 17.3% receiving placebo.[37] In the long-term extension (LTE) of the TULIP 1 and TULIP 2 trials, the risk of non-opportunistic infections was similar between the anifrolumab and placebo groups over a 3-year observation period, and the risk of serious adverse events was lower in the treated group.[38] The potential of anifrolumab in patients with severe LN was assessed in phase II randomized

trial. In the intensified regimen group (3 initial doses of 900 mg followed by 300 mg maintenance doses), 45.5% of patients achieved a complete renal response (CRR), compared to 31.1% in the placebo group. Furthermore, sustained corticosteroid reduction was observed in 55.6% of patients in the anifrolumab group compared to 33.3% in the placebo group.[39] Long-term extension provided similar results, also showing the safety of the treatment, with adverse event rates of 6.9% and 8.7% in the anifrolumab and placebo groups, respectively.[40]

Obinutuzumab

The cell-surface antigen CD20, expressed on mature B cells and most malignant B cells, is an excellent target for the treatment of B-cell malignancies as well as autoimmune disorders.[41] Numerous studies have shown that B-cell depletion therapy with anti-CD20 monoclonal antibodies (mAbs), such as rituximab, have notably improved symptoms and clinical remission in patients with rheumatoid arthritis.[42] Moreover, administration of anti-CD20 mAb in the population of SLE patients has been associated with a substantial decrease in plasma cell population[43] subsequently reducing several SLE antibodies including anti-dsDNA, anti-nucleosome and anti-cardiolipin.[44] Anti-CD20 antibodies can exhibit functional activity in 3 different ways: signaling in target cells leading to growth inhibition and non-classical apoptosis, described as "direct cell death", complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC), mediated by cells displaying Fcγ receptors (FcγRs).[41,45]

Two types of effector function profiles of CD20 antibodies have been described, referred to as type I and type II. They have been distinguished by the CD20 epitope, to which the antibodies bind and/or their binding mode.[46] According to the study by Cragg and Glennie, the therapeutic efficacy of type I mAbs is directly related to the classical pathway of complement activation through their binding to the C1q component. On the other hand, type II mAbs do not utilize complement or NK cells for their function. Instead, their therapeutic activity is achieved through potent induction of direct apoptosis.[46]

In randomized controlled trials, rituximab, a type I mAb, was not effective in the treatment of SLE and LN.[47] However, observational studies, large retrospective studies and meta-analyses of observational studies have shown efficacy in the treatment of SLE and LN with complete response estimates of 46–57% and 36–51%, respectively.[48] Furthermore, a study conducted by van der Kolk et al. has shown that most of the side effects of anti-CD20 type I mAbs treatment were correlated with the activation of complement.[49] Therefore, it is suggested that type II antibodies could potentially offer the benefit of lower toxicity. These beneficial qualities have led to increased interest in anti-CD20 type II mAbs, resulting in the development of new monoclonal antibodies, such as obinutuzumab.

Obinutuzumab, also known as GA101, is a humanized, Fc-engineered type II IgG1 antibody targeted against CD20. It was originally engineered and characterized by Mössner et al. in 2010.[50] Obinutuzumab was compared in the preclinical studies with another anti-CD20 mAb, rituximab, a humanized, chimeric type I IgG1 antibody.[51] GA101 showed in vivo efficacy superior to rituximab in all tested parameters. In the aggressive human B-cell lymphoma xenograft models, tumor growth inhibition was more effectively achieved by obinutuzumab compared to rituximab. Moreover, GA101 exhibited a dose-dependent enhancement in performance leading to a complete tumor regression at a dose of 30 mg/kg, while rituximab failed to achieve this at any dose used. In addition, obinutuzumab demonstrated superior B-cell depleting activity in the blood in the cynomolgus monkeys in comparison with rituximab. Studies also showed that B-cell depletion by GA101 was greater in spleen and lymph nodes.[50] According to the study by Beers et al., type II anti-CD20 antibody complexes tend to remain on the B-cell surface for extended periods of time, resulting in a more efficient depletion of the B-cells compared with type I. This could possibly be linked to a more potent effectiveness of obinutuzumab, as indicated in the previous study.[52] Those promising results from the preclinical studies suggest that obinutuzumab has the potential to be used in the SLE treatment.

A phase II trial (NOBILITY), conducted in 2021, compared obinutuzumab with a placebo in the management of LN in association with standard therapies involving mycophenolate mofetil and steroids. A total of 125 patients with SLE, aged between 18 and 75 years and presenting with class III or IV LN, were included in the study. The participants were randomly divided into 2 equal groups to receive either obinutuzumab 1,000 mg or placebo infusions on day 1 and week 2, 24 and 26. A total of 115 patients completed 52 weeks and 103 patients completed 104 weeks of follow-up. At week 52 of the protocol, the primary endpoint, CRR, was achieved to a greater extent by the obinutuzumab group (35%) compared to the placebo group (23%). Although the last dose of the drug was administered at week 26, the advantage persisted throughout the study reaching 41% of CRR in the obinutuzumab group and remaining unchanged in the placebo group. Moreover, the results in the test group outperformed the results in the control group, reaching a higher overall renal response (ORR), consisting of CRR and partial renal response (PRR), with a rate of 55% compared to 35% in the control group at week 56, and 54% compared to 29% at week 104. The positive effects of obinutuzumab were especially noticeable in patients with class IV LN and those with a baseline urine protein-to-creatinine ratio (UPCR) ≥3. In addition, obinutuzumab led to a more substantial increase in C3 and C4 levels as well as improvement in eGFR. Moreover, anti-dsDNA antibody levels decreased significantly with applied treatment. Compared

to placebo, UPCR levels also showed a greater reduction. In terms of safety, the use of obinutuzmab did not show any correlation with an increase in serious adverse events over a period of 2 years, nor with serious infections or fatalities. Furthermore, there were no instances of severe reactions related to the infusion or severe cases of thrombocytopenia or neutropenia.[53] The data gathered has prompted the initiation of a phase III trial (REGENCY) to test obinutuzumab in patients with class III or IV LN.

Another study, conducted by Arnold et al., verified the validity of obinutuzumab in SLE patients with secondary non-depletion nonresponse (2NDNR) to rituximab. Nine patients who had been previously treated with cycles of rituximab 2 × 1,000 mg and developed 2NDNR were switched to obinutuzumab 2 × 1,000 mg infusions alongside methylprednisolone 100 mg. Six months after the treatment, there were substantial reductions in median Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) and total British Isles Lupus Assessment Group 2004 (BILAG-2004) score as well as significant improvements in C3 and dsDNA levels. Six patients achieved complete B-cell depletion, of whom 4 reached a SLE low disease activity state (LLDAS) with reduced methylprednisolone dosage. Also, no adverse, infusion-related events were observed.[54]

In addition, the efficacy of obinutuzumab in renal and non-renal SLE is currently being evaluated in a phase III trial (OBILUP, NCT04702256) and a phase III trial (ALLEGORY, NCT04963296), both launched in 2021.

Dapirolizumab

CD40 ligand (CD40L), mainly found on activated T lymphocytes and platelets, together with its receptor CD40, are involved in regulating interactions between T cells and other cells. These actions lead to increased B-cell proliferation and differentiation, antibody production, as well as the formation of germinal centers in lymph nodes. Given its pivotal role in activating the immune system, and thus influencing the development of SLE, the CD40 ligand has become a potential target for the treatment of this condition.[54,55]

Dapirolizumab is a polyethylene glycol-conjugated antigen-binding (Fab') fragment that targets CD40L.[56] Unlike its predecessors directed against CD40L, dapirolizumab lacks a functional Fc domain, which has been reported to carry a risk of thromboembolism.[56] In order to verify the safety of dapirolizumab, 2 phase I clinical trials were conducted. In the 1st one, healthy volunteers and SLE patients were administered a single dose of dapirolizumab or placebo. The 2nd trial examined the response to receiving multiple doses of the drug. In the final analysis, in both cases, dapirolizumab was shown to be well-tolerated and no thromboembolic events occurred.[57,58]

A randomized, placebo-controlled phase II clinical trial of dapirolizumab in patients with active SLE has failed to demonstrate a pre-specified dose-response relationship. One hundred and eighty-two patients were randomly assigned to receive placebo or dapirolizumab at a dose of 6 mg, 24 mg or 45 mg every 4 weeks until week 20. The overall treatment efficacy was assessed with British Isles Lupus Assessment Group–based Composite Lupus Assessment (BICLA) at week 24. Despite not reaching the primary endpoint, dapirolizumab-treated patients experienced significant improvements in Systemic Lupus Erythematosus Responder Index (SRI-4), BICLA, SLEDAI-2K, Physician Global Assessment (PGA), BILAG, and Cutaneous Lupus Erythematosus Disease Area and Severity Index (CLASI) scores relative to the placebo group. Moreover, the tested drug appeared to effectively reduce anti-dsDNA levels and increase C3 and C4 levels. In addition, dapirolizumab lowered the risk of severe flares compared to placebo (5 vs 7). Considering the safety of dapirolizumab, it was rated as acceptable, with a similar incidence of adverse events among all groups. Similarly to phase I trials, treatment with dapirolizumab did not increase the risk of thromboembolism. On the other hand, the tested drug raised the frequency of infections, mainly those affecting the upper respiratory tract.[59] The overall results of this study have contributed to a phase III clinical trial to further evaluate the efficacy of dapirolizumab.

Deucravacitinib

Janus kinases (JAKs) are a group of enzymes comprised of JAK1, JAK2, JAK3, and TYK2, involved in transmitting information from the cytokine receptors to the cells. The JAKs phosphorylate STATs (signal transducers and activators of transcription), enabling them to form dimers and translocate into the cell nucleus, where they bind to DNA and activate the transcription of specific genes. This JAK-STAT pathway plays a crucial role in hematopoiesis, inflammation and immune response.[60,61] TYK2 protein binds with JAK1 and JAK2 to mediate the signaling of several cytokines involved in the pathogenesis of SLE, especially type I IFNs, interleukin (IL)-10, IL-12 and IL-23.[62–64]

Deucravacitinib is an oral, allosteric, highly selective inhibitor of TYK2. Unlike the other kinase inhibitors, it binds to the catalytically inactive regulatory pseudokinase JH2 domain of the TYK2, thereby blocking the enzyme in its inactive state and preventing downstream signal transduction.[65–67] The FDA has approved deucravacitinib in the treatment of adults with moderate-to-severe plaque psoriasis who are candidates for systemic therapy or phototherapy. The drug's safety profile was documented by Catlett et al. in a phase I clinical trial involving 100 healthy participants, 75 of whom received the medication. Deucravacitinib was rapidly absorbed and had a half-time of 8–15 h. The drug was found to be safe and well tolerated. No serious adverse events (AEs) were

reported. The incidence of non-serious AEs in test group (64%) was not higher than in the placebo group (68%). The most reported adverse events were headache, nausea, rash, acne, and upper respiratory tract infections.[68]

A randomized, double-blind, placebo-controlled phase II clinical trial (PAISLEY) tested the efficacy and safety of deucravacitinib in adult patients with active SLE with SLEDAI-2K score ≥6 and at least 1 BILAG A or >2 BILAG B manifestations from the musculoskeletal or mucocutaneous domain. Respondents were randomly divided into 4 equal groups receiving deucravacitinib 3 mg twice daily, 6 mg twice daily, 12 mg once daily, or placebo for 48 weeks. The primary endpoint of SRI-4 was evaluated at week 32. A significantly higher response rate of SRI-4 was observed in the group taking 3 mg deucravacitinib than placebo (58.2% vs 34.4%). In the 6 mg and 12 mg dose groups, the primary endpoint was achieved by 49.5% and 44.9% of patients, respectively. Moreover, this correlation was maintained among all groups until the end of the study at week 48. Secondary endpoints assessed at week 48 were met by more patients in all test groups compared to placebo but only the group taking the 3 mg dose achieved a statistically significant difference. Deucravacitinib demonstrated higher BICLA responses (47.3% vs 25.6%), the organ-specific end points for skin (CLASI-50 response, 69.6% vs 16.7%), the treat-to-target end point LLDAS (36.3% vs 13.3%), and a substantial mean change from baseline in the joint count (–8.9 vs –7.6). Furthermore, there was a noticeable enhancement in the C3 and C4 levels, along with a reduction in anti-dsDNA antibodies in patients who were administered deucravacitinib throughout the study. Additionally, a significant decrease in the expression of the *IFN* gene was observed starting from the 4th week. As for safety, adverse events occurred at similar levels in the test groups as in the placebo group, with the most frequent being upper respiratory tract infections, headaches, nasopharyngitis, and urinary tract infections. Nonetheless, acne and rash occurred more frequently with deucravacitinib treatment, with a significantly higher percentage at 6 mg and 12 mg dose. No deaths, systemic opportunistic infections, active tuberculosis, hematologic malignancies, or major cardiovascular incidents occurred.[69] The encouraging outcomes of the PAISLEY trial have prompted the creation of 2 phase III trials (POETYK SLE-1, NCT05617677, and POETYK SLE-2, NCT05620407) that will explore the potential of deucravacitinib in treating extra-renal SLE.

Litifilimab

Plasmacytoid dendritic cells, derived from bone marrow, constitute a specialized subset of DCs.[70] They represent a minor part of peripheral blood leukocytes and organized lymphoid tissue that secrete large amounts of type I IFNs in response not only to various bacterial and viral stimuli but also to SLE immune complexes.[71,72] Numerous studies have indicated that patients with SLE have increased levels of pDC in both skin lesions and affected organs, such as the kidneys, putting them in the spotlight for developing new therapies for SLE.[73–75]

Litifilimab is a humanized IgG1 mAb that binds to the blood dendritic cell antigen 2 (BDCA2), a receptor expressed on pDC cells.[76] As a consequence, litifilimab contributes to a significant suppression of IFN, other cytokines and chemokine production.[77] The first phase I clinical trial conducted to evaluate the safety, tolerability and pharmacokinetics of litifilimab involved 54 healthy volunteers and 12 patients with SLE. It consisted of 3 parts, in which volunteers were administered either single or multiple doses of the drug or placebo. Litifilimab was found to be safe and well tolerated, as well as effective in reducing BDCA2 levels on pDCs, lowering CLASI-A scores, inhibiting INF-1 production and normalizing IFN-response markers, including the expression of myxovirus resistance protein A (MxA) in skin lesions.[76]

Furie et al. continued to further assess the efficacy of litifilimab in patients with SLE and CLE in a 2-part, randomized, placebo-controlled phase II trial. A total of 110 participants were enrolled in part A, which was focused on managing active SLE. Patients were randomized in a 1:1 ratio to receive either a placebo or 450 mg of litifilimab in addition to their standard of care. The primary endpoint based on the reduction from baseline in the number of active joints at week 24 demonstrated superiority of litifilimab over placebo (–15 ±1.2 vs –11.6 ±1.3). In addition, more patients treated with litifilimab achieved a decrease of at least 7 points on the CLASI-A score (56% vs 34%), as well as a greater change in the SLEDAI-2K score. Moreover, the litifilimab group included a higher number of SRI-4 responders in comparison with placebo group (56% vs 29%). However, litifilimab did not appear to significantly reduce the number of SLE-associated autoantibodies or increase C3 and C4 levels.[78] Part B focused on the efficacy of litifilimab in the treatment of CLE and included 132 participants. Patients were randomized to receive placebo or litifilimab 50 mg, 150 mg or 450 mg until week 12. Litifilimab treatment demonstrated a significant advantage over placebo in CLASI-A scores resulting in least-squares mean differences of –24.3 percentage points for the 50 mg dose, –33.4 percentage points for the 150 mg dose and –28.0 percentage points for the 450 mg dose compared to placebo at week 16. In terms of safety, litifilimab appeared to be safe and well-tolerated, with more patients experiencing adverse events in the placebo group (68%) compared to the litifilimab group (59%). The most frequent side effects of the tested drug included diarrhea, nasopharyngitis and urinary tract infections.[79]

Three clinical trials are currently underway: phase III trial (TOPAZ-1), phase III trial (TOPAZ-2) and a 2-part phase II/III trial (AMETHYST) that will provide more information on the efficacy and safety of litifilimab in SLE patients.

Ustekinumab

Interleukin 12 and IL-23 have been identified as crucial cytokines involved in SLE pathogenesis. The former promotes inflammation and triggers the differentiation of Th cells into Th1 cells and stimulates B cells to produce autoantibodies. In addition, IL-12 has a significant function in microbial response by activating NK cells. It consists of a heterodimeric structure composed of 2 subunits, p40 and p35, and is released by monocytes, macrophages and dendritic cells (DCs). Interleukin 23 plays a key role in chronic inflammation. It suppresses the production of IL-2 and is essential for the differentiation of Th cells into Th17 that secrete IL-17 and subsequently induce inflammation by targeting endothelial cells, macrophages, fibroblasts, and keratinocytes. Interleukin 23 has a similar structure to IL-12, composed of a p19 subunit and a shared p40 subunit. It is secreted by antigen-presenting cells, mainly macrophages, DCs and keratinocytes.[80] Both IL-23 and IL-12 levels have been found to be notably increased in patients with SLE compared to control groups.[81,82] The associated pathways of the IL-12 and IL-23/Th17 axis in the pathogenesis of SLE have contributed to the development of a new drug targeting these cytokines.[83]

Ustekinumab is a human IgG1κ monoclonal antibody directed against the p40 shared subunit of IL-12 and IL-23.[84] It has been previously approved in the treatment of plaque psoriasis, psoriatic arthritis, Crohn's disease, and ulcerative colitis.

In a phase II trial conducted by van Vollenhoven et al., 102 participants with seropositive active SLE were randomized (3:2) to either receive 90 mg of ustekinumab or placebo every 8 weeks. The placebo group started receiving ustekinumab 90 mg at week 24. The last dose of the drug was administered to both groups at week 40. The primary endpoint of SRI-4 was evaluated at week 24 and was achieved by 62% patients from the ustekinumab group compared to 33% from the placebo group. In terms of safety, ustekinumab did not increase the risk of adverse events, with the most common being upper respiratory and urinary infections and nasopharyngitis.[85]

The study was extended to week 120 and 46 participants were enrolled, 29 in the ustekinumab group and 17 in the placebo group, with a final dose at week 104. Interestingly, the SRI-4 response assessed at week 112 was achieved to a greater extent by the placebo crossover group (92%) compared to ustekinumab group (79%). Furthermore, both the ustekinumab and the placebo crossover group had significant improvements in SLEDAI-2K score (92% in both), PGA score (79% and 93%, respectively) and active joint count (86% and 91%, respectively). No deaths, malignancies, opportunistic infections, or tuberculosis cases occurred in the study.[84] On the contrary, a phase III trial failed to meet the expectations of the previous study and was terminated as both the primary endpoint; therefore, secondary endpoints were not met.[86]

Moreover, it appears that administration of ustekinumab may increase the risk of new-onset SLE or its flares. A case report indicated that a 68-year-old patient with chronic plaque psoriasis was started on ustekinumab, resulting in the development of subacute cutaneous lupus erythematosus (SCLE).[87]

Targeting of plasma cells

Plasma cells are differentiated B-lymphocyte white blood cells capable of secreting immunoglobulin or antibodies. They are divided into long- and short-lived cells.[88] B-cell-focused treatments may impact the plasma cell section, specifically plasmablasts, by eliminating plasma cell precursors (rituximab – anti-CD20), inhibiting plasma cell differentiation (belimumab – anti-BAFF, atacicept – anti-BAFF/APRIL) or a combination of both (ianalumab – anti-BAFF receptor).[89] However, these therapies generally have no effect on the long-lived plasma cells, as demonstrated on the example of rituximab.[90,91] This shows that plasma cell-directed therapy may become an alternative strategy in the future, particularly for patients who are refractory to the B-lymphocyte-directed therapy. The following strategies are currently under consideration: proteasome inhibition, therapeutic antibodies, chimeric antigen receptor T-cell therapy (CAR-T), or antigen-specific targeting.

Proteasomes function as integral components within a pivotal cellular mechanism, facilitating the regulation of specific protein concentrations and the degradation of misfolded proteins. The identification and targeting of proteins for degradation involves the attachment of a protein known as ubiquitin.[92] Bortezomib is the 1st-generation, reversible proteasome inhibitor. It selectively blocks the function of the 26S proteasome, resulting in a lack of proteolysis of the ubiquitin-proteasome complex. This leads to the accumulation of both misfolded and unfolded proteins, which results in the endoplasmic reticulum stress and the unfolded protein response, leading to an increased susceptibility to apoptosis. Proteasome inhibitors also inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, an important pathway for long-term plasma cell survival.[93]

Bortezomib was evaluated in animal models of SLE and has shown efficacy in depleting both short- and long-lived plasma cells in SLE-prone mice. As a consequence, the depletion of the plasma cells producing anti-dsDNA antibodies was observed, as well as the alleviation of nephritis and a significant increase in survival.[94] Bortezomib has also been tested in patients with SLE. The study showed a significant reduction in the disease activity, attributable to the therapeutic intervention, characterized by a marked reduction in anti-dsDNA antibodies (approx. 60%), which exceeded the reduction in vaccine-induced protective antibody titers (approx. 30%). There was also a reduction in the population of plasma cells in the peripheral blood and bone marrow (approx. 50%).[95,96] Bortezomib is not specific

for plasma cells and therefore causes a number of side effects in the treatment, leading in many cases to the discontinuation of the treatment. Potential side effects include an increased risk of peripheral neuropathy as well as cardiovascular and muscular complications.[97] In another, small, randomized trial involving people with SLE, a high rate of the treatment discontinuation due to serious side effects was found. Additionally, contrary to the previously cited study, there was only a minimal effect on dsDNA titers. The change in anti-dsDNA antibody titer did not support the effectiveness of bortezomib as a therapeutic intervention for SLE. Despite this result, the elevated SRI-4 among the treatment group suggests that bortezomib may have the potential to engage mechanisms beyond the suppression of the anti-dsDNA antibody production.[98]

An alternative approach involves the use of antibodies directed against surface markers that are upregulated at different stages of plasma cell development, a therapeutic strategy currently used in the treatment of multiple myeloma. Notable antibodies in this category include daratumumab (anti-CD38), elotuzumab (anti-SlamF7) and belantamab (anti-B-cell maturation antigen (BCMA)). CD38 and SlamF7 have non-exclusive expression patterns in plasma cells.[89] The therapeutic use of SlamF7 expression seems promising in the treatment of SLE. However, targeting CD38- or SlamF7-positive cells should be approached with caution, as this may inadvertently affect other immune cell populations, including B and T regulatory cells in the case of anti-CD38, and NK cells in the case of anti-SlamF7.[99,100] In contrast, BCMA expression is more specific to plasma cells, which has led to various methods for targeting it in the treatment of multiple myeloma. However, the effectiveness of these methods on plasma cells from different sources is still under investigation in this therapeutic approach. Instances of successful therapeutic outcomes in patients with life-threatening, refractory SLE after receiving daratumumab have been documented.[101] Nevertheless, it is important to note that their administration resulted in a concomitant reduction in tetanus-specific and total IgG antibodies.

Chimeric antigen receptor T-cell therapy

The CAR-T therapy involves taking a patient's T-cells, which are genetically engineered to express chimeric antigen receptors (CARs) that target specific antigens, and then reintroducing these engineered cells into the patient. The result is the activation of a potent immune response against targeted cells (Fig. 2).[102] Recent scientific studies have reported on the application of anti-CD19 CAR-T cell-based therapy in individuals with treatment-resistant SLE. Preclinical studies in a mouse model have shown that therapeutic intervention in SLE with anti-CD19 CAR-T cells results in a reduction in the B-lymphocyte population, cessation of autoantibody formation and reversal of organ-related symptoms.[103] Mougiakakos et al. reported the case of a 20-year-old patient with refractory SLE complicated by active nephritis who underwent the therapeutic intervention described above. After the administration of CAR-T therapy, the patient exhibited a rapid reduction in dsDNA autoantibodies and achieved clinical remission.[104] Building on previous publications, the study by Mackensen et al. reports the results of 5 refractory SLE patients receiving CAR-T therapy. The results revealed a significant reduction in B-cell counts, normalization of clinical parameters and improvement in laboratory results, including a reduction in anti-dsDNA and anti-Sm antibody below detectable levels. Following a 3-month period, all enrolled patients demonstrated sustained SLE remission. The intervention's safety profile shows a positive trend, with only mild cytokine release syndrome observed in some treated patients. However, larger placebo-controlled trials are needed to obtain comprehensive follow-up data.[105] In another case study, a patient with a long (20 years) history of SLE complicated by stage IV diffuse large B-cell lymphoma was treated with a CAR-T construct expressing both anti-BCMA and anti-CD19. After an extended post-treatment period, sustained plasma cell depletion and durable remission were consistently observed, accompanied by undetectable anti-nuclear antibody titers.[106] Chimeric antigen receptor cells offer a pivotal breakthrough in SLE therapy. However, further preclinical investigations and clinical trials are necessary to fully evaluate their potential.

Hematopoietic stem cells

Research endeavors targeting the treatment of SLE frequently incorporate stem cell transplantation, with a particular focus on hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The potential of hematopoietic stem cells transplantation (HSCT) as a therapeutic option for patients with SLE has been the subject of investigation over the past 2 decades. The cited literature review has identified limitations to this therapeutic approach that prevent its widespread clinical use. These constraints include the possibility of adverse effects, a significant tendency for relapse and higher financial burdens compared to biologic medications.[107,108]

Scientific studies have identified changes in the characteristics of MSCs in individuals with SLE. Mesenchymal stem cells derived from SLE patients exhibit deficiencies, including aberrant cytokine secretion, compromised phenotypic features, diminished proliferation, and impaired immunomodulatory capacities.[109] The therapeutic effectiveness of allogeneic mesenchymal stem cell therapy (MSCT) depends mainly on its systemic immunoregulatory effect on various immune regulatory cell populations, including T cells, B cells, plasma cells, dendritic cells, macrophages, and others.[110] In addition, MSCs secrete a range of anti-inflammatory cytokines, which act as mediators in regulating immune responses. In addition, MSCs have the capacity to localize in kidney, lung, liver, and spleen tissues, where they may play a role in the regulation of local inflammatory processes.[111] Over half of the patients with SLE experienced complete and partial clinical remission

following MSCT. Mesenchymal stem cell therapy has been shown to induce remission in multi-organ dysfunction, such as LN. It is worth noting that mild side effects, such as dizziness and a feeling of warmth, were experienced by only a small number of patients.[112] Nevertheless, it is imperative to emphasize the need for additional evidence from large clinical trials to validate the results observed in preclinical studies, while fully elucidating the therapeutic mechanisms underlying MSC treatment.

Limitations

It is important to acknowledge that cited studies and the therapies they propose have their limitations. The heterogeneity of SLE presents challenges at the trial design stage. Achieving a homogenous population is exceptionally difficult. Furthermore, the issue also lies in the small number of patients participating in the reviewed studies. Many scales are used to assess disease activity and treatment response, but there is often a lack of consistency between them. In addition, it is difficult to compare results between studies because the clinical endpoints of the studies are different. Finally, experimental therapies, especially HSC transplantation, carry a high risk of adverse events for a patient, which can lead to serious complications such as serious infections or even death.

Conclusions

In this review, we summarized recent achievements in the treatment of SLE. It is a heterogeneous disease, with a complex pathogenesis and an unpredictable course. Therefore, it is important to develop novel treatment modalities that address these challenges. For patients with suboptimal disease management, the inclusion of anifrolumab and voclosporin in treatment guidelines opens up new opportunities. Anifrolumab is the first biological drug that modulates an interferon signaling pathway that plays a major role in the SLE pathogenesis. At the moment, its results are noninferior to other recommended biologics. On the other hand, voclosporin is the first calcineurin inhibitor specifically indicated in the treatment of LN.

Other biological medications described in our review remain in phase II and III clinical trials, but they have already shown some promising potential. Plasma depletion therapy, CAR-T and HSCT remain an experimental therapy in SLE. Further research is needed to assess the safety and efficacy of the proposed treatment, as well as long-term results and side effects.

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Otolaryngological manifestations in patients with obstructive sleep apnea and continuous positive airway pressure users: A systematic review

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Abstract

Sleep disorders have emerged as a significant public health issue, adversely affecting quality of life and precipitating severe complications. The association between obstructive sleep apnea syndrome (OSAS) and otolaryngological manifestations appears to be underrecognized. This study posits that manifestations in the ear, nose and throat (ENT) among patients with OSAS and users of continuous positive airway pressure (CPAP) therapy are relatively common. Utilizing the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement, this systematic review, registered at PROSPERO (No. CRD42023452473), involved a comprehensive search of the MEDLINE databases. We included studies published in English from 1979 to March 2021 that explored the linkages between OSAS, CPAP and otolaryngological manifestations. A total of 29 articles were reviewed, with findings indicating 12 studies on ear dysfunctions, 11 on nose dysfunctions and 6 on pharynx dysfunctions. Reported symptoms included hearing dysfunction, vestibular function disorders, cerebrospinal fluid leak, Eustachian tube (ET) dysfunction, rhinosinusitis, olfaction and taste disorders, dysphagia, dry mouth, and gastroesophageal reflux. The etiology of these ailments varies, yet an understanding of these symptoms can improve the diagnosis to confirm or rule out OSAS. Early identification of ENT symptoms related to OSAS may facilitate prompt diagnosis and mitigate serious complications.

Key words: continuous positive airway pressure, obstructive sleep apnea, hearing dysfunction, cerebrospinal fluid leak, Eustachian tube dysfunction

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Introduction

Sleep disorders have become a significant public health concern, leading to a reduction in quality of life and serious complications such as stroke, myocardial infarction, heart failure, and overall diminished life quality. Obstructive sleep apnea syndrome (OSAS) is the most prevalent sleep-related breathing disorder, affecting an estimated 936 million adults aged 30–69 globally with mild-to-severe OSAS, and 425 million adults aged 30–69 with moderate-to-severe OSAS.[1] Characterized by recurrent episodes of partial or complete upper airway collapse during sleep, OSAS is typically treated with continuous positive airway pressure (CPAP), the "gold standard" for sleep apnea treatment.

Although OSAS predominantly impacts the cardiovascular and cerebrovascular systems, it can also manifest a range of otolaryngological symptoms. These issues are often overshadowed by more life-threatening problems, thus receiving less attention from both patients and healthcare providers. The association between OSAS, CPAP therapy and otolaryngological manifestations is frequently overlooked during initial examinations. Therefore, identifying ear, nose and throat (ENT) symptoms in patients with undiagnosed OSAS is crucial. This study aims to provide a systematic review of current knowledge on OSAS-related issues in the head and neck region and to assess symptoms during CPAP use.

Objectives

The objectives of this study are:

1. To systematically review and summarize the current knowledge on otolaryngological manifestations associated with OSAS.
2. To assess the prevalence and types of ENT symptoms in patients diagnosed with OSAS.
3. To evaluate the impact of CPAP therapy on the otolaryngological symptoms in OSAS patients.
4. To identify gaps in existing research and suggest areas for future studies on the relationship between OSAS, CPAP therapy and ENT symptoms.

Methods

This review was based on the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines for systematic reviews[2] and was registered in PROSPERO (The International Prospective Register of Systematic Reviews) under No. CRD42023452473.

Search strategy and data sources

A systematic search was performed by screening the MEDLINE database. This database was searched using the terms "obstructive sleep apnea", "OSAS, "CPAP" in conjunction with "otorhinolaryngological manifestation", "ear", "nose", "throat", "oral cavity", "pharynx", "larynx", "hearing", "vertigo", "head and neck cancer", "olfaction", "voice", "infection", "sinusitis", "tinnitus", "tympanosclerosis", "myringosclerosis", "halitosis", "epistaxis", "candidiasis", "xerostomia", "taste", and "facial pain". Boolean operators (NOT, AND, OR) were also searched in succession to narrow and broaden the search. Articles that met the inclusion criteria were examined (Fig. 1).

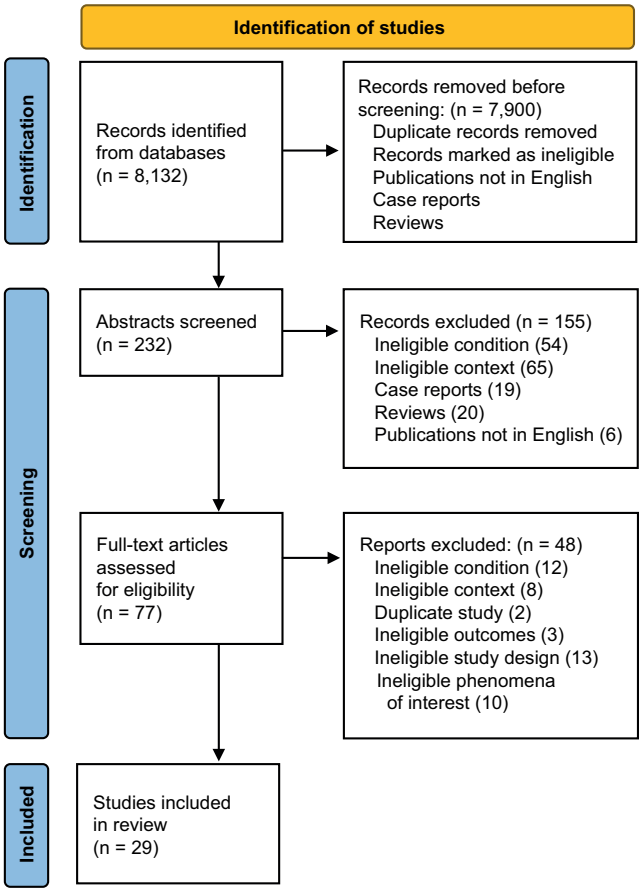

Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) diagram depicting the selection of articles

Inclusion and exclusion criteria

We included all studies published in English between January 1979 and March 2021 that addressed the links between OSAS, CPAP and otolaryngological manifestation. Obstructive sleep apnea syndrome had to be diagnosed by polysomnography. There were no age restrictions.

Due to the focused scope of this review, ENT symptoms in conditions leading to OSAS, such as obesity, hypertension and facial deformities, were not discussed. The search was limited to publications in English. Articles that did not cover the topic areas, case reports, reviews, and duplicate articles were excluded.

Data extraction and assessment of bias risk

The title, abstract, keywords, authors' names, journal names, and year of publication of the identified records were exported to a Microsft Excel spreadsheet (Microsoft Excel 2023; Microsoft Corp., Redmond, USA). Two independent reviewers (J.C. and K.S.) screened the titles and abstracts of the records, and papers that clearly did not address the topic areas were discarded. Then, the 2 reviewers performed an eligibility assessment by carefully screening the full texts of the remaining papers independently. During this phase, disagreements between the reviewers were discussed and resolved by consensus. In the event of disagreement, the views of a 3rd reviewer (M.F.) would have been considered.

Results

Study selection

The search resulted in 8,132 studies, of which 232 abstracts were reviewed. Of the abstracts screened, 54 were ineligible condition, 65 were ineligible context, 19 were case reports, 20 were reviews, and 6 were not in English. Seventy-seven studies were subject to a full eligibility assessment. Our review ultimately included 29 articles; 12 studies showed ear dysfunction, 11 described nose dysfunction and 6 showed pharynx dysfunction. Included articles described ENT symptoms of hearing dysfunction, vestibular function disorders, cerebrospinal fluid leak, Eustachian tube (ET) dysfunction, rhinosinusitis, olfaction and taste disorders, dysphagia, dry mouth symptom, and gastroesophageal reflux disease (GERD). A summary of the included studies and quality of ENT symptoms in OSAS and relevant findings are presented in Table 1 and Table 2.[3–31]

Risk of bias in studies

To evaluate the quality of the identified studies, a tool proposed by Kmet et al.[32] has been applied separately for qualitative and quantitative studies. This instrument for quality assessment takes into consideration the following criteria: the precision of the research aims; the report of study design, materials and methods, justification of study's relevance, sampling strategy, and reflexivity. Each component was rated using a 3-point response scale (2 points for "yes", 1 point for "partial" and 0 points for "no"). If the criterion was not applicable for a study, then its score was excluded from the computation of the overall score. From the 5 possible cutoff points proposed by Kmet et al.[32] (75%, 70%, 65%, 60%, and 55%), the 65% threshold was selected to indicate moderate quality. Overall, the quality of the 29 selected studies was evaluated. All of the studies met the 65% threshold and were included in the analyses.

Discussion

Ear

Hearing dysfunction

The cochlea is particularly sensitive to low partial pressure of oxygen (pO_2) due to its high energy demands, making ischemic hypoxia a significant factor in sudden hearing loss.[3] It has been shown that OSAS reduces blood pO_2 levels, adversely affecting cochlear hair cells and central auditory structures, including the auditory pathway. A study by Li et al. demonstrated high-frequency hearing loss in pure-tone threshold audiometry among patients with severe OSAS.[3] Similar findings were observed in patients with moderate OSAS, who also exhibited deteriorated speech discrimination.[4,5] Pure-tone thresholds and speech discrimination thresholds correlated positively with apnea–hypopnea index (AHI) and desaturation index, and negatively with minimum O_2 saturation ($p < 0.001$).[5] Notably, hearing impairment in the initial phase of OSAS may go unnoticed by patients and might not be detected through pure-tone audiometry. However, there is a significant reduction in the amplitude of the distortion product otoacoustic emissions (DPOAE).[3] The DPOAE, an objective hearing test commonly used in clinical settings to assess the health of the inner ear (outer hair cells), shows changes in patients with OSAS as an early indicator of cochlear damage before it manifests itself in reduced hearing thresholds. In the study, 71% of OSAS patients showed abnormal DPOAE results at 750 Hz, compared to 0% in the control group ($p < 0.001$). Similarly, abnormalities were observed at other frequencies: 47% at 1 kHz, 26% at 2 kHz, 33% at 4 kHz, 28% at 6 kHz, and 34% at 8 kHz, while no abnormalities were detected in the control group ($p < 0.001$ for all frequencies).[3] Damage to hair cells at the cochlear base, where higher frequencies were encoded according to tonotopy, was more pronounced, which explained the observed deterioration in high-frequency hearing in pure-tone audiometry.[6] Additionally, 42% of OSAS patients experience tinnitus within the 4–8 kHz frequency range, further confirming the frequent damage to hair cells at the cochlear base.[5]

Another objective hearing test that exhibits abnormalities in patients with sleep apnea is the auditory brainstem response (ABR).[3] The ABR assesses the accuracy of transmission from the auditory nerve to the auditory radiation in the forebrain, with results consisting of 7 waves, labeled I–VII, whose latencies, interlatencies and morphology are analyzed. At a stimulation rate of 11 times per second, the binaural wave I latency in OSAHS patients was 1.51 ±0.13 ms, compared to 1.33 ±0.07 ms in the control group ($p < 0.001$). The wave V latency in OSAHS patients was 5.65 ±0.23 ms, compared to 5.53 ± 0.23 ms in the control group ($p = 0.0016$). At the higher stimulation rate of 51 times per second, the wave I latency was

Table 1. Summary of the reviewed studies

Author	Year	Sample size	Procedure	Results
Xin et al.[3]	2019	78	Patients underwent: PTA, ABR, DPOAE.	High-frequency hearing loss in adults with severe OSAS, wave I and V latencies were prolonged in ABR.
Kayabasi et al.[4]	2019	120	Studies correlated: apnea–hypopnea index, desaturation index and min oxygen saturations with pure-tone thresholds, speech recognition thresholds, and speech discrimination scores.	High-frequency hearing functions is affected in moderate OSAS. Severe OSAS affected all hearing functions negatively
Martines et al.[5]	2015	160	Patients underwent: polysomnography, multi-frequency audiometry, acufenometry, TEOAE.	Chronic hypoxia develops early cochlear damage and it is more marked in severe OSAS (high-frequency hearing loss)
Casale et al.[6]	2011	39	Patients underwent: polysomnography, pure tone audiometry, ABR, TEOAE.	The mean latencies of waves I, III and V were prolonged in OSAS patients. The IPL of I–V was significantly prolonged.
İriz et al.[7]	2017	31	All participants underwent: polysomnography pure tone audiometry, speech discrimination analyses, auditory time processing, and sequencing tests.	OSAS group had a significant loss in speech discrimination rates compared to the control group.
Kayabasi et al.[8]	2014	50	Participants assessed: PTA, VNG, caloric vestibular response tests, and DHI.	Moderate-to-severe OSAS patients had higher scores in the DHI. Nystagmus and canal paresis rates were significantly higher in the moderate-to-severe OSA group.
Micarelli et al.[9]	2017	32	OSAS patients underwent: PG testing, Video vHIT and SPT.	Studied group had significant decay of VOR gain and an increase in both frequency spectra PS values, especially within the low-frequency interval, and in classical posturographic SP parameters.
Schutt et al.[10]	2015	32	Frequency of OSA, BMI, and presence of tegmental defects in patients with SSCD were compared to the control cohort.	The patients with SSCD demonstrated higher rates of OSA (SSCD 29.03% vs no SSCD 7.00%) and rates of tegmental defects (SSCD avg. 64.5% vs no SSCD 16%) in comparison to the control cohort.
Yancey et al.[11]	2020	94	Comparison of a sCSFL and nsCSFL in patients undergoing lateral cranial base repair. BMI, OSA, CPAP, and intracranial hypertension were compared.	OSAS and CPAP use was more prevalent among spontaneous CSFL.
Magliulo et al.[12]	2018	40	Eustachian tube function was assessed with eustachian tube score-7 (ETS-7) (tubomanometry, tympanometry, Valsalva's and Toynbee's symptoms), rhinomanometry, mucociliary transport time.	Twenty percent of the patients with OSA had ETS-7 ≤ 7 which confirmed diagnosis of eustachian tube dysfunction.
Thom et al.[13]	2015	10	Evaluating serial tympanometry on sleeping adult patients during polysomnography was performed. MEP was recorded awake at 1-h intervals during diagnostic polysomnography and at all CPAP levels during titration.	The mean MEP during sleep without CPAP was 26 daPa, which was significantly lower than the mean MEP during sleep with CPAP (5–10 cm H_2O).
Sivri et al.[14]	2013	78	The middle ear pressure of CPAP users and CPAP no users (control group) was evaluated with tympanometry at the beginning of the study and 6 months later.	The middle ear peak pressure values in CPAP users were significantly increased from –63.04 ±55.82 daPa to –39.6 ±27.72 daPa after 6 months.
Deniz et al.[15]	2014	122	Mucociliary clearance was measured by saccharin test in patients divided into 3 groups with mild, moderate and severe OSAS.	Severe OSAS patients had a statistically significant prolonged mucociliary clearance.
Saka et al.[16]	2012	25	Before and 3 months after the start of CPAP treatment, a patient with OSAS had a measurement of mucociliary clearance measured with a saccharin test and tissue samples from the inferior turbinate, middle turbinate and septal mucosa.	Mucociliary transport times before and after treatment were not significant. CPAP treatment significantly influenced histological changes in the nasal mucosa.
Constantinidis et al.[17]	2000	10	Patients with OSAS who had undergone nCPAP-therapy had mucociliary clearance measured with a saccharin test and tissue samples from the inferior turbinate – the specimens were examined by electron microscopy.	In all patients the nasal epithelium underwent fundamental changes u CPAP-therapy (modifications in the shape of epithelial cells, conglutination and clumping of the microvilli).
Ji et al.[18]	2019	41	Comparison of SNOT-22 scores between CRS and OSA groups.	CRS group demonstrated higher scores in nasal, extra-nasal, and ear/facial symptoms, while OSA group displayed higher psychological and sleep domain scores.

Table 1. Summary of the reviewed studies – cont.

Author	Year	Sample size	Procedure	Results
Arens et al.[19]	2010	54	Magnetic resonance imaging was used to evaluate radiographic changes within the: paranasal sinuses, middle ear and mastoid air cells, and the nasal passages in children with OSAS.	Children with OSAS had significantly more opacification of maxillary sinuses, sphenoid sinuses and mastoid air cells, middle ear effusions.
Kaya et al.[20]	2020	26	The CCCRC odor test was done in OSAS patients before treatment and 4 months after CPAP therapy.	The odor test average scores of the patients after 4-month CPAP therapy compared to pretreatment scores were increased and the difference was statistically significant.
Versace et al.[21]	2017	20	The study's objective was to assess the cholinergic function, as measured using SAI, a paired-pulse TMS protocol in OSAS patients with olfactory impairment.	This study revealed a significantly reduced SAI, a putative marker of cholinergic dysfunction in study participants with OSAS and olfactory dysfunction.
Liu et al.[22]	2020	120	Study participants were divided into 3 groups according to polysomnography results: snoring group, mild-to-moderate OSAS group and severe OSAS group. Olfactory and gustatory functions were evaluated by the Sniffin' Sticks test and the triple-drop method.	There was a significant difference in OT, OD, OI, TDI and total taste score in all 3 groups.
Walliczek-Dworschak et al.[23]	2017	44	Olfactory and gustatory function was measured (Sniffin' Sticks test battery and "taste strips") in OSAS patients diagnosed via polysomnography and eligible for CPAP treatment before and after treatment.	Baseline olfaction decreased in OSA patients and improved significantly after CPAP therapy olfactory scores (TDI, ID).
Koseoğlu et al.[24]	2017	30	All participants underwent polysomnography. The "Sniffin' Sticks" test was used for the analysis of olfactory function before the CPAP therapy and about 4 months after the therapy.	OT, OD, OI and TDI scores significantly increased after PPAP therapy.
Yenigun et al.[25]	2019	65	Participants were divided into 4 groups according to apnoea–hypopnoea index, obtained on polysomnography. Smell CCCRC and taste (taste strips) tests were performed on these patients.	The smell threshold in severe OSAS group was significantly lower than that in control group. The sweet, sour and salty taste threshold in severe OSAS group was significantly lower than that in the control group.
Schindler et al.[26]	2013	72	OSAS patients without symptoms of dysphagia underwent a FEES examination using 5, 10 and 20 mL of liquids and semisolids and solids.	The results of the FEES examination demonstrated that 28% of examined patients had piecemeal deglutition with the 10 mL liquid trials and 64% had spillage with the 20 mL liquid trials.
Jäghagen et al.[27]	2003	80	Videoradiography was performed to examine the oral and pharyngeal swallowing function in patients with OSAS.	Pharyngeal swallowing dysfunction was observed in 50% of patients with an AHI > 30, 61% with an AHI of 5–29, and 43% with an AHI < 5.
Hanning[28]	2005	39	Vibratory sensation threshold and 2-point discrimination were determined in the pharynx using Vibratron II clinical unit in OSAS patients.	There was a significant impairment in sensory detection threshold for OSA versus control subjects in the oropharynx.
Heiser et al.[29]	2013	26	OSAS participants had checked: mechanical sensitivity (two-point discrimination test at the soft palate and air puffs at the posterior pharyngeal wall) and chemosensitivity (sensitivity to capsaicin at the posterior pharyngeal wall) at the pharynx.	There was decreased sensitivity to capsaicin and air puffs in OSAS patients. Two-point discrimination at the soft palate was reduced with statistical significance in the OSA group.
Oksenberg et al.[30]	2006	631	The participants were asked to answer the following question: "During the last month, did you experience waking up in the morning with a dry mouth?" The scale consisted of 5 categories: "never", "rarely", "sometimes", "often", or "almost always".	The prevalence of dry mouth upon awakening was two-fold higher in patients with OSA (31.4%) than in primary snorers (16.4%) and increased linearly from 22.4% to 34.5%, and 40.7% in mild, moderate and severe OSA.
Green et al.[31]	2003	181	Patients with OSAS graded their frequency of nGER symptoms on a scale of 1 (never) to 5 (always). All patients were prescribed CPAP. At follow-up, the frequency of nGER symptoms was obtained.	The patients compliant with CPAP had a significant improvement in nGER score, from a mean of 3.38 before CPAP treatment to 1.75 after treatment (48%) improvement; while patients not using CPAP (control subjects) showed no improvement (mean, 3.56–3.44). There was a strong correlation between CPAP pressure and improvement in nGER score, patients with higher CPAP pressures demonstrating a more significant improvement in nGER score.

ABR – auditory brainstem response; AHI – apnea hypopnea index; BMI – body mass index; CCCRC – Connecticut Chemosensory Clinical Research Centre; CPAP – continuous positive airway pressure; CRS – chronic rhinosinusitis; DHI – dizziness handicap inventory; DPOAE – distorted products otoacoustic emission; ET – Eustachian tube; FEES – fiberoptic endoscopic evaluation of swallowing; IPL – interpeak latency; MEP – middle ear pressure; nGER – nocturnal gastroesophageal reflux; nsCSFL – non-spontaneous cerebrospinal fluid leak; OD – odor discrimination; OI – odor identification; OT – odor thresholds; PG – polygraphic; PTA – pure-tone audiometry; SAI – short latency afferent inhibition; sCSFL – spontaneous cerebrospinal fluid leak; SNOT-22 – 22 Item Sinonasal Outcomes Test; SPT – static posturography testing; SSCD – superior semicircular canal dehiscence; TDI – thresholds-discrimination-identification; TEOAE – transient-evoked otoacoustic emissions; TMS – transcranial magnetic stimulation; vHIT – video head impulse testing; VNG – videonystagmography.

Table 2. Quality of ENT symptoms in OSAS and relevant findings

Symptom	Quality changes in OSAS patients
Hearing dysfunction[3–7]	High-frequency hearing loss, prolonged ABR latencies, significant loss in speech discrimination.
Vestibular dysfunction[8,9]	Nystagmus and canal paresis rates were significantly higher, significant decay of VOR gain.
Spontaneous cerebrospinal fluid leak[10,11]	OSAS and CPAP use was more prevalent among spontaneous CSFL.
Eustachian tube dysfunction[12–14]	Eustachian Tube Dysfunction, The middle ear peak pressure values in CPAP users were significantly increased.
Rhinosinusitis[15–19]	Prolonged mucociliary clearance, CPAP treatment significantly influenced histological changes in the nasal mucosa.
Olfaction disorders[20–25]	Reduced odor threshold and discrimination.
Dysphagia[26–29]	Pharyngeal swallowing dysfunction.
Dry mouth syndrome[30]	The prevalence of dry mouth upon awakening was twofold higher in patients with OSA.
Nocturnal gastroesophageal reflux[31]	There was a strong correlation between CPAP pressure and improvement in nGER score.

ABR – auditory brainstem response; VOR – vestibulo-ocular reflex; OSAS - obstructive sleep apnea syndrome; CPAP – continuous positive airway pressure; CSFL – cerebrospinal fluid leak; nGER – nocturnal gastroesophageal reflux.

1.64 ±0.12 ms in OSAHS patients compared to 1.44 ±0.06 ms in the control group (p = 0.0001). The latency of wave V was 5.92 ±0.26 ms in the OSAHS group compared to 5.80 ±0.18 ms in the control group (p = 0.0077).[3] Li et al. reported increased wave I and V latencies in patients with OSAS, indicating conduction disturbances in the central auditory pathway, which was corroborated by İriz et al. through speech discrimination tests.[3,7] While Li et al. demonstrated increased wave I and V latencies, they also noted that air-conduction thresholds in patients with moderate-to-severe OSAHS were similar to those of healthy controls. This suggests that while central auditory pathways are affected, peripheral hearing remains relatively unaffected.[3]

Peripheral vestibular system damage

Obstructive sleep apnea syndrome, by affecting the inner ear, also disrupts the peripheral vestibular system, which is crucial for maintaining balance, stability and spatial orientation. The primary symptoms of peripheral vestibular system damage include vertigo and nystagmus, accompanied by visceral autonomic symptoms such as nausea and vomiting. The primary test used to assess labyrinthine function is videonystagmography (VNG), which reveals a significantly higher occurrence of nystagmus and canal paresis in patients with moderate-to-severe OSAS compared to those with mild OSAS.[8] Furthermore, patients with moderate-to-severe OSAS exhibit higher scores on the Dizziness Handicap Inventory (DHI) survey, indicating the prevalence and impact of dizziness on daily life.[8] Micarelli et al. demonstrated vestibular dysfunction in patients with no evidence of vestibular dysfunction on otologic examination using the vestibulo-ocular reflex (VOR), which stabilizes gaze during head movements by activating the vestibular system. In the video Head Impulse Test (vHIT), the mean VOR gain was significantly lower in OSA patients (0.42 ±0.06) compared to healthy controls (1.05 ±0.16).[9] This masking of clinical evidence of vestibular disorder can be explained by the central vestibular system compensating for disequilibrium caused by peripheral vestibular system damage.[9] Impaired vestibular function should prompt physicians to refer patients for polysomnography, especially when the etiology of vestibular organ damage is not fully understood.

Cerebrospinal fluid leak

The etiology of cerebrospinal fluid leak (CSFL) is often unclear and frequently cannot be identified. The most common cause is trauma to the dura mater due to surgery or accidents. However, this condition may also be associated with OSAS, where it is linked to increased intracranial pressure (ICP). Apneic episodes can lead to hypoxia and hypercapnia, resulting in increased cerebral blood flow and elevated ICP.[10] Additionally, OSAS frequently coexists with obesity, a potential risk factor for spontaneous CSF (sCSF) leak, as obesity can lead to idiopathic intracranial hypertension (IIH).[10,11] Consequently, weight loss may reduce both OSAS severity and the incidence of elevated ICP.[11] However, increased ICP in OSAS patients is independent of concurrent obesity and is correlated with apneic episodes and decreased SaO_2 levels.[10] In this context, Yancey et al. noted that 47% of sCSF patients had OSAS, suggesting that polysomnography (PSG) should be considered in patients with sCSF.[11]

Eustachian tube dysfunction

Maintaining normal middle ear pressure is vital to middle ear health and depends on the proper functioning of the ET. Obstructive sleep apnea syndrome has been identified as an independent risk factor for ET dysfunction.[12] Negative pressure in the upper airway of OSAS patients may lead to tissue collapse around the ET, resulting in dysfunction. Eustachian tube dysfunction causes negative middle ear pressure (MEP) and may contribute to various otologic conditions such as conductive hearing loss, tympanic membrane retraction and chronic otitis

media.[13] Continuous positive airway pressure therapy provides a pneumatic stent for the upper airway and also prevents the collapse of the ET ostia, thereby reducing the negative consequences for the middle ear. Thom et al. noted that the average MEP during sleep in CPAP users increase to more than half its value, thus mitigating the effects of decreased pressure in the middle ear.[13] This relationship was further supported by Sivri et al., who observed a conversion from a type B or C (abnormal MEP) tympanogram to a type A (normal MEP) in a number of CPAP users.[14] Continuous positive airway pressure therapy can also be successfully used in tympanic membrane retraction.[13] However, increases in MEP in CPAP therapy can cause otalgia, ear fullness, pharyngitis, and in rare cases even tympanic membrane rupture, pneumocephalus and tension pneumocranium.[13]

Nose

Rhinosinusitis

One of the most important functions of the nasal mucosa is mucociliary clearance, which cleanses the sinuses. In the severe OSAS group, Deniz et al. found that the mean mucociliary clearance time was significantly prolonged.[15] These findings suggest that OSAS may lead to deterioration of mucociliary transport, further causing chronic rhinosinusitis (CRS).[15] The impact of CPAP on mucociliary transport is significant. Some studies showed no adverse effect of CPAP therapy on mucociliary function, while others revealed negative changes in the nasal epithelium caused by dry and cold air.[16,17] Reported adverse effects of CPAP include epithelial flattening, decreased cilia number and prolonged mucociliary clearance. Cold and dry air provokes the release of inflammatory factors, causing inflammation of the nasal mucosa.[17] However this study used non-humidified CPAP, whereas humidified CPAP is now the standard, as it redusces negative side effects.

Symptoms such as fatigue, difficulty sleeping, nasal obstruction, facial pain, and headaches are commonly reported by OSAS patients and can mimic CRS symptoms.[18] The 22-item Sinonasal Outcome Test (SNOT-22) is commonly used in patients with CRS. Ji et al. showed that SNOT-22 is a valuable tool for identifying undiagnosed OSAS.[18] Their study concluded that OSAS patients more often cited sleeping difficulty as the most troublesome symptom compared to CRS patients, who reported nasal and ear-related complaints (thick nasal drainage, ear pain and anosmia). Therefore, patients with high sleep -and psychological domain scores on SNOT-22 should undergo PSG.[18]

Pediatric rhinosinusitis may also be associated with OSAS. According to Arens et al., OSAS in children leads to sinus inflammation, particularly in the maxillary sinus.[19] However, in most cases, pediatric OSAS occurs due to adenoid hypertrophy, which itself generates inflammation, defects in mucociliary clearance and nasal obstruction.

Olfaction and taste disorders

Similar to hearing, the sense of smell is sensitive to hypoxia. This sensitivity is evident in studies showing that the cholinergic neurotransmitter system is susceptible to hypoxemia, affecting brain activity in the thalamus, hippocampus, prefrontal, and posterior parietal cortex.[20,21] These neuroanatomical structures influence cognitive abilities and may be implicated in dysfunctional smell identification and odor differentiation.[20] Liu et al. demonstrated significant reductions in odor thresholds, odor discrimination (OD), odor identification (OI), and total taste score in patients with snoring and OSAS in subjective odor and taste measurements.[22]

Sleep apnea has been shown to increase pro-inflammatory effects on the nasal mucosa by markers such as interleukin (IL)-8 and tumor necrosis factor alpha (TNF-α).[20] Inflammation of the olfactory epithelium disrupts odor transmission in the olfactory nerve.

Numerous clinical studies have confirmed that CPAP therapy can improve olfaction in patients with OSAS through several mechanisms.[20,23,24] First, by increasing oxygen saturation, which has a positive effect on cognitive function and by normalizing cholinergic neurotransmitters.[20,23] Second, positive airway pressure reduces the amount of inflammatory factors in the airways, which improves the condition of the olfactory epithelium and improves odor identification.[20,23]

Taste in OSAS patients may also be affected by neuropathy resulting from vibrations in the upper respiratory tract tissue. However, anosmia itself also impairs the gustatory sensations.[25]

Pharynx

Dysphagia

Dysphagia frequently occurs in patients with OSAS.[26] The fiberoptic endoscopic evaluation of swallowing (FEES) has revealed swallowing abnormalities that were not self-reported by patients.[26] During the FEES, a flexible endoscope records the swallowing process using boluses of different textures (thin liquid, semisolid and solid) and volume to assess the signs and symptoms of oropharyngeal dysphagia.[26] The FEES exam demonstrated that 28% of patients had piecemeal deglutition with the 10 mL liquid trials and 64% had spillage with the 20 mL liquid trials, confirming dysphagia.[26] Furthermore, another study indicated a disturbance in the protective role of the epiglottis.[27] In half of the study participants with OSAS, the epiglottis remained elevated and the airway open after taking a bolus of food, indicating an increased risk of aspiration when the patient inhaled rapidly or was speaking at the same time.[27] However, the severity of dysphagia did not correlate with the severity of OSAS.[26]

Vibration trauma during snoring in OSAS causes local neuronal damage, resulting in failure of the swallowing reflex response. A larger volume of food in the pharynx is required to provoke this reflex compared to healthy patients.[26,28] Heiser et al. showed a decreased mechanical sensitivity of the pharynx using a 2-point discrimination test at the soft palate and air puffs at the posterior pharyngeal wall in OSAS patients.[29] This research also found impaired chemosensitivity of the pharynx during stimulation with capsaicin and CO_2 at the posterior pharyngeal wall, which suggest a possible link between local neuropathologies and OSAS.[29]

Dry mouth symptom

Saliva performs essential functions such as protecting dentition and the oral mucosa, aiding digestion, providing antibacterial activity, enhancing taste, and lubricating oral tissues. Studies have found that approx. 74% of OSAS patients report dry mouth and the necessity for water intake during the night or immediately upon waking.[30] Moreover, dry mouth may reduce the function of taste receptors.[25]

Other issues reported by OSAS patients include painful burning and/or tingling sensations of the oral mucosa, lips, tongue, gingiva, and teeth, as well as difficulties with chewing, halitosis and dental impairment. Halitosis and dental impairment may also be associated with laryngopharyngeal reflux, which is more common in OSAS.[33]

Gastroesophageal reflux

Some studies have noted a reduction in OSAS symptoms during GER treatment and an improvement in GER symptoms during OSAS treatment.[31] Continuous positive airway pressure therapy has also been observed to decrease nocturnal GER symptoms.[31] Green et al. suggested that OSAS may lead to GER, rather than GER causing OSAS, though the direction of causality remains controversial.[31]

Laryngopharyngeal reflux (LPR) is clinical entity different from classic GER.[31] The main symptoms of LPR are dysphonia, chronic cough, sore throat, and pharyngeus globus.[31] These symptoms are caused by the irritating effect of acid which can affect tissue superior to the hypopharynx and larynx. Obstructive sleep apnea syndrome can induce GER and LPR by creating greater negative intrathoracic pressure, thereby impairing esophageal sphincter function.[31] Gastric acid and other gastric contents, such as pepsin, cause inflammation, hypertrophy and sensory disturbances in the larynx and pharynx that contribute to the progression of OSAS.[31] Given that the associations between GER and OSAS are still unclear, further clinical trials are necessary to elucidate their respective mechanisms.

Limitations

This study has several limitations that should be acknowledged:

1. Language restriction: Only articles published in English were included, potentially excluding relevant studies in other languages.
2. Search limitation: The search was conducted only in the MEDLINE database, which may have resulted in the omission of relevant studies available in other databases.
3. Exclusion of certain conditions: ENT symptoms related to conditions leading to OSAS, such as obesity, hypertension and facial deformities, were not discussed, which might have provided a more comprehensive understanding of the topic.
4. Quality of included studies: While all included studies met the 65% quality threshold, varying levels of quality and potential biases within these studies could influence the overall findings of this review.

Conclusions

This paper has established that otolaryngologic manifestations are common symptoms of OSAS, particularly in severe cases. It is often the case that ENT symptoms, which include hearing dysfunction, rhinosinusitis, olfaction disorders, and gastroesophageal reflux, are linked with OSAS. This can result in significant complications. Recognizing these symptoms is crucial for differential diagnosis in patients presenting with sleep-related issues. The presence of these manifestations should prompt the use of polysomnography to confirm an OSAS diagnosis, especially when they occur with other sleep disorder symptoms. Continuous positive airway pressure therapy, the primary treatment for OSAS, has been shown to alleviate many ENT symptoms associated with the condition. Future research should focus on further elucidating the connections between ENT symptoms and OSAS to enhance diagnostic accuracy and treatment outcomes. This will underscore the role of otolaryngology in managing this pervasive disorder.

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