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Contents

Evidence-based healthcare: Bridging the gap between research and practice

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Abstract

The establishment of the first JBI Affiliated group in Poland at Wroclaw Medical University marks a significant advancement in evidence-based healthcare (EBHC) nationally. This editorial explores the evolution of EBHC and the critical role of JBI in driving its progress. Founded in 1996 as a research institute at the Royal Adelaide Hospital in South Australia and now based at the University of Adelaide, JBI has emerged as an international leader in evidence synthesis, transfer and implementation. Its Feasibility, Appropriateness, Meaningfulness, and Effectiveness (FAME) framework highlights the feasibility, appropriateness, meaningfulness, and effectiveness of healthcare practices, ensuring that decisions are patient-centered and contextually relevant. JBI's global collaboration network encompasses over 85 entities, with 23 located in Europe, emphasizing the importance of cultural inclusivity and international partnerships. Recent initiatives include translating the JBI Model of into Polish, German and Czech, linking global knowledge to local contexts, and enhancing understanding for professionals and students alike. This editorial also underscores the collaborative achievements of JBI entities in Wroclaw, Brandenburg an der Havel, Prague, and Olomouc. These partnerships have propelled regional implementation, research and education, fostering a shared vision for elevating healthcare quality. Launching a new EBHC section in the *Advances in Clinical and Experimental Medicine* journal is a significant step forward, inviting global contributions and stimulating innovation and knowledge sharing in EBHC. The presence of a JBI Affiliated group at Wroclaw Medical University symbolizes a transformative commitment to excellence and collaboration. It sets new benchmarks for healthcare in Poland and beyond while reinforcing the global mission of evidence-based practice.

Key words: collaborative research, evidence-based practice, internationality, translational medical research

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Introduction

Establishing the first JBI Affiliated Group in Poland at Wroclaw Medical University marks a pivotal moment in advancing evidence-based healthcare (EBHC) nationally. By aligning with JBI, previously known as the Joanna Briggs Institute, Wroclaw Medical University commits to the highest standards of EBHC. It signifies a commitment to innovation, excellence and patient-centered care, catalyzing positive change in the area and setting a new benchmark for healthcare quality in Poland.

Today, professional practice is acknowledged as requiring a foundation of sound evidence combined with expertise gained from education and experience. Healthcare professions and education now emphasize research-based evidence, a shift that began only after the 1950s. Previously, practices relied heavily on handed-down knowledge from experienced professionals, often unevaluated. Early attempts to test treatment effectiveness lacked the scientific rigor now considered essential.

The JBI Collaboration is comprised of over 80 entities that collaborate worldwide. The closest JBI entities to Wroclaw are Brandenburg an der Havel (Germany) and Prague and Olomouc (Czech Republic). These entities are the closest not only geographically but also because of their ongoing collaboration. The present editorial focuses on a comprehensive exploration of EBHC and highlights the collaborative efforts and insights from the JBI centers in Wroclaw, Brandenburg an der Havel, Prague, and Olomouc.

The evolution of evidence-based healthcare

Evidence-based healthcare evolved from evidence-based medicine (EBM) in the early 1990s. In 1990, Professor Dr. Gordon Guyatt from McMasters University (Hamilton, Canada) introduced Scientific Medicine, a bedside teaching method inspired by Professor Dr. David Sackett. His colleagues reacted negatively, rejecting the idea that current practices were unscientific. In 1991, Guyatt renamed the curriculum Evidence-Based Medicine and published it. Evidence-based medicine asserts that medical procedures and medications must be supported by evidence of effectiveness, cost-efficiency and safety. While the term emerged in 1991, foundational work began earlier.[1]

The EBM movement also owes much to the pioneering work of Scottish physician and epidemiologist Dr. Archibald Cochrane, who advocated basing medical decisions on rigorous research and data. In 1972, Cochrane's lecture introducing his monograph "Effectiveness and Efficiency: Random Reflections on Health Services" highlighted the lack of evidence for medical treatment effectiveness in the UK, criticizing it as a waste of resources and a cause of unnecessary suffering.[2] The monograph, known by its short title "Effectiveness and Efficiency," became a bestseller and captured the attention of physicians, medical administrators and public health officials in the UK, the USA and many other countries. That seminal text supported the development of evidence-based practice ideals in medicine and other health disciplines.[3]

Building on this foundation, Professor Alan Pearson greatly enhanced the concept by expanding the scope of evidence-based practices from medicine to encompass all areas of healthcare. As a leading figure in the development of EBHC, Pearson advocated for the inclusion of all health professions in the evidence-based approach, ensuring that EBHC became a comprehensive model for enhancing care across disciplines, from nursing to allied health fields.

Pearson's concerns about the lack of an evidence base for patient care began in the late 1970s and involved nursing, as he was a nurse working at that time in Ward 33 of North Tees General Hospital in Stockton-on-Tees in the UK. The high incidence of pressure sores among orthopedic patients in his ward led him to research the latest treatment methods and implement the most effective one. He observed that many colleagues preferred seeking peer advice over using well-researched evidence. In the early 1980s, as head of UK's first nurse-led unit for post-acute patients, Pearson became even more convinced that nursing practice should be evidence-based. At that time, little research on clinical nursing problems was being utilized, and care was primarily based on the clinical opinions of senior nurses and passed down through generations.[4]

While Cochrane and Pearson were exploring evidence-based practice, the prevalent clinical trials utilized comparison groups. Despite efforts to improve measurement and reduce bias, the focus remained on matching patients with similar characteristics, which limited the accuracy of the findings. Cochrane argued that the only way to improve accuracy was through randomized controlled trials (RCTs), namely, randomly assigning patients to groups to eliminate bias. He called RCTs "a very beautiful technique" for testing whether one treatment is better than another, expressing results as probabilities, and placing RCTs at the top of his evidence hierarchy.[4]

It has to be highlighted that the ideas behind the RCTs that Cochrane promoted in "effectiveness and efficiency" were not new. The necessity of a research-based approach to medicine and the significance of randomization in assessing effectiveness was already emphasized in the mid-19th century by French scientist Pierre Louis. While modern clinical trials are more advanced and rely on statistical analysis rather than Louis's simple arithmetic, the core principle of randomization remains unchanged.[5] Nevertheless, Louis's random method for enhancing efficiency did not spark interest in the medical community until 1946 when the *British Journal of Medicine* announced the first UK RCT on human subjects for a new drug, streptomycin, initiated by Sir Austin Bradford Hill.[6] Still, it is widely considered that the first RCT was conducted by James Lind, a Scottish physician. Through his pioneering experimental study, Lind

demonstrated the therapeutic value of fruits containing what was later identified as vitamin C in treating scurvy, setting a foundational example for clinical research methodology.

Although Cochrane strongly advocated for RCTs as the best way to determine treatment effectiveness, he recognized their limitations and challenges. He acknowledged that while double-masked trials reduced bias, RCTs were not the only valuable research design. Still, he believed they offered the most accurate information for medical administrators choosing between alternatives. However, Cochrane emphasized that conducting more trials alone was insufficient; their findings needed to be readily accessible to clinicians to impact practice.[4]

Cochrane recognized that research findings would more effectively influence clinical practice if systematically reviewed and aggregated. Starting in 1970, Dr. Ian Chalmers conducted the first systematic review of controlled trials (to our current standards), which Cochrane hailed as a milestone in evaluating care. This success led to 2 influential books in 1989, profoundly impacting policy and practice. The findings became a basis for decision-making by professionals and laypeople, while scientists benefited from insights guiding further research.[7]

Following the success of the review, Chalmers and others argued that funding was needed to establish a Cochrane Centre to undertake a range of systematic reviews. While some clinicians questioned the value of secondary research, the Cochrane Collaboration was founded in the 1990s, building on Cochrane's work and influence. It has since led the development and promotion of evidence-based medical care and remains a leader in designing methodologies for systematic reviews of RCTs. The establishment of the current Cochrane Collaboration can be credited to 3 people: Dr. Tom Chalmers, Dr. Ian Chalmers and Dr. Murray Enkin. Today, the collaboration connects review groups worldwide and provides training and support.[4] For example, Cochrane Poland – a branch of the Nordic Cochrane Centre in Copenhagen, hosted by the Systematic Reviews Unit – Polish Cochrane Branch, was established in 2015 at the Faculty of Medicine of the Medical College of the Jagiellonian University in Cracow.[8]

The evidence-based movement began gaining momentum in the early 1990s when several agencies became involved in systematic reviews. Additionally, the Campbell Collaboration, established in the USA in 2000, should be mentioned. The international collaboration focuses on evidence of the effects of social and educational policies and practices by service providers, policymakers, educators, their students, and professional researchers. The Cochrane Collaboration established the standard for most other international collaborations. Thus, the focus was primarily on the systematic review of trials for specific medical conditions, client groups or particular interventions by health professionals.[4]

When discussing those who played a crucial role in formalizing and expanding the concept of EBM, Sackett must be acknowledged in more detail. As one of the key figures in developing EBM at McMaster University, Sackett was instrumental in establishing evidence-based practice as a core principle in medical education and healthcare. His work provided the framework for integrating clinical expertise with the best available research evidence.[9] In 1981, articles from the *Canadian Medical Association Journal* by Sackett, Dr. Brian Haynes, Dr. Peter Tugwell, and Dr. Victor Neufeld introduced a method for physicians called "critical appraisal." They aimed to teach not only how to understand literature but also how to apply new information at the bedside.[10]

Other notable contributors to the development of EBM include numerous researchers and clinicians whose efforts have greatly advanced the field.[10–15]

The role of JBI in advancing evidence-based healthcare

The JBI was established as a research institute by Pearson in 1996. It was based at the Royal Adelaide Hospital in South Australia and made possible through a grant from the Royal Adelaide Hospital Research Foundation. In recognition of this support, the institute was named after Mrs. Joanna Briggs, who served as the hospital's first Matron in 1855. Since 2010, JBI has been integrated into the Faculty of Health and Medical Sciences at the University of Adelaide.[4]

JBI, based at the University of Adelaide, is an international collaboration of health scientists, professionals and researchers dedicated to promoting and supporting evidence-based decision-making that improve health and healthcare service delivery. The JBI's vision is "Better evidence. Better outcomes. Brighter future." Its mission is to promote and support EBHC.[16] What distinguishes JBI is that it is an organization involved not only in evidence synthesis but also in the transfer and utilization of evidence for the clinical decision-making process. Pearson's vision has always been to bridge academia and practice, making a reality of what the theoreticians are saying.

From the very beginning, international collaboration and global partnership were at the forefront of Pearson's mind. Through the JBI Collaboration (JBIC), JBI collaborates with universities and hospitals worldwide, ensuring that the research evidence we synthesize, transfer and implement is culturally inclusive and relevant to the diversity of healthcare internationally. The JBIC is comprised of JBI Centres of Excellence and JBI Affiliated Groups. Currently, it includes over 85 collaborating entities worldwide, with 23 located in Europe.

According to Pearson, the Founding Executive Director, and Professor Zoe Jordan, the current Executive Director, the main aim of JBI is to "get the appropriate information into the hands of those who determine health policy and deliver healthcare, as this is fundamental to improving healthcare delivery and health outcomes."[17]

Pearson's path to founding JBI began well before the rise of the so-called evidence-based movement. His inspiration

stemmed from his time as a clinician in the UK, where he was influenced by philosophies that connected research with practice, sparking meaningful changes in nursing care.[4] His ambitious vision for JBI encompassed establishing global collaborating entities, training systematic reviewers, creating "Practice Information Sheets" (now known as Best Practice Information Sheets) for clinicians, and developing and delivering short courses on evidence-based nursing.[4] JBI aims to improve global health by providing point-of-care access to evidence databases, decision support systems, implementation, evaluation, and continuous improvement tools.

JBI defines EBHC as decision-making that considers the feasibility, appropriateness, meaningfulness, and effectiveness of healthcare practices.[18] This process is informed by the best available evidence, the context in which care is delivered, the individual patient, and the professional judgment and expertise of the health professional.[18] This approach is encapsulated in JBI's FAME framework, which stands for Feasibility, Appropriateness, Meaningfulness, and Effectiveness. Feasibility is the extent to which an activity or intervention is practical and practicable. Clinical feasibility is whether or not an activity or intervention is physically, culturally or financially practical or possible within the given context. Appropriateness is the extent to which an activity or intervention fits with context or situation. Clinical appropriateness is about how the activity or intervention relates to the context in which the care is given. Meaningfulness refers to the significance a patient associates with an activity or intervention based on their experience with it. It encompasses personal experiences, opinions, values, thoughts, beliefs, and interpretations expressed by patients or clients. Effectiveness is the extent to which an activity or intervention achieves the intended effect.[18–20]

The logo of JBI effectively embodies its ideas and activities. Designed to represent the evidence-based movement and its connection to clinical practice, the logo was entrusted to Simone Lee. It was ultimately decided that a pebble dropping into water, creating ripples, is the apt metaphor for knowledge sharing and practice change. Today, the JBI red "pebble of knowledge" and its surrounding ripples are recognized worldwide as the organization's trademark. This logo is prominently featured in all JBI publications, products and promotional materials, symbolizing the profound impact a single pebble can have. Additionally, each JBI entity has its logo that includes both JBI and the entity's name (Fig. 1–3).

The original JBI Model of EBHC was published in 2005 and defined evidence-based practice as "clinical decision-making that considers the best available evidence, the context in which the care is delivered, client preference and the professional judgment of the health professional."[19] Furthermore, this model has emerged as a crucial benchmark, showcasing JBI's distinct and innovative strategy for conceptualizing and operationalizing EBHC. Consecutively, based on the results of citation analysis and a stakeholder engagement process, the model was updated.[20]

Fig. 1. Logo of the evidence-based healthcare in Wroclaw: A JBI Affiliated Group

Fig. 2. Logo of the evidence based practice in Brandenburg: A JBI Affiliated Group

Fig. 3. Logo of the Czech Republic: A JBI Centre of Excellence

Pearson et al. emphasized the importance of EBHC as a process that not only identifies and addresses clinical or policy questions but also ensures that the knowledge generated is appraised, synthesized and effectively translated into practice. This approach focuses on delivering healthcare that is not only evidence-based but also effective, feasible, and meaningful for specific populations, cultures and settings.[21]

JBI highlights the importance of translational science, which bridges the gap between evidence and practice, ensuring that evidence is implemented in ways that positively impact health outcomes, health systems and professional practice. Moreover, JBI acknowledges the critical need to bridge an often-overlooked gap – the gap between the need for knowledge and its discovery – ensuring that the evidence generated aligns with real-world healthcare challenges and priorities.

In 2024, EBHC in Wroclaw (EBHC-W): A JBI Affiliated Group, undertook the translation of the JBI Model into Polish (Fig. 4). Recently, the Evidence Based Practice in Brandenburg (EBB) – A JBI Affiliated Group also translated the model into German (Fig. 5). Earlier, entities in the Czech Republic had translated the JBI model into Czech (Fig. 6). These initiatives allow healthcare professionals, researchers and policymakers in these 3 countries to engage with the JBI Model of EBHC principles without language barriers, bridging the gap between global knowledge and local practice. By facilitating integration into the Polish, German and Czech healthcare context, the translation enhances understanding and ensures accurate implementation of the model. Translating the JBI Model into other languages has significant educational benefits, particularly for future healthcare professionals. Providing the model in their native language improves comprehension, encourages critical thinking and fosters

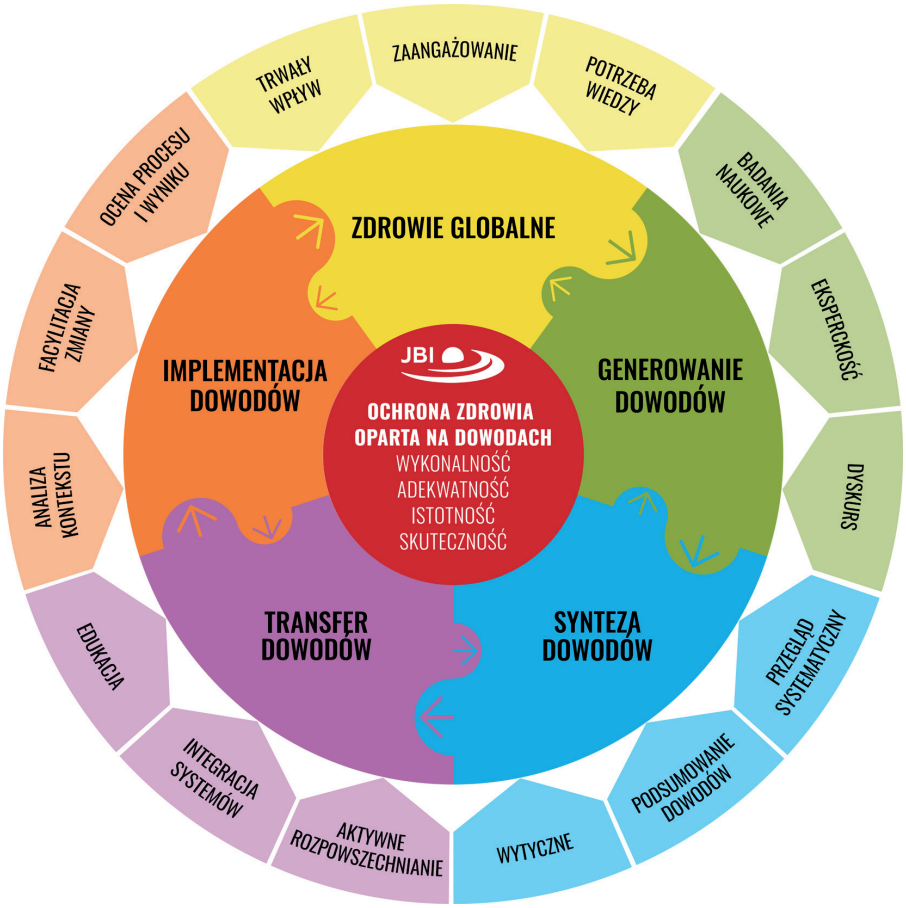

Fig. 4. Polish translation of the JBI model of evidence-based healthcare

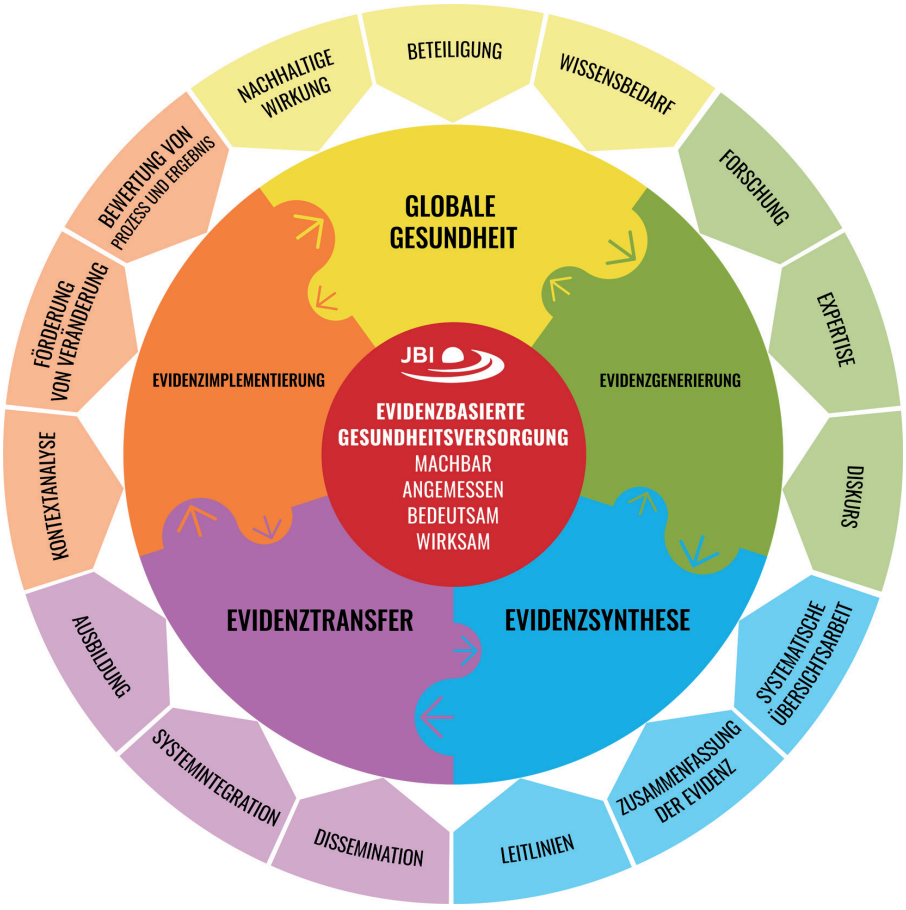

Fig. 5. German translation of the JBI model of evidence-based healthcare

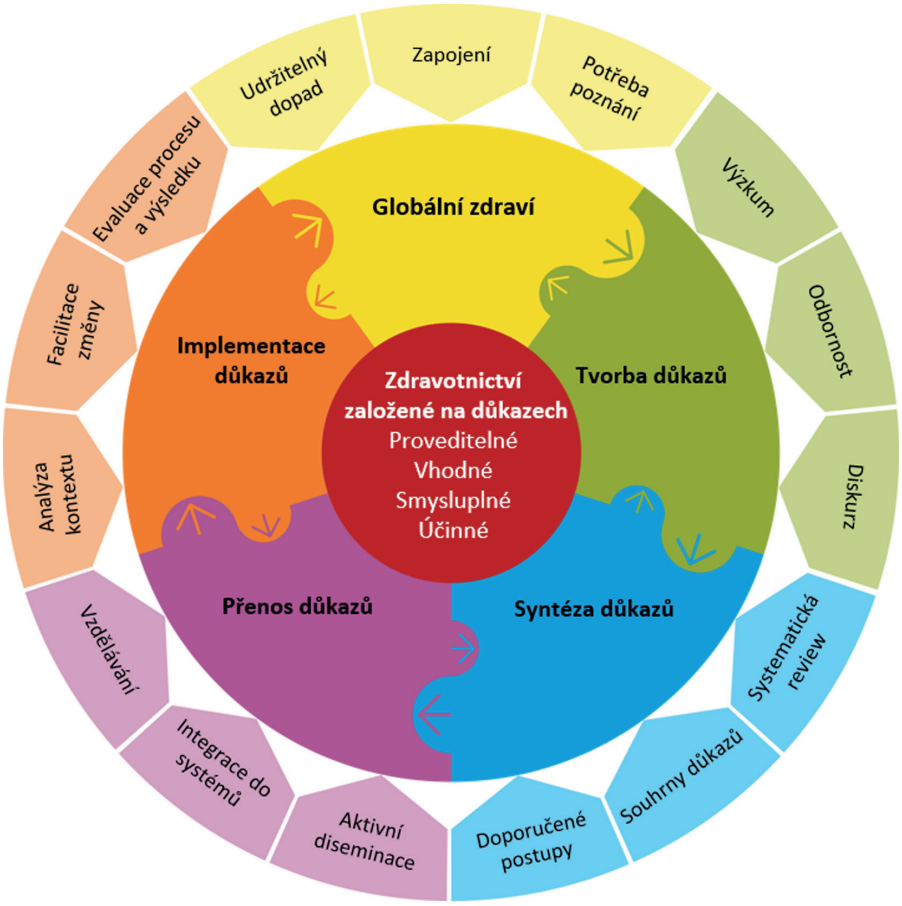

Fig. 6. Czech translation of the JBI model of evidence-based healthcare

engagement, enabling students to grasp its nuances and applications better. This inclusive education and professional development approach supports the JBI's mission to advance evidence-based practice and drive global healthcare improvement.

Regional perspectives: The JBI entities in Wrocław, Brandenburg an der Havel, Prague, and Olomouc

The establishment of a JBI Affiliated Group at Wroclaw Medical University has been one of the tasks financed under the university's 2024–2026 Development Strategy, titled "Wroclaw Medical University in the Light of Scientific Excellence 2024–2026".

The Strategy mentioned above prioritizes enhancing the quality and international scope of scientific research, improving teaching standards and elevating the global recognition of Wroclaw Medical University. As part of these efforts, establishing a JBI Affiliated group aligns with the University's objective of building partnerships with leading international research institutions. In 2024, dr. hab. Aleksandra Królikowska, a university professor, became the Proxy of the Rector of Wroclaw Medical University for EBHC, leading to the establishment of the EBHC in Wroclaw (EBHC-W) Group. That year, the EBHC-W Group applied for and joined the JBI Collaboration, a process started by Królikowska, who also serves as the the Group's convenor.

The EBHC-W: A JBI Group is dedicated to synthesizing, transferring and implementing evidence to enhance patient care and outcomes across diverse healthcare disciplines. The group's primary strength lies in its diverse and multidisciplinary team, which effectively fosters innovative interprofessional collaboration to tackle complex healthcare challenges. The EBHC:W: A JBI Group focuses on conducting systematic reviews, developing innovative methodologies and creating tailored dissemination strategies. Its primary goal is to apply the best available evidence, particularly within the Jan Mikulicz-Radecki University Hospital in Wrocław, while striving to broaden its international impact continuously.[22]

The Evidence-Based Practice in Brandenburg (EBB): A JBI Affiliated Group was established in 2023. The convenors – Dr. Robert Prill and Professor Dawid Pieper – lead a multidisciplinary group focusing on evidence synthesis and evidence implementation. Both have been involved in the European Network Grant COST CA17117, focusing on evidence-based research.[23,24] Within the project, they began collaborating with Dr. Miloslav Klugar. Prill finished the JBI Comprehensive Systematic Review Training Program at an entity led by Klugar. Both successfully applied

together with universities from Cracow and Split for an Erasmus+ Strategic Partnership Project focusing on Evidence Implementation under the leadership of the German team at Brandenburg Medical School. Despite pandemic-related difficulties, the "Evidence Implementation in Clinical Practice" (EICP) Project was successfully finished and the EBB team has been already involved in 32 implementation projects using the JBI Implementation Framework. Examples of successfully published best practice implementation projects are "Nutrition as Therapy – the Role of Dietitian Counseling", "Intra-Articular Knee Injections in Patients with Primary Osteoarthritis in a Tertiary Clinical Setting", "Cross-Disciplinary Advance Care Planning in Oncology and Palliative Care Amidst a Pandemic", "Promoting Running as the Best Treatment for Lower Back Pain in Physiotherapy Practice", or "Education of Adult Type I Diabetes Patients in a Diabetes Ward Setting".[25–29] In 2024, the EBB organized a JBI Comprehensive Systematic Review Training Programme for the first time, with participants from Brandenburg an der Havel and Wrocław. The participants of the course, coming from various disciplines within healthcare, have already started working on their reviews.

The Czech Republic: A JBI Centre of Excellence (Czech JBI) was established in 2013 at Palacký University Olomouc. It was promoted to the status of "Centre of Excellence" in 2016 due to its activities in evidence synthesis, evidence implementation and research methodology. Dr. Miloslav Klugar and Dr. Jitka Klugarová, leaders of the Czech JBI, are members of several JBI method groups. They have trained over 200 health professionals in evidence synthesis methods and more than 100 in implementation methods, not only in the Czech Republic but also globally. Czech JBI contributed to the establishment and mentoring of other JBI groups in the region, including the Center of Evidence-Based Education and Arts Therapies: A JBI Affiliated Group at the Faculty of Education, Palacký University Olomouc (2021) and Evidence-Based Practice in Brandenburg – A JBI Affiliated Group (2023). The Czech JBI co-initiated an important discussion in the Czech Republic regarding the "National Trustworthy Guidelines," which led the leaders of Czech JBI to establish the Cochrane Czech Republic and the Czech GRADE Network in 2018. 2018 was also the year when the pilot project "Clinical Practice Guidelines" received financial support from the Ministry of Health. Then, in 2023, the Ministry of Health of the Czech Republic established the National Institute for Quality and Excellence in Healthcare (NIKEZ), under which the Czech JBI, Cochrane and GRADE centers are currently hosted as 3 international pillars and guarantors of the robustness and trustworthiness of Czech National Guidelines.[30] The 3 Czech international centers won a bid in 2018 to host the most significant and largest event in the field of EBHC, the Global Evidence Summit (GES), which was successfully held in Prague in September 2024.[31] Klugar served as the Chair of the Scientific Committee of GES. The center was successful in several research grants that supported 52 JBI implementation projects across 8 European countries. The center contributed to establishing the International Living Map for COVID-19 recommendations during the COVID-19 pandemic.[32] Currently, the center collaborates with numerous entities worldwide regarding evidence implementation, evidence-based practice and evidence-based research.

Implementation and collaborative research achievements

The JBI entities from Germany, the Czech Republic and Poland have already participated in 1 collaborative implementation project, the results of which were published in 2024 in the *JBI Evidence Implementation* journal.[28] This implementation project aimed to promote running as an effective treatment for lower back pain (LBP) in an outpatient physiotherapy setting, addressing gaps in understanding the role of intervertebral disc water management. Conducted following the JBI Evidence Implementation Framework, the project employed an evidence-informed clinical audit and feedback strategy, resulting in significant improvements in compliance with key criteria and enhancing patient confidence in managing their condition through running.[28] The entities also published protocols in JBI Evidence Synthesis of the systematic review they initiated on the effects of physiotherapy interventions for home-based rehabilitation on physical function following primary total knee arthroplasty.[33] Currently, they are working on new joint scoping and systematic reviews and are planning further collaborative implementation projects.

With the professional background of the convenors from Wrocław and Brandenburg an der Havel, their collaboration has been deeply focused on advancing research in orthopedics, traumatology, sports medicine, and rehabilitation. In 2023, they co-authored an editorial titled "Why There is a Need to Improve Evaluation Standards for Clinical Studies in Orthopedic and Sports Medicine," which launched a series of articles aimed at enhancing evaluation standards in these fields.[34] The series was published in 2023–2025 in *Knee Surgery, Sports Traumatology, Arthroscopy* (KSSTA) and the *Journal of Experimental Orthopaedics* (JEO), both official journals of the European Society of Sports Traumatology, Knee Surgery and Arthroscopy (ESSKA), a leading pan-European organization for knee surgery, arthroscopy and sports traumatology. By launching this series written by experts in given fields, the 2 journals sought to equip researchers with the tools and knowledge to produce high-quality studies that drive meaningful advancements in the field.

The first editorial by Prill, Królikowska and leading experts in orthopaedics and sports medicine emphasized the need for transparency, rigor and adherence to reporting guidelines, such as the Consolidated Standards of Reporting Trials (CONSORT), to address common issues

like inadequate randomization, unclear methodologies and poor result reporting.[34] Other co-authored articles offered valuable, practical insights on tackling these issues, such as how to prepare and register a study protocol, which checklists and guidelines help report research, how to verify the reliability of the measurement tool intended for study purposes, and how to apply patient-reported outcome measures (PROMs).[35–38] As an extension of their activities on the improvement of research in orthopedics, traumatology, sports medicine, and rehabilitation, Królikowska, Prill et al. recently explored and mapped the reporting practices and methodological quality in RCTs published in the KSSTA journal, focusing on identifying gaps in adherence to reporting guidelines and transparency.[39]

One of the editorials from the series mentioned above, co-authored by the representatives of JBI entities from Germany, Poland and the Czech Republic, precisely Prill, Królikowska and Klugarová, addressed the challenges and importance of implementing EBM in everyday clinical practice, particularly in orthopedics and sports medicine. It highlighted the need for integrating clinical expertise, patient preferences and high-quality evidence while overcoming barriers such as resource limitations, communication gaps among stakeholders and varying healthcare contexts.[40] Prill, Królikowska and Klugar have made significant efforts to promote systematic and scoping reviews, emphasizing the value of evidence-based research in rehabilitation, orthopedics and sports medicine, while advocating for new research that systematically builds on previous studies to address critical knowledge gaps and prevent unnecessary redundancy.[23,41,42]

Another highly significant activity in the context of knowledge translation was the involvement of Prill (Chair), Królikowska and Klugarová (Steering Group) in the Formal EU-US Meniscus Rehabilitation Consensus: An ESSKA-AOSSM-AASPT initiative (2022–2024). The goal of the consensus, which was a collaborative effort by ESSKA, the American Orthopaedic Society for Sports Medicine (AOSSM) and the American Academy of Sports Physical Therapy (AASPT), was to provide recommendations for the rehabilitation of patients undergoing either conservative or surgical treatment for meniscus lesions or acute meniscus tears.[43] Consensus initiatives are crucial components of EBHC, as they combine expert opinions and research findings to develop unified, reliable guidelines that ensure consistent, high-quality care throughout the healthcare spectrum settings.[44] The convenors of the described JBI entities are currently involved in consecutive ESSKA and other expert consensuses.

Future directions and collaborative vision

Considering the professional backgrounds and experiences of the JBI entities from Wrocław, Brandenburg an der Havel, Prague, and Olomouc, their collaboration demonstrates significant potential to shape the future of evidence-based practice, particularly but not limited to rehabilitation within orthopedics, sports medicine and traumatology. The collaboration aims to promote interdisciplinary approaches that enhance care across the continuum – from prevention to treatment and recovery. By emphasizing rigorous methodologies, innovative research and the integration of high-quality evidence into clinical practice, this partnership is poised to tackle key challenges and create impactful advancements.

Currently, Prill chairs the ESSKA Rehabilitation Committee, with Królikowska set to take over from 2026 to 2028, showcasing their dedication to the field. Since its establishment, the ESSKA Rehabilitation Committee has been crucial in promoting interdisciplinary collaboration among healthcare professionals, fostering innovation and bridging gaps between diverse specialties.[45] Looking ahead, the Committee aims to enhance clinical outcomes and advance patient care throughout Europe.[46]

In addition, the collaboration between Wrocław, Brandenburg an der Havel, Prague, and Olomouc intends to strengthen partnerships with organizations such as ESSKA, expand educational initiatives and advocate for evidence-based practice among healthcare stakeholders. These activities will prioritize integrating patient or population perspectives, ensuring the real-world applicability of research findings and addressing healthcare disparities.

In a related development, the *Advances in Clinical and Experimental Medicine* journal has introduced a new section called "Evidence-Based Healthcare". This section, overseen by editors Aleksandra Królikowska and Robert Prill, invites submissions from researchers worldwide. We strongly encourage authors to contribute their work to this initiative, which will foster further growth and innovation in the field of EBHC.

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Chronic pain in the elderly: A constant challenge

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Abstract

Chronic pain is a common, long-standing and bitter experience affecting a huge percentage of the still increasing elderly population. Owing to the multifactorial etiopathology and complex clinical presentation with a lot of severe consequences, management of the permanent pain should be varied and tailored to the particular patient. This approach comprises multimodal pharmacotherapy, including all analgesics and adjuvants, likewise selected interventions, physical therapy and rehabilitation, as well psychological counselling.

Key words: elderly, chronic pain, neuropathic pain, multimodal pain treatment, opioids

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Introduction

Pain is defined as both sensory and emotionally feeling connected with current or potential tissue damage.[1] Persistent pain means pain that continues beyond the expected time of healing, or for minimum 3–6 months.[2] Patophysiologically, chronic pain is categorized as nociceptive (from tissue injury), neuropathic (from nerve injury, like in diabetes) or nociplastic (from a sensitized nervous system, like in fibromyalgia).[2,3]

As the population of elderly people grows, the number of the oldest, frailest and pain-ridden is increasing at the fastest rate. Numerous comorbidities, as well as psychological, social and environmental factors may contribute to pain severity and effectiveness of treatment applied.

Clinical presentation

In the Polish study PolSenior 2, the incidence of pain was reported by 52% of women and 41% of men aged ≥60 years. Chronic pain was reported in 47.6% of the examined seniors. Of the 4.5 million older people with pain, 25% suffer from severe pain. Chronic pain management with medication was reported by 38% of Polish seniors.[4]

The most common pain complaints in the elderly are related to osteoarthritis, neurodegenerative and musculoskeletal conditions, peripheral vascular diseases, rheumatoid arthritis, polymyalgia rheumatica, giant cell angiitis, as well to often misdiagnosed myofascial pain syndrome, low back pain, lumbar spinal stenosis, and fibromyalgia.[1–3,5]

Interestingly, older people manifest an altered pain experiencing, which is a result of the changed pain processing mechanisms involving a structural and functional brain plasticity. This phenomenon is probably associated with the degeneration of circuits modulating the descending pain inhibitory pathways, with the periaqueductal gray (PAG) constituting a key node.[6]

Prolonged pain may impair physical and cognitive functions manifested by falls, kinesiophobia, immobility, problems with appetite and sleep, depression, anxiety, and increased risk of dementia and delirium.[1–3,5]

It should be emphasized that there are bidirectional interrelations among pain and depression, insomnia and anxiety. Finally, permanent pain can contribute to worsened life quality, social isolation, impaired physical activity, and institutionalization.

Management of chronic pain

Currently, the importance of a multidisciplinary model of pain treatment is emphasized strongly. This approach comprises multimodal pharmacotherapy, selected interventions, physical therapy and rehabilitation, as well psychological counselling. Cuomo et al. proposed the "multimodal trolley approach" that takes into account the physical, psychological and emotional causes of pain and underlies the necessity for personalized therapy. According to this approach, a dynamic management of pain by combining several pharmacologic and non-pharmacologic strategies is possible.[7]

The principles of the analgesic ladder (as outlined by the World Health Organization (WHO) in 2019) should be followed when introducing analgesics. The 1st step for mild pain is acetaminophen, metamizole or nonsteroidal anti-inflammatory drugs (NSAIDs). For moderate pain, it is recommended to use weak opioids such as tramadol, codeine or dihydrocodone. For severe and persistent pain, the 3rd step involves potent opioids (morphine, buprenorphine, oxycodone, tapentadol, fentanyl).[8–10] It is important to note that many older adults are reluctant to use opioids due to concerns about addiction. However, proper education can help them accept opioids when medically necessary.

Because of the complex nature of pain perception, there is a wide range of drugs from different classes that can be beneficial in different pain conditions. These so-called adjuvants or co-analgesics include antidepressants: tricyclic antidepressants (amitriptyline, nortriptyline), serotonin-norepinephrine reuptake inhibitors (duloxetine, venlafaxine), anticonvulsants (pregabalin, gabapentin), topical agents (lidocaine or capsaicin patches), corticosteroids, bisphosphonates, miorelaxants and also medicinal cannabinoids. Interestingly, although adjuvants are co-administered with analgesics, they are indicated as a first-line treatment option for treating specific pain conditions, like neuropathic pain and fibromyalgia.[6,8–10]

It is important to emphasize that there is a generally accepted gold standard for pharmacotherapy in geriatric patients that should always be followed: "Start low and go slow".

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A systematic review and meta-analysis of serum cystatin C levels and acute ischemic stroke outcomes

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Abstract

Acute ischemic stroke (AIS) has a high rate of death and causes long-term disability, leading to a global economic burden annually. Therefore, discovering biomarkers to improve AIS patient prognosis is critical. Previous studies reported an association between serum cystatin C (CysC) levels and outcomes in AIS patients, but the results remain controversial. This systematic review and meta-analysis aimed to explore the relationship between serum CysC and AIS patient outcomes using currently available studies. The literature search included PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure (CNKI), VIP, and Wan Fang databases. Outcomes included poor functional recovery, cognitive dysfunction and death. Weighted mean difference (WMD) with 95% confidence interval (95% CI) was used as an effect index for measurement data. Results demonstrated that serum CysC was significantly higher in AIS patients with poor functional recovery (WMD = 0.18, 95% CI: 0.08–0.28), cognitive dysfunction (WMD = 0.16, 95% CI: 0.09–0.23) and death (WMD = 0.32, 95% CI: 0.02–0.62) than in the control groups when follow-up time was <1 month. These findings show that high serum CysC levels were associated with poor AIS patient outcomes. Further studies are needed to examine whether reducing serum CysC can prevent poor outcomes in AIS patients.

Key words: risk factors, biomarkers, cognitive dysfunction, cystatin C, ischemic stroke

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Introduction

Stroke has one of the highest global morbidity rates, with ischemic stroke accounting for approx. 87% of such events.[1] Acute ischemic stroke (AIS) refers to disorders of the cerebral circulation which cause irreversible damage to local brain tissues, leading to brain tissue ischemia and hypoxic necrosis in the acute stage.[2] Acute ischemic stroke has a high rate of death and causes long-term disability, resulting in a considerable annual economic burden worldwide.[3]

Acute ischemic stroke is caused by focal cerebral hypoperfusion, mainly from atherosclerotic diseases.[2] During a transient ischemic attack, arterial flow to brain tissue is temporarily interrupted, leading to focal neurologic symptoms (such as hemiparesis), but spontaneous flow recovery can alleviate symptoms without permanent tissue damage.[2] However, if arterial flow does not recover promptly, ischemia may develop into irreversible infarction, and patients may experience an AIS.[2]

Several neural substrates are thought to be involved in AIS, such as kynurenine, methylxanthine and N-methyl-D-aspartate,[4–6] and have been shown to be associated with cognitive functions, motor skills and emotions.[7–13] Post-stroke cognitive dysfunction is a common symptom affecting 20–80% of patients and impacting neurological function recovery.[14] Depression is a common emotional syndrome after a stroke that substantially reduces patients' quality of life and occurs in approx. 30% of cases.[15] Quantitative analyses of electroencephalogram data have identified the psychophysiological correlates of cognition. However, current international guidelines do not endorse the use of electroencephalogram biomarkers in neuropsychiatric disorders and depression.[16,17] To improve the prognosis of AIS patients, it is necessary to identify other methods, such as simple and economical biochemical detection indicators, that may have additional clinical application value.

Serum cystatin C (CysC) is a member of the endogenous cysteine protease inhibitor family that can inhibit the activity of endogenous protein cysteine[18]; it is released from all nucleated cells at a constant rate, and can freely pass through the glomerular filtration membrane due to its low molecular weight.[18,19] Previous studies demonstrated that CysC was an effective biomarker for assessing kidney function.[19] Kidney disease is a crucial risk factor for cerebrovascular diseases, and the proposed underlying mechanisms for this association include cerebral hypoperfusion.[20] Recent studies found an association between CysC and cardiovascular diseases and showed its involvement in the pathophysiology of atherosclerosis in AIS patients.[21,22]

Serum CysC levels were significantly elevated in patients with AIS.[23] Zeng et al. found it to be an independent prediction biomarker for ischemic stroke,[24] and a meta-analysis by Wang et al. reported an association between high serum CysC levels and increased ischemic stroke risk.[25] However, the correlation between CysC and AIS patient outcomes also deserves attention, though the specific mechanisms by which CysC affects AIS patient outcomes remain unclear.

Cystatin C is an endogenous cathepsin inhibitor that plays a central role in regulating vascular wall proteases and antiproteases,[26] and unbalanced expression of CysC and cysteine cathepsin may lead to atherosclerosis.[26] In addition, increased serum CysC levels may indicate renal dysfunction in AIS patients,[27] and those with chronic renal failure generally experience oxidative stress and systemic inflammation, which accelerates the progression of atherosclerosis.[27]

Cystatin C participates in inflammatory reactions and injures nervous system cells, creating a vicious cycle.[28] Existing studies show that CysC plays a role in neuronal damage and dysfunction, and may be related to clinical manifestations of cognitive impairment in neurodegenerative disorders.[29,30] Meanwhile, other studies found that CysC can inhibit cerebral amyloid protein aggregation and potentially prevent cognitive dysfunction in neurodegenerative disorders.[31,32] Previous studies reported an association between CysC and AIS patient outcomes, but the results are inconsistent.[29,33–35] Zeng et al. found that higher serum CysC levels were associated with a higher risk of cognitive dysfunction,[36] whereas Guo et al. reported a protective effect of increased CysC levels in cognitive dysfunction.[33] The reason for this discrepancy may be the difference in serum CysC levels among the populations included in their studies (1.07 compared to 0.77).[33,36]

Liu et al. found that a higher CysC level was related to an increased risk of recurrent stroke in AIS patients.[34] Dong et al. also found that the risk of recurrence was elevated with increasing serum CysC in AIS patients.[37] However, Zhu et al. found no association between CysC level and recurrence in AIS patients.[35] Such a discrepancy may be due to the difference in follow-up time of their studies (1 year compared to 2 years).[34,35,37]

A meta-analysis is a powerful tool that combines the results of 2 or more separate studies to demonstrate good evidence strength and contribute to healthcare decision-making.[38,39] Considering the controversial results in previously reported studies, we aimed to conduct a systematic review and meta-analysis to comprehensively explore the association between CysC level and AIS patient outcomes to guide unfavorable outcome management in AIS patients.

Objectives

The study analyzed the association between CysC levels and outcomes in AIS patients.

Methods

The meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement (PRISMA) guidelines.[40]

Literature search

The literature search included 4 English-language (PubMed, Web of Science, Cochrane Library, and Embase) and 3 Chinese databases (VIP, China National Knowledge Infrastructure (CNKI) and Wan Fang) from inception to August 21, 2023. Supplementary File 1 shows the search terms. The literature search was conducted by 2 independent researchers (CGH and SBC), and the 3rd person (Guosen Bu) provided consultation if conflicts arose.

Study selection

The inclusion criteria were 1) patients: patients with AIS, 2) reporting: serum CysC levels (at baseline); 3) outcomes: functional recovery, cognitive dysfunction, death, hemorrhagic transformation, vascular events, depression, and recurrence; 4) cohort studies or case-control studies; and 5) language: published in Chinese or English. Studies meeting 1 of the following criteria were excluded: 1) duplicated publication; 2) animal studies; 3) incomplete or inaccessible data, 4) not matching the topic; and 5) reviews or meta-analyses, conference abstracts, case reports, and letters.

Functional recovery was evaluated using the modified Rankin Scale (mRS)[41] and the National Institute of Health Stroke Scale (NIHSS).[42] According to the mRS, patients were divided into good recovery (mRS ≤2 points) and poor recovery (mRS >2 points) groups.[41] Based on the NIHSS, patients were divided into basic recovery (91–100% decrease in NIHSS score), significant improvement (46–90% decrease in NIHSS score), improvement (18–45% decrease in NIHSS score), and ineffective/deterioration (≤17% decrease in NIHSS score) groups.[42]

The Montreal Cognitive Assessment (MoCA) evaluated cognitive function from a total score of 30 points, with <26 points defined as cognitive dysfunction.[29]

Data extraction and quality assessment

Two researchers (CGH and SBC) independently conducted data extraction, including the study authors, publication year, country, study design, patients, treatments, groups, sample size, gender, age, body mass index (BMI), comorbidities (hypertension, diabetes mellitus and hyperlipidemia), smoking, CysC levels, and follow-up time.

The Newcastle–Ottawa Scale (NOS) was used to assess the quality of cohort studies and case-control studies[43] on a 9-point scale, with 0–3 points indicating poor quality, 4–6 points fair quality and 7–9 points good quality.[43]

Statistical analyses

Weighted mean difference (WMD) acted as an effect index for measurement data, with effect size expressed as 95% confidence intervals (95% CIs). The choice between using a fixed-effect or random-effect meta-analysis was performed a priori, with a random-effect model selected to analyze the association between CysC levels and functional recovery assessed using mRS, functional recovery assessed using NIHSS, cognitive dysfunction, and death. Heterogeneity was assessed for each outcome using Cochrane I2 statistics, with I^2 ranging 0–100%, and divided into <50% (low heterogeneity) and ≥50% (high heterogeneity). Subgroup analysis was performed based on follow-up time to explore the source of heterogeneity for outcomes (functional recovery assessed using mRS) with high heterogeneity. The robustness of the pooled results for each outcome was assessed using sensitivity analysis by excluding each study independently. Publication bias was assessed using Begg's test when the outcome included 10 or more studies.[44] Funnel plots were provided for each outcome (functional recovery assessed using mRS, functional recovery assessed using NIHSS, cognitive dysfunction, and death). All statistical analysis employed Stata v. 15.1 software (StataCorp, College Station, USA), with $p < 0.05$ regarded as statistically significant.

Results

Selection and characteristics of studies

There were 535 studies identified from English-language databases and 410 from Chinese databases, with 232 duplicates excluded and 653 excluded due to being reviews or meta-analyses (n = 38), conference abstracts (n = 47), case reports (n = 4), letters (n = 4), animal experiments (n = 34), articles not in English or Chinese (n = 2), or the topic not meeting the requirements (n = 524). After screening full texts, 36 studies were excluded for not having access to the full text (n = 2) and the topic not meeting the requirements (n = 34). Finally, 24 eligible studies were included (Fig. 1). Table 1 describes the characteristics of these studies, of which 23 were cohort studies[15,26,29,33,34,42,45–61] and 1 was a case-control study.[36] A total of 7,567 AIS patients were assessed.

Risk of bias assessment

The NOS evaluated the risk of bias by evaluating 3 methodological domains, including selection, comparability and outcomes (for cohort studies)/exposure (for case-control studies) assessment. In this meta-analysis, 1 study was assessed as having poor quality, 17 fair quality and 6 good quality (Supplementary Table 1).

Meta-analysis of the association between CysC levels and AIS patient outcomes

Functional recovery assessed by mRS showed no significant difference in CysC levels between the poor and

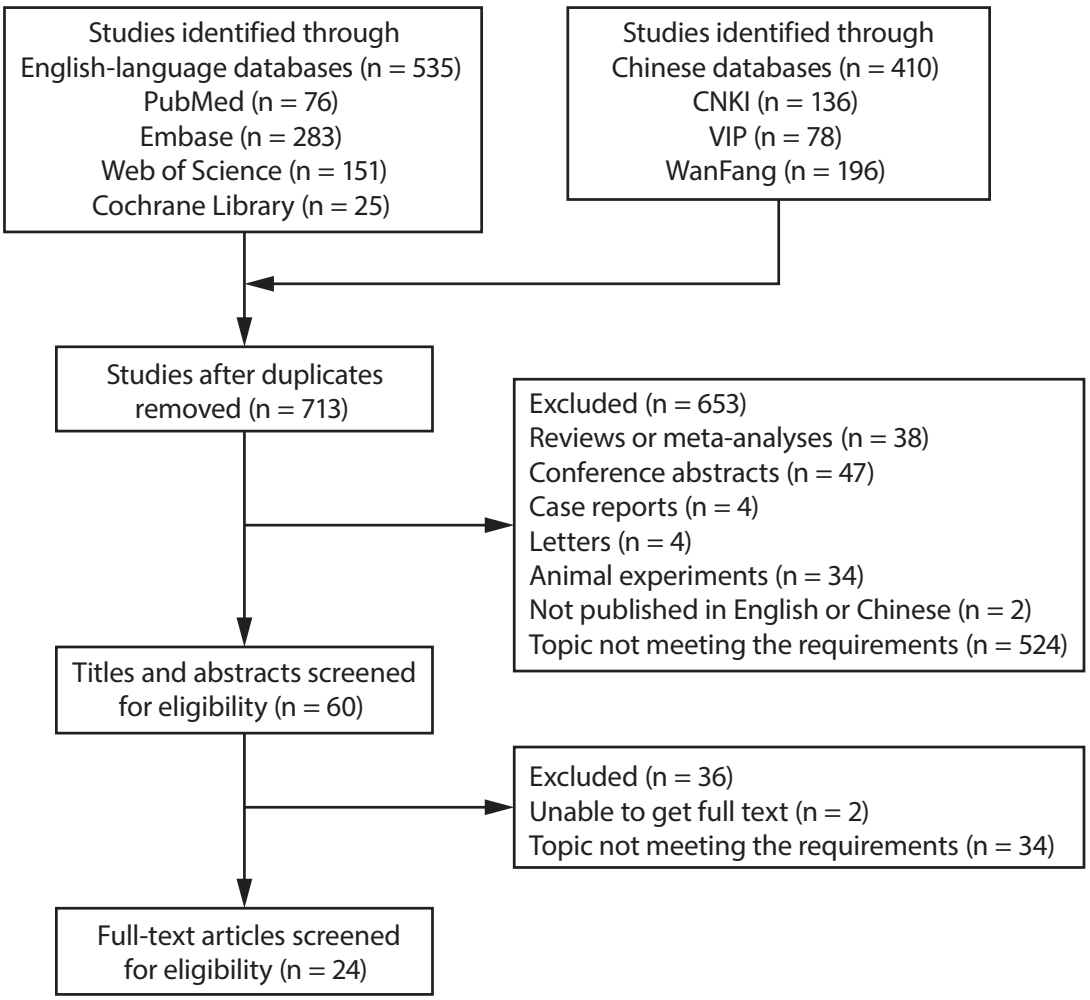


Fig. 1. Flowchart of study selection

good functional recovery groups (WMD = 0.36, 95% CI: −0.18–0.91, I^2 = 99.9 %) (Fig. 2A). Follow-up time was identified as the source of heterogeneity (follow-up time <1 month: WMD = 0.18, 95% CI: 0.08–0.28; follow-up time ≥ 1 month: WMD = 0.43, 95% CI: −0.29–1.14). For functional recovery assessed with the NIHSS, compared to the basic recovery group, the CysC levels were higher in the significant improvement group (WMD = 0.15, 95% CI: 0.06–0.24), improvement group (WMD = 0.27, 95% CI: 0.16–0.37) and ineffective/deterioration group (WMD = 0.37, 95% CI: 0.27–0.47) (Fig. 2B). Furthermore, CysC was higher in AIS patients with cognitive dysfunction (WMD = 0.16, 95% CI: 0.09–0.23) (Fig. 2C). In addition, high CysC levels were found in the death group (WMD = 0.32, 95% CI: 0.02–0.62) (Fig. 2D). The results of the meta-analysis are summarized in Table 2.

Systematic review of the association between CysC levels and AIS patient outcomes

Dong et al. divided the CysC level by quartile and found no statistical difference between the CysC and functional recovery in the 4 groups.[47] Regarding cognitive dysfunction, Guo et al. reported no association between CysC and cognitive dysfunction,[33] while Li et al. found that high CysC was a risk factor for cognitive dysfunction.[50] We also observed hemorrhagic transformation, vascular events, depression, and recurrence, with 1 study showing that AIS patients with hemorrhagic transformation had higher CysC levels than those without hemorrhagic transformation (1.28 ±0.27 mg/L compared to 1.13 ±0.27 mg/L, $p < 0.01$).[56] Xing et al. reported high CysC levels as an independent risk factor for depression in AIS patients.[15] For stroke recurrence, Ding et al. found that CysC was higher in the recurrence group than in the non-recurrence group.[46] Meanwhile, Liu et al. reported no association between CysC and recurrence but found a higher risk of vascular events in patients with increased CysC.[34]

Sensitivity analysis and publication bias

The influence of a single study on the effect estimate was assessed using sensitivity analysis by removing each study in turn. The results showed no significant influence of any individual study on the results of the meta-analysis. Begg's test was used to evaluate the publication bias for functional recovery assessed with the mRS, showing no

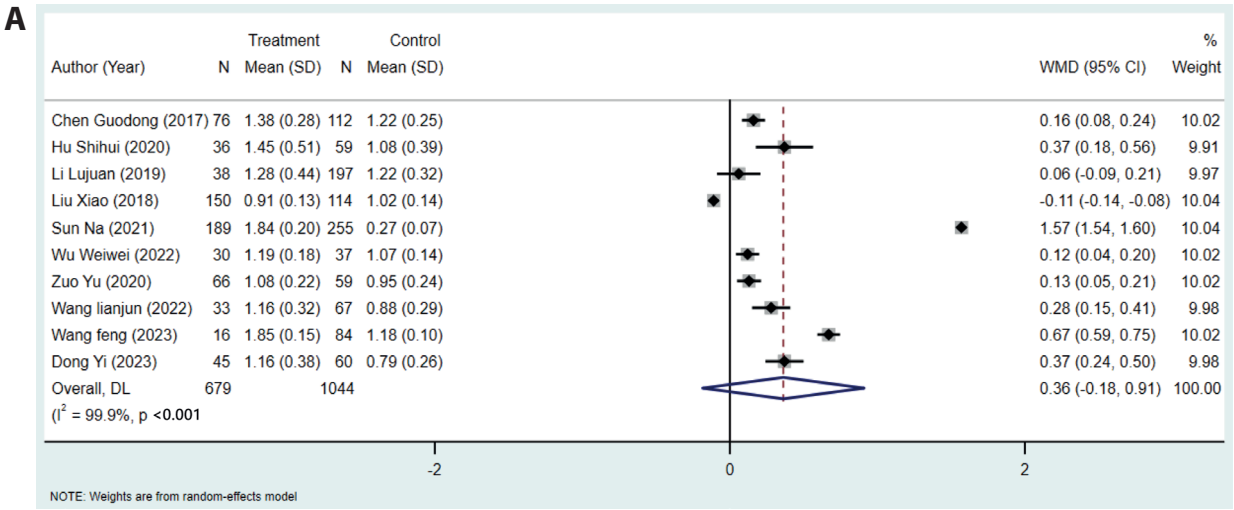
Table 1. Characteristics of included studies

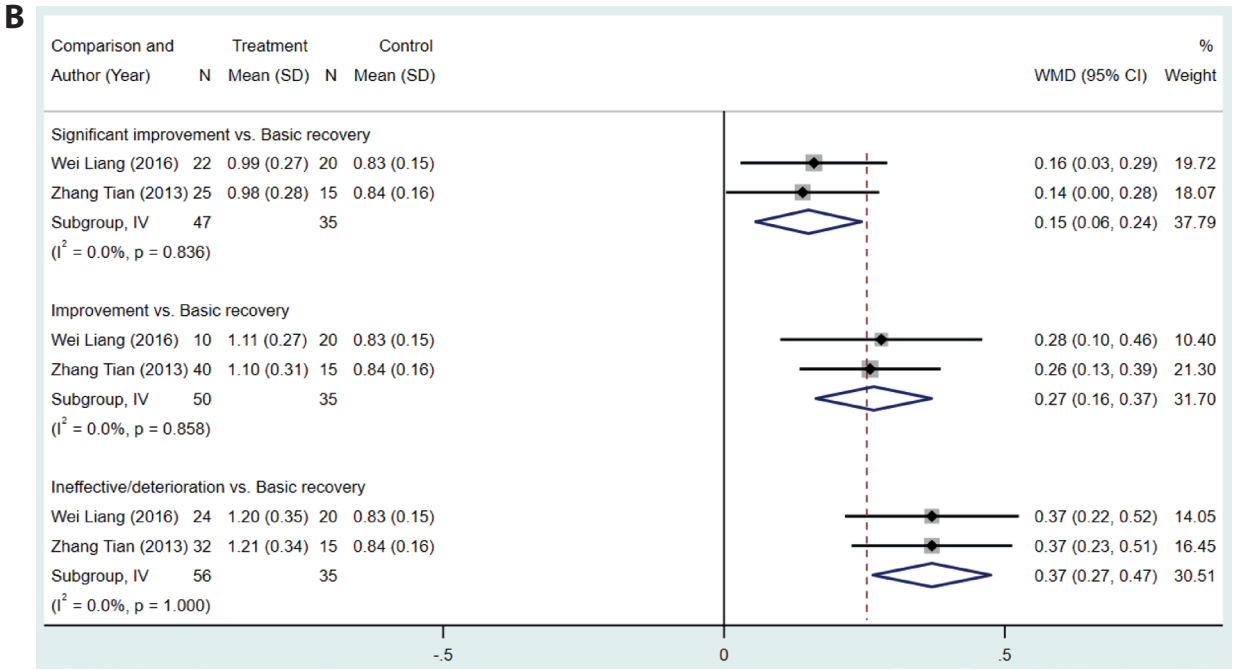
Author, year	Study design	Patients	Treatments	Groups	Sample size, n	Male, n	Age [years]	BMI [kg/m^2]	Hyper-tension, n	Diabetes mellitus, n	Hyper-lipidemia, n	Smoking, n	Cystatin C [mg/L]	Follow-up	Quality
Xing et al., 2020[15]	cohort	AIS	N/A	non-PSD	64	49	61.56 ±8.30	N/A	39	22	28	21	0.662 ±0.110	1 month	6
				mild PSD	19	4	65.89 ±10.36	N/A	6	4	6	3	0.717 ±0.075		
				moderate-severe PSD	18	10	64.67 ±11.20	N/A	4	7	7	8	0.928 ±0.084		
				non-CMBs	74	43	59.43 ±10.80	N/A	30	12	20	11	0.79 ±0.23		
Zuo et al., 2020[26]	cohort	AIS	endovascular therapy	mRS > 2	66	36	66.9 ±13.0	N/A	41	10	N/A	18	1.08 ±0.22	3 months	6
				mRS ≤ 2	59	40	59.8 ±16.4	N/A	38	9	N/A	24	0.95 ±0.24		
Yan et al., 2022[29]	cohort	AIS	without endovascular therapy or intravenous thrombolysis	with PSCI	164	117	62.31 ±13.78	N/A	102	41	N/A	59	1.2 ±0.39	3 months	7
				without PSCI	117	77	57.74 ±12.2	N/A	73	43	N/A	41	1 ±0.38		
Guo et al., 2019[33]	cohort	AIS	receiving immediate blood pressure reduction or use of hypoglycemic treatment	–	582	405	60.5 ±10.4	24.9 ±3.1	448	97	42	220	0.77 (0.65–0.91)	3 months	6
Liu et al., 2021[34]	cohort	AIS	receiving antihypertensive medications or lipid-lowering medications	high CysC	1528	1022	64.8 ±11.0	24.9 ±3.2*	1246	260	96	571	≥0.78	24 months	6
				low CysC	1946	1196	59.6 ±10.1	24.9 ±3.1**	1490	349	149	706	<0.78		
Zeng et al., 2019[36]	case-control	AIS	N/A	VCI	71	45	76.28 ±15.16	N/A	61	30	N/A	20	1.07 ±0.28	N/A	5
				CIND	81	61	71.40 ±11.32	N/A	61	38	N/A	29	0.97 ±0.27		
Zhang and Wang, 2013[42]	cohort	AIS	N/A	–	112	71	66.96 ±12.90	N/A	N/A	N/A	N/A	N/A	1.07 ±0.32	N/A	3
Chen et al., 2017[45]	cohort	AIS	antihypertensive drugs, antiplatelet drugs, anticoagulants, and statins	mild stroke	120	37	64.3 ±9.15	N/A	95	46	77	41	1.21 ±0.23	2 weeks	6
				moderate and severe stroke	68	58	65.8 ±9.43	N/A	60	38	41	21	1.36 ±0.29		
Ding et al., 2020[46]	cohort	AIS	anti-platelet aggregation, improve cerebral blood supply, nutritional brain cells and other symptomatic treatment	recurrence	38	21	61.25 ±8.12	27.12 ±3.08	25	19	35	34	3.95 ±1.07	12 months	8
				no recurrence	153	82	59.98 ±8.04	26.58 ±2.94	84	88	162	167	2.78 ±0.86		
Dong and Nao, 2018[47]	cohort	AIS	without intravenous thrombolysis	–	109	69	63.25 ±12.99	N/A	80	34	N/A	48	1.33 ±0.52	3 months	7
Hu et al., 2020[48]	cohort	AIS	N/A	–	95	44	75.0	N/A	N/A	N/A	N/A	N/A	1.25 ±0.45	10 days	5
Li et al., 2019[49]	cohort	AIS	without intravenous thrombolysis or arterial thrombectomy	–	235	126	639 ±14.8	N/A	170	98	175	135	1.24 ±0.42	3 months	6

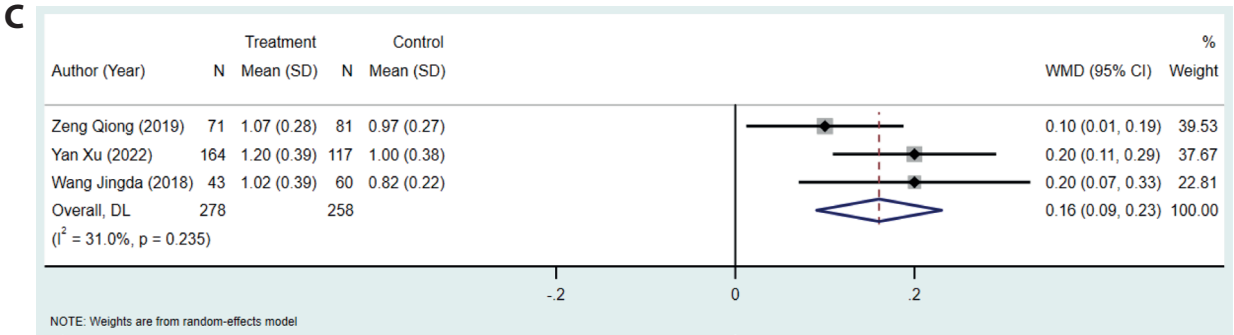
Table 1. Characteristics of included studies – cont.

Author, year	Study design	Patients	Treatments	Groups	Sample size, n	Male, n	Age [years]	BMI [kg/m^2]	Hyper-tension, n	Diabetes mellitus, n	Hyper-lipidemia, n	Smoking, n	Cystatin C [mg/L]	Follow-up	Quality
Li and Yuan, 2019[50]	cohort	AIS	N/A	–	297	168	68.24 ±6.33	N/A	64	96	N/A	N/A	1.49 ±0.31	10 days	5
Liu et al., 2018[51]	cohort	AIS	N/A	mRS >2	150	96	60.5 (54.0, 71.0)	N/A	37	50	84	78	0.9 (0.82, 1.00)	3 months	8
				mRS ≤2	114	62	74.0 (60.0, 80.3)	N/A	30	36	56	49	1.03 (0.92, 1.10)		
Luan et al., 2019[52]	cohort	AIS	N/A	–	40	23	63.52 ±2.04	N/A	N/A	N/A	N/A	N/A	1.24 ±0.27	3 months	5
Lv, 2019[53]	cohort	AIS	intravenous thrombolysis	–	60	35	65.78 ±3.33	N/A	N/A	N/A	N/A	N/A	1.46 ±0.31	2 weeks	4
Sun et al., 2021[54]	cohort	AIS	intravenous thrombolysis	mRS >2	189	99	70.50 ±14.76	25.05 ±2.12	90	78	N/A	55	1.84 ±0.20	1 month	7
				mRS ≤2	255	156	68.95 ±14.90	24.80 ±2.05	135	54	N/A	57	0.27 ±0.07		
Wang and Tan, 2018[55]	cohort	AIS	N/A	with PSCI	43	25	64.37 ±6.24	N/A	32	21	N/A	17	1.02 ±0.39	2 weeks	5
				without PSCI	60	37	61.82 ±5.95	N/A	46	17	N/A	25	0.82 ±0.22		
Wang, 2017[56]	cohort	AIS	without anticoagulants or thrombolysis and anticoagulation	hemorrhagic transformation	65	31	62.09 ±10.49	N/A	30	25	N/A	27	1.28 ±0.27	2 weeks	5
				non-hemorrhagic transformation	127	63	58.54 ±10.01	N/A	55	41	N/A	57	1.13 ±0.27		
Wei and Wang, 2016[57]	cohort	AIS	N/A	–	76	44	64.1 ±11.9	N/A	N/A	N/A	N/A	N/A	1.06 ±0.31	N/A	4
Wu et al., 2022[58]	cohort	AIS	intravenous thrombolysis	–	67	45	57.93 ±12.96	N/A	N/A	12	N/A	N/A	1.12 ±0.17	3 weeks	5
Wang and Li, 2022[59]	cohort	acute cerebral infarction	rt-PA intravenous thrombolysis	mRS > 2	33	20	63.03 ±6.90	23.12 ±2.41	18	N/A	N/A	9	1.16 ±0.32	3 months	6
				mRS ≤ 2	67	37	61.30 ±6.01	23.43 ±2.10	47	N/A	N/A	26	0.88 ±0.29		
Wang et al., 2023[60]	cohort	acute cerebral infarction	N/A	–	100	73	64.18 ±3.51	N/A	52	48	N/A	N/A	1.82 ±0.15	3 months	5
Dong et al., 2023[61]	cohort	acute cerebral infarction	rt-PA intravenous thrombolysis	mRS > 2	45	26	64.24 ±5.18	22.46 ±2.02	18	15	7	13	1.16 ±0.38	1 month	7
				mRS ≤ 2	60	36	61.55 ±4.78	21.97 ±1.95	21	18	9	10	0.79 ±0.26		

Continuous variables were expressed as mean ± standard deviation (mean ± SD), or as median (interquartile range (IQR)); AIS – acute ischemic stroke; BMI – body mass index; END – early neurological deterioration; VCI – vascular cognitive impairment; CIND – cognitive impairment no dementia; CysC – cystatin C; PSCI – post-stroke cognitive impairment; mRS – modified Rankin Scale; CMBs – cerebral microbleeds; rt-PA – recombinant tissue plasminogen activator; PSD – post stroke depression; N/A – not available.







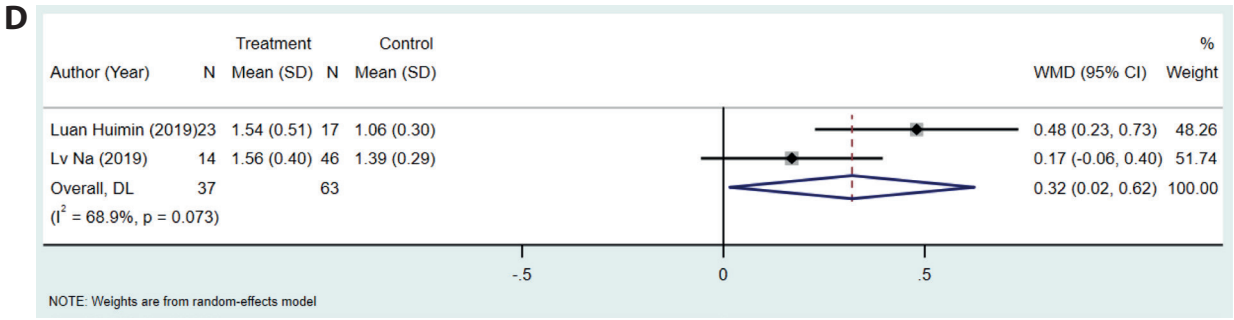


Fig. 2. Forest plots for the association between cystatin C (CysC) level and functional recovery assessed using the modified Rankin Scale (mRS) (A), function recovery assessed with the National Institute of Health Stroke Scale (NIHSS) (B), cognitive dysfunction (C), and death (D)

Table 2. Association between CysC level and outcomes of AIS patients

Outcomes		Number of studies	WMD (95% CI)	p-value	I^2
Function recovery assessed with mRS	sensitivity analysis	10	0.36 (–0.18, 0.91)	0.194	99.9
	publication bias		Z = 0.18	0.858	–
	follow-up, <1 month	3	0.18 (0.08, 0.28)	<0.001	63.5
	follow-up, ≥1 month	7	0.43 (–0.29, 1.14)	0.241	99.9
Function recovery assessed with NIHSS	significant improvement, sensitivity analysis	2	0.15 (0.06, 0.24)	0.002	0.0
	improvement, sensitivity analysis	2	0.27 (0.16, 0.37)	<0.001	0.0
	ineffective/deterioration, sensitivity analysis	2	0.37 (0.27, 0.47)	<0.001	0.0
Cognitive dysfunction, sensitivity analysis		3	0.16 (0.09, 0.23)	<0.001	31.0
Death, sensitivity analysis		2	0.32 (0.02, 0.62)	0.039	68.9

95% CI – 95% confidence interval; WMD – weighted mean difference; mRS – modified Rankin Scale; NIHSS – National Institute of Health Stroke Scale.

publication bias (Z = 0.18, p = 0.858) (Table 2). Also, funnel plots indicated no evidence of publication bias for functional recovery assessed using the mRS (Supplementary Fig. 1A), functional recovery assessed with the NIHSS (Supplementary Fig. 1B), cognitive dysfunction (Supplementary Fig. 1C), and death (Supplementary Fig. 1D).

Discussion

Acute ischemic stroke is a cause of long-term disability and has a high mortality rate, which leads to a significant annual economic burden globally.[3] Previous studies reported controversial results on the association between CysC and AIS patient outcomes. Therefore, this systematic review and meta-analysis comprehensively explored the association between CysC and AIS outcomes based on currently available studies. Results showed that CysC level was higher in AIS patients with cognitive dysfunction, significant improvement of function recovery, improvement of function recovery and ineffective/deterioration of function recovery, and in patients who died. Although there was no significant difference between CysC levels and functional recovery assessed using mRS in the total population, subgroup analysis showed a significant difference during follow-up time <1 month. For the systematic review, we found that high CysC levels were associated with hemorrhagic transformation,[56] vascular events,[34] depression,[15] and recurrence.[46] The overall results suggest that high CysC levels may be one of the risk factors for poor outcomes in AIS patients.

Cystatin C has been reported to influence endogenous neuroprotection and may be a candidate drug for treating stroke through lysosomal membrane integrity maintenance.[62] However, some studies reported CysC as a risk factor for stroke.[25,27] Compared to patients without stroke, CysC was higher in AIS patients, indicating that CysC may be an AIS risk prediction factor.[25] Cognitive dysfunction is a common symptom after stroke, and the incidence reaches up to 56.6% in China within 3 months after stroke,[63,64] though current studies on the association between CysC and cognitive dysfunction remain inconsistent.[29,33,36,65] Nonetheless, our meta-analysis found higher CysC in AIS patients with cognitive dysfunction. Some pathways may explain the mechanisms by which CysC affects cognitive function after AIS. The stroke event may elevate serum CysC level, and a high CysC level is associated with dementia and cognitive dysfunction.[29,36] Furthermore, there is an association between CysC and symptomatic common carotid artery stenosis, which is related to cognitive impairment in patients with or without stroke.[21,66–68] Moreover, CysC is involved in the pathogenesis of cerebral amyloidosis, which may result in early cerebral hemorrhage.[69] Previous research indicated higher serum CysC in AIS patients complicated with cerebral microbleeds than in those without, highlighting CysC as a risk factor for cerebral microbleeds.[70] Cerebral microbleeds are closely associated with cognitive dysfunction, resulting in damage to memory, abstract thinking and visual, spatial and executive functions.[71]

Functional recovery is of substantial concern to patients. The 7-level mRS covers a range of functional outcomes, from no symptoms to death, and the categories are intuitive and easy for clinicians and patients to understand.[41] Results of our meta-analysis showed that, although there was no association between CysC levels and functional recovery assessed using mRS in the total AIS population, a significant association was found with a follow-up time <1 month. There are no studies on serum CysC levels at different time points, and the mechanism behind this finding remains unclear. Further studies are needed to explore the relationship between serum CysC and functional recovery at different time points to help understand the mechanisms underlying these associations.

Cystatin C was higher in significant improvement, improvement and ineffective/deterioration groups than the basic cure group, and there are several explanations for this. Cystatin C participated in the inflammatory reaction, destroyed nerve cells and aggravated nervous system damage, which affected the recovery of patients with cerebral infarction.[72] In addition, CysC was involved in blood vessel

wall remodeling and aggravated atherosclerosis, causing cerebral infarction.[73] These mechanisms are supported by our finding that high CysC levels were associated with vascular events.[34]

Stroke is the 2nd leading cause of death and the primary reason for long-term disability worldwide.[3] Our results showed that serum CysC was higher in the death group than in the survival group. Luan et al. and Li et al. reported that serum CysC was higher in the death group than in the AIS patient survival group.[52,53] Serum CysC level may increase in AIS patients with disease aggravation,[53] as it may cause neuronal cell apoptosis and neuron loss, leading to delayed neuronal damage and resulting in death.[53]

We included available studies on the relationship between CysC levels and AIS patient outcomes, and found consistent and meaningful results through the meta-analysis. Publication bias was not found, and sensitivity analyses showed stable overall effect sizes. To minimize factors that may affect the results and explore potential sources of heterogeneity, we applied subgroup analyses and found that follow-up time may be a source of heterogeneity. Furthermore, high CysC levels in AIS patients with cognitive dysfunction, unfavorable functional recovery and death suggest that it may be related to AIS patient outcomes. Our meta-analysis highlighted the importance of early monitoring and management of CysC and provided evidence for improving AIS patient outcomes. Future studies should explore whether decreasing serum CysC is a therapeutic target for preventing poor outcomes in AIS patients.

Limitations

Limitations of this meta-analysis include the small number of studies on some outcomes, which may affect the stability of the results. All included studies were conducted in China, meaning the results cannot be generalized to global populations. As such, the findings of this meta-analysis should be verified by studies in other countries. Cystatin C is a highly accurate measure of kidney decline, and since data on kidney function were not recorded in the included studies, we were unable to perform analysis based on renal function. In addition, patient age, AIS course and severity, and comorbidities may affect the prognosis, but current data are insufficient to support further analysis. As such, further meta-analyses with more studies are needed to explore the impact of these factors on the association between serum CysC and AIS patient outcomes.

Conclusions

The current systematic review and meta-analysis found that higher levels of serum CysC are associated with poor outcomes of patient with AIS. The results demonstrate that higher serum CysC may be a risk factor for poor outcomes in patients with AIS, which provides a new direction for preventing such outcomes. However, more prospective studies are needed to confirm these findings and reduce study limitations.

Since AIS patient outcomes are affected by many factors, it is not clear whether CysC is an independent predictor. Additional research is needed to explore whether single or combined measurements of this biomarker, with or without other demographic, clinical and biochemical variables, can further increase early risk stratification and clinical decisions in this population. Close attention should also be paid to studies of CysC as a therapeutic target for preventing poor outcomes in patients with AIS. Further clinical studies are important for examining whether decreasing serum CysC can prevent or treat poor AIS patient outcomes.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10674816. The package includes the following files:

Supplementary File 1. Search terms.

Supplementary Table 1 Risk of bias summary.

Supplementary Fig. 1. A. Funnel plot for publication bias regarding function recovery assessed with mRS; B. Funnel plot for publication bias regarding to function recovery assessed using NIHSS; C. Funnel plot for publication bias regarding cognitive dysfunction; D Funnel plot for publication bias regarding death.

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An artificial intelligence model for Lhermitte's sign in patients with pediatric-onset multiple sclerosis: A follow-up study

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Abstract

Background. Lhermitte's sign (LS) is an important clinical marker for patients with multiple sclerosis (MS). Research on pediatric-onset MS (POMS) and LS is limited. To date, there has been no research conducted on the clinical and artificial intelligence (AI)-based radiological correlation of LS.

Objectives. This follow-up study aims to investigate the relationship between LS and clinical findings according to AI-based radiological characteristics of patients with POMS.

Materials and methods. Basic descriptive statistics of patients with POMS according to sociodemographic, clinical and radiological findings were collected. Variables were evaluated at a 95% confidence level (95% CI), and a value of $p < 0.05$ was accepted as statistically significant. The LS in patients with MS was classified according to its presence in the past and at the time of the study screening: group A: absent; group B: positive in the past but absent at screening; group C: present both in the past and at the screening; group D: absent in the past but present at the screening. In addition, patients were grouped according to the duration of their MS, with the following classifications: <10 years and at least 10 years.

Results. A total of 1,298 records were identified in the database search. Ninety-two patients who met the inclusion criteria were included in the study. The frequency of upper cervical lesions (C1–4 vertebral segmental levels) was higher in group B and C than in group A ($p = 0.017$). Among patients with an MS duration of 10-years, C1–4 lesions were least frequent in group A.

Conclusions. Spinal imaging with AI-based programs can be used at least as much as brain magnetic resonance imaging (MRI) for early diagnosis, prognosis and treatment response. We have for the first time investigated LS in a large sample of patients with POMS. It is, however, recommended to conduct further multicenter studies to more specifically identify LS in patients with POMS.

Key words: artificial intelligence, multiple sclerosis, Lhermitte's sign, pediatric onset multiple sclerosis, spinal lesions

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Background

Pediatric-onset multiple sclerosis (POMS), formerly referred to as juvenile multiple sclerosis (MS), has been increasingly diagnosed.[1] Despite heightened awareness, POMS remains a rare condition. The estimated annual incidence rates range from 1 to 5.4 cases per 100,000 people. This rate was determined to be 5.4 per 100,000 in a German cohort that included children under the age of 18.[2] It has been reported that at least 5% of patients with MS experience the clinical symptoms onset of disease prior to the age of 18 years.[3,4] The vast majority of children presenting with a 1st clinical demyelinating attack have a monophasic course, and fewer than 20% of children presenting with a 1st demyelinating attack are diagnosed with POMS, which has worse long-term physical and cognitive disabilities.[5] In the 2010 and 2017 McDonald criteria, the requirements for confirming the diagnosis of MS in the 1st demyelinating attack with pediatric onset were determined. Accordingly, children presenting with acute disseminated encephalomyelitis (ADEM), defined by polyfocal deficits and encephalopathy,[6] must have experienced a non-ADEM attack plus evidence for dissemination in time and space prior to MS diagnosis.[7,8] High relapse rates and rapid accumulation of lesion burden are typical features that distinguish patients from adult-onset multiple sclerosis (AOMS).[9] The positivity of oligoclonal bands (OCBs) in cerebrospinal fluid (CSF) increased diagnostic performance.[10] Although spinal cord lesions are common, their diagnostic performance is poor due to the high frequency of juxtacortical and periventricular lesions.[11] Therefore, detailed brain magnetic resonance imaging (MRI) seems to be sufficient in patients without spinal cord complaints. Developing diagnostic criteria for patients with spinal cord complaints has become important, especially in those whose brain imaging is insufficient for diagnosis.

High levels of proinflammatory cytokines have been reported in the CSF and serum of POMS patients. Cytokine density has diagnostic value for both 1st attack and relapsed patients.[12] Biomarkers found in the serum or CSF of POMS patients that indicate diagnosis, disease activity/active neuroinflammation, or response to treatment include matrix metalloproteinase-9 (MMP-9),[13] some miRNAs (such as *miR-125a-5p* and *miR185-5p*)[14] and serum neurofilament light chain (sNfL).[15] In particular, the increase in serum neurofilament light chain levels is an important indicator of disease activity and response to treatment, in addition to its early diagnostic value. Additionally, glial fibrillary acidic protein (GFAP) levels were found to be high in the serum of patients with neuromyelitis optica spectrum disorder (NMOSD). A high sGFAP/sNfL ratio may help distinguish NMOSD from MS.[16]

The clinical features of POMS are comparable to those of AOMS, with recurrent episodes of optic neuritis, diplopia or transverse myelitis. However, isolated brainstem syndrome, optic neuritis or encephalopathy symptoms are more likely to occur in children.[17]

The Lhermitte's sign (LS) was first described in 1917 by Pierre Marie and Chatelin in a soldier who had suffered a head injury during World War I.[18] The following year, it was described in spinal concussions by Babinski and Dubois,[19] and then the observations of J. Ribeton in 12 cases with head or neck trauma captured the first attention for this phenomenon. Jean J. Lhermitte reported this symptom in 1920. In 1924, Lhermitte, Bollak and Nicholas published the seminal paper on the subject entitled "Les douleurs à type de décharge électrique consécutives à la flexion céphalique dans la sclérose en plaques: Un cas de forme sensitive de la sclérose multiple."[20] Thus, in addition to the observations of J. Ribeton, they provided the first description in the medical literature of an electrical discharge following neck flexion in a patient with MS.

It is believed that the LS is caused by ectopic firing and hyperexcitability of demyelinated sensory neurons located in the cervical spinal cord. It is also thought that increased spinothalamic nociceptive signal transmission and impaired function of inhibitory GABAergic interneurons affect this process.[21] Molecular mechanisms include downstream activated microglia that enhance proinflammatory cytokine signaling, activation of proteins such as bradykinin by B1 and B2 receptors, upregulation of Wnt signaling, cAMP-response element binding protein (CREB) phosphorylation, and other transcription factors that increase hyperexcitability and pain in the central nervous system (CNS).[22]

The LS is accepted both as a symptom and a sign of physical examination, and results from irritation of the spinal cord, especially the posterior and lateral columns.[23] The LS is not specific to MS. It may occur in autoimmune diseases such as Behçet's disease[24] and systemic lupus erythamosus,[25] as well as in various conditions in which the cervical spinal cord is affected, e.g., atlantoaxial subluxation, cervical myelitis, cisplatin toxicity, ionizing radiation affecting the cervical cord, pernicious anemia affecting the dorsal columns, prolapsed cervical disc, spinal cord tumor, syringomyelia, cervical spine trauma, tuberculous arachnoiditis, alcoholic myelopathy, intramedullary spinal cord hemorrhage (hematomyelia), and paroxetine withdrawal.[26–29] Hence, the overall prevalence of LS seems difficult to estimate or investigate. The LS may be observed in pediatric- or adult-onset MS.[26]

Studies on early diagnosis and prognosis of MS using multi-dimensional data with computer-aided diagnosis and deep learning (DL)/machine learning (ML) methods have focused mostly on brain imaging. Artificial intelligence (AI)-based automatic analyses of lesion classification provide a time advantage over manual measurements.[30,31] Machine learning refers to the concept of computers acquiring knowledge without being programmed directly, while DL involves software training via algorithmic exposure to extensive data sets through multi-layered neural

networks. In short, DL is a subset of ML. Recent studies have demonstrated the efficacy of AI techniques, including DL and ML, in the segmentation of white matter lesions and the evaluation of novel imaging markers, such as paramagnetic rim lesions and central vein sign.[32,33]

For AI-based spinal lesion definition in the radiological differentiation of conditions such as myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), NMOSD, ADEM and clinically isolated syndrome (CIS) with POMS, the characteristics of these lesions must be correctly recognized. Research into the clinical course of LS, or the association of LS with clinical or radiological features in MS, has been limited.

It is important to note that LS is indicative of neuronal damage, particularly in the posterior and lateral columns of the cervical spine. Therefore, it could be a useful early indicator of neuroinflammation in patients with POMS.

Objectives

This retrospective study aimed to investigate the relationship between LS and clinical findings according to AI-based radiological characteristics of POMS patients.

Materials and methods

Study design

Patients diagnosed with MS and admitted to the neurology clinics of Izmir University of Economics Faculty Hospital (Turkey) between 2012 and 2023 were included in this retrospective study.

Participants

Patients who were diagnosed with MS in childhood (age at diagnosis <16) were included in the study. The diagnosis and typing of MS was based on the criteria defined in the previous guidelines.[6,34] Participants for whom data were missing, those whose signs and symptoms were attributed to a diagnosis other than MS, or for whom a diagnosis of MS was confirmed after 16 years of age were excluded from the study. Patients with possible factors associated with LS other than MS, such as cervical disc herniation, vitamin B12 deficiency, systemic lupus erythematosus, spinal cord compression, spondylitis, or radiculopathy were excluded.

Test methods and radiological work-up

Demographic (age at the diagnosis of MS, age at screening and sex), and clinical parameters (type of MS, past and present history of the presence of LS, confirmation of LS by self-detection (symptom) or on physical examination (sign), the presence of vibration, position and tactile sensation, or Romberg's test on physical examination) and radiological findings were recorded using electronic and written patient files.

Types of MS were defined as CIS, RR (remitting–relapsing), SP (secondary progressive), and PP (primary progressive). We grouped the patients according to the presence of LS as follows: group A: absent; group B: positive in the past but absent at screening; group C: present both in the past and at the screening; group D: absent in the past but present at the screening. We also grouped the patients according to MS duration: <10 years vs >10 years.

Cervical and cranial MRI of all patients were performed upon the diagnosis of MS and at the screening. Gadolinium-based contrast agents were used during the imaging. The data presented in the result section represent ecomprehensive analysis of the aggregated data obtained from both sets of MRI scans. On the cervical MRI, localization of spinal cord lesions both at the level of the vertebra and localization in the spinal cord, identification of spinal cord lesions on T1 or T2-weighted images, gadolinium enhancement, and atrophy or expansion of the spinal cord lesions were assessed. The presence of intracranial lesions was evaluated on cranial MRI.

Statistical analyses

The data obtained in the study were statistically analyzed using SPSS v. 25.0 software (IBM Corp., Armonk, USA). The conformity of the univariable data to normal distribution was evaluated using the Shapiro–Wilk–Francia test. Homogeneity of variance was evaluated with the Levene's test. When comparing more than 2 independent groups according to qualitative variables, the Kruskal–Wallis test with Monte Carlo simulation was used. The Monte Carlo simulation is regarded as a crucial tool in modeling uncertainty and supporting decision-making processes, as it allows for the evaluation of all possible outcomes and the estimation of their probabilities. The Monte Carlo simulation method was incorporated into IBM SPSS v. 25.0 through the installation of supplementary tools. Once added, it appears as an option in the relevant menus. When comparing 2 independent groups according to qualitative variables, the Mann–Whitney U test with Monte Carlo simulation was used with the Benjamin–Hochberg correction for the post hoc analysis. When comparing categorical variables to each other, the Fisher exact test and the Fisher–Freeman–Halton test were used. Nonparametric methods were used for the analysis of variables with a sample size smaller than 10. Quantitative variables were stated as n/median (min–max) (Q1–Q3) values and categorical variables as number (n) and percentage (%) in the tables. Variables were evaluated at a 95% confidence level (95% CI), and a p-value of <0.05 was accepted as statistically significant.

Ethics

This study was conducted in accordance with the World Medical Association Declaration of Helsinki. The study was approved by the İzmir Bakırçay University Non-Interventional Transactions Ethics Committee (approval No. 926/906 issued on March 15, 2023). Written informed consent was obtained from all of the participants.

Results

A total of 92 patients were included in this study. The flow chart of the study is shown in Fig. 1. The female-to-male ratio was 2.17 (63/29). The mean age at diagnosis was 14.67 (±2.27) years, and the mean age at screening was 25.99 (±8.08) years. The LS was absent in 63 patients (68.5%) (group A), while 15 patients (16.3%) had a positive history of LS without current manifestation (group B). The LS was present in both the past and at the screening in 9 patients (9.8%) (group C), while 5 patients (5.4%) developed LS during the screening despite no prior history (group D).

The mean duration of MS was 123.65 (±107.69) months, with 33.7% of patients having an MS duration longer than 10 years. The majority (87%) of patients had relapsing–remitting (RR) MS. Detailed demographic, clinical and radiological features are presented in Table 1.

Table 2 provides a comprehensive breakdown of spinal cord lesions categorized according to the levels of cervical or thoracic vertebrae. The data are presented for the total cases and are further stratified based on the duration of MS (<10 years and >10 years). Percentages were

Table 1. Demographic, clinical and radiological features of the patients

Characteristics		n (%)
Lhermitte's sign	group A	63 (68.5)
	group B	15 (16.3)
	group C	9 (9.8)
	group D	5 (5.4)
MS duration	≤10-year	61 (66.3)
	>10-year	31 (33.7)
Sex	female	63 (68.5)
	male	29 (31.5)
Lhermitte's sign in the past	absent	68 (73.9)
	present	24 (26.1)
Lhermitte's sign at the screening (symptom and physical sign)	absent	78 (84.8)
	present	14 (15.2)
Lhermitte's symptom	absent	81 (88)
	present	11 (12)
Lhermitte's sign	absent	82 (89.1)
	present	10 (10.9)
MS type	CIS	3 (3.3)
	RR	80 (87)
	SP	7 (7.6)
	PP	2 (2.2)
C1–4 spinal lesion	absent	40 (43.5)
	present	52 (56.5)
C5–8 spinal lesion	absent	66 (71.7)
	present	26 (28.3)
Thoracal spinal lesion	absent	69 (75)
	present	23 (25)
Localization of cervical spinal lesion	lateral	6 (6.5)
	posterior	17 (18.5)
	posterolateral	36 (39.1)
	absent	33 (35.9)
Cervical lesion on T2-weighted MRI	absent	33 (35.9)
	present	59 (64.1)
Cervical lesion on T1-weighted MRI	absent	83 (90.2)
	present	9 (9.8)
Gadolinium enhancement on cervical MRI	absent	72 (78.3)
	present	20 (21.7)
Atrophy on cervical MRI	absent	86 (93.5)
	present	6 (6.5)
Expansion on cervical MRI	absent	76 (82.6)
	present	16 (17.4)
Intracranial lesion	absent	2 (2.2)
	present	90 (97.8)
Right superficial sensation	absent	86 (93.5)
	present	6 (6.5)
Left superficial sensation	absent	86 (93.5)
	present	6 (6.5)
Vibration sensation abnormality on upper extremity	absent	71 (77.2)
	present	21 (22.8)
Vibration sensation abnormality on lower extremity	absent	39 (42.4)
	present	53 (57.6)
Position sensation abnormality on upper extremity	absent	91 (98.9)
	present	1 (1.1)
Position sensation abnormality on lower extremity	absent	91 (98.9)
	present	1 (1.1)
Romberg test	negative	65 (70.7)
	positive	27 (29.3)
Age at diagnosis [years], mean (SD)	14.67 (2.27)	
Age at screening [years], mean (SD)	25.99 (8.08)	
MS follow-up duration [months], mean (SD)	123.65 (107.69)	

MRI – magnetic resonance imaging; MS – multiple sclerosis; CIS – clinically isolated syndrome; RR – relapsing–remitting; SP – secondary progressive; PP – primary progressive; SD – standard deviation; min – minimum; Q1 – percentile 25; Q3 – percentile 75; max – maximum.

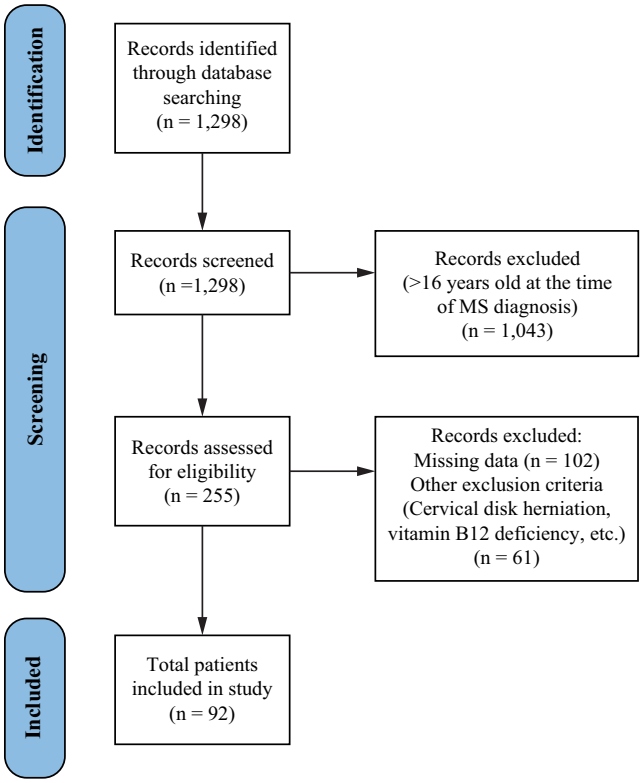

Fig. 1. The flowchart of the study

MS – multiple sclerosis.

Table 2. Localization of spinal cord lesions

Localization of spinal lesion	Total n = 249	MS duration (<10 years) n = 173	MS duration (>10 years) n = 76
	n (%)	n (%)	n (%)
C1	8 (3.2)	5 (2.9)	3 (3.9)
C2	27 (10.8)	19 (11)	8 (10.5)
C3	45 (18.1)	33 (19.1)	12 (15.8)
C4	38 (15.3)	26 (15)	12 (15.8)
C5	18 (7.2)	13 (7.5)	5 (6.6)
C6	18 (7.2)	12 (6.9)	6 (7.9)
C7	10 (4)	5 (2.9)	5 (6.6)
C8	1 (0.4)	1 (0.6)	–
T1	10 (4)	7 (4)	3 (3.9)
T11	2 (0.8)	2 (1.2)	–
T12	2 (0.8)	2 (1.2)	–
T2	7 (2.8)	4 (2.3)	3 (3.9)
T3	6 (2.4)	4 (2.3)	2 (2.6)
T4	3 (1.2)	2 (1.2)	1 (1.3)
T5	5 (2)	5 (2.9)	–
T6	8 (3.2)	7 (4)	1 (1.3)
T7	4 (1.6)	3 (1.7)	1 (1.3)
T8	2 (0.8)	2 (1.2)	–
T9	2 (0.8)	2 (1.2)	–
Absent	33 (13.3)	19 (11)	14 (18.4)

MS – multiple sclerosis.

calculated in relation to the total number of cases in each respective category.

Table 3 presents a thorough comparison of demographic, clinical and radiological characteristics among the different patient groups (group A, B, C, and D). The total number of patients included in the analysis was 92. Group A comprised 63 (68.5%) patients; group B 15 (16.3%); group C 9 (9.8%); and group D 5 (5.4%). There was a statistically significant difference in the distribution of C1–4 lesions among groups A, B, C, and D ($p = 0.017$). The frequency of C1–4 lesions in group B and C was higher compared to group A ($p < 0.05$). The frequency of the presence of cervical cord lesions on T2-weighted MRI was higher in group B when compared to that in group A ($p = 0.038$). The frequency of the presence of cervical cord lesions on T1-weighted MRI was higher in group C when compared to that in group A ($p = 0.021$) (Fig. 2).

In this segment, we present a thorough comparison of demographic, clinical and radiological features among distinct patient groups (group A, B, C, and D) within the subgroup of MS patients with a disease duration shorter than 10 years. A total of 61 patients are included in this analysis. Among those with an MS duration of less than 10 years, our investigation reveals striking similarities in demographic, clinical and radiological parameters across the various groups, as detailed in Table 4.

Table 5 presents a detailed comparison of demographic, clinical and radiological features among subgroups of MS patients with a disease duration exceeding 10 years, labeled as group A, B and C. The analysis encompasses a total of 31 patients. The localization of cervical spinal lesions was higher in group B than in group A ($p = 0.010$) (Fig. 3). The C1–4 lesions were more frequent in group B than in group A ($p = 0.030$). The frequency of the presence of cervical cord lesions on T2-weighted MRI, and atrophy and expansion on cervical MRI were higher in group B when compared to those in group A ($p = 0.042$, $p = 0.042$ and $p = 0.042$, respectively). Vibration abnormality in the upper extremity was found to be significantly more pronounced in group B compared to group A ($p = 0.003$). A significant disparity was noted in the Romberg test, with 100% of group B exhibiting abnormalities compared to 24% in group A ($p = 0.009$) (Fig. 4). The findings presented in the tables are derived from the results of current MRIs. The MRIs taken during the study for screening purposes were conducted for patient monitoring and were used for patient and lesion follow-up.

Discussion

We have studied a considerable number of patients with POMS. About 1/3 of the patients had LS during the course of MS. We showed that the presence of LS was associated with cervical spinal lesions.

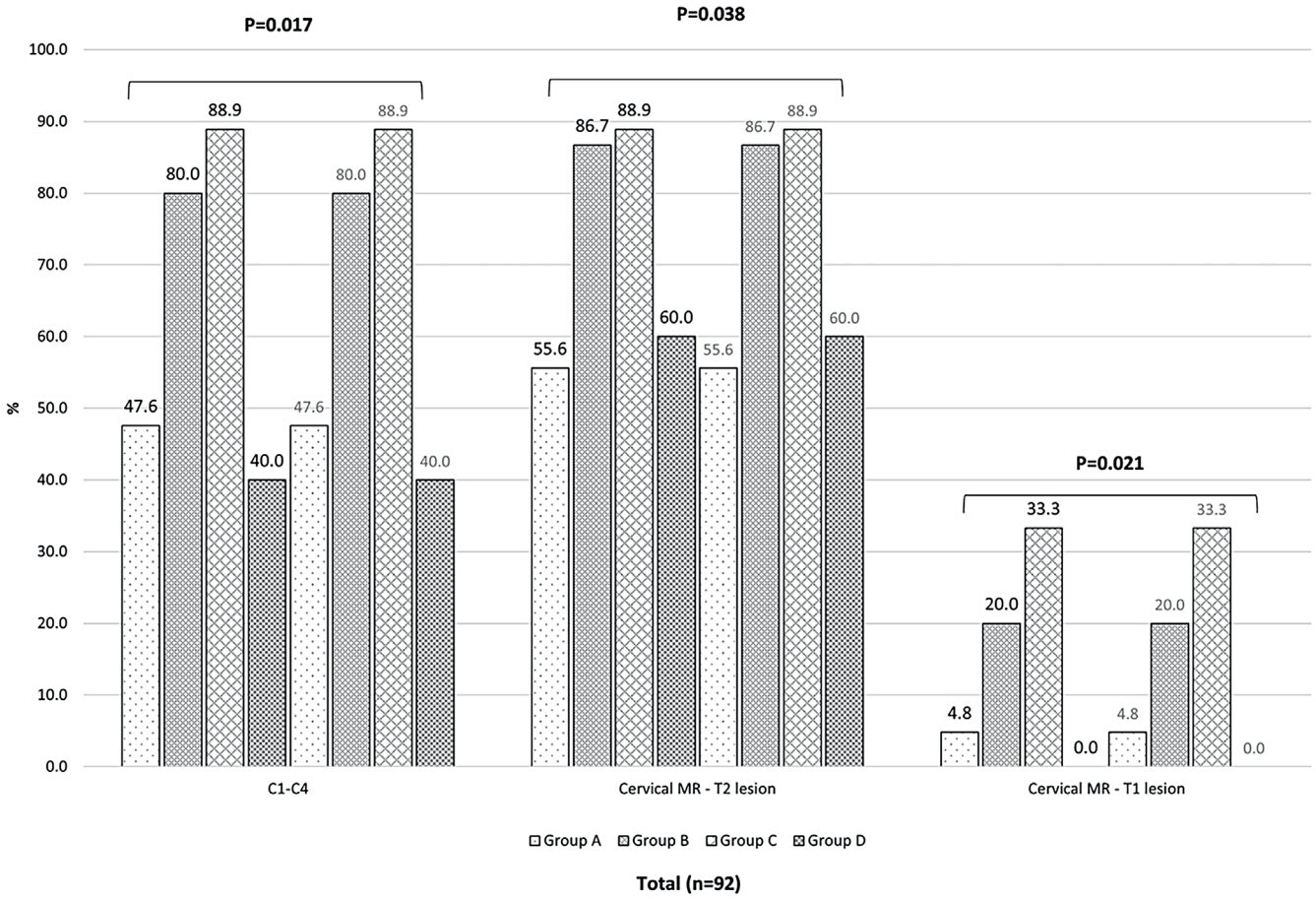

Fig. 2. Comparison of the Lhermitte's sign (LS) and radiological features. Group A: LS is absent. Group B: LS is positive. Group C: LS is present in both the past and at the screening. Group D: Developed LS during the screening despite no prior history. The frequency of C1–4 lesions was higher in groups B and C when compared to that in group A (p = 0.017). Group B exhibited a higher frequency of cervical cord lesions on T2-weighted magnetic resonance imaging (MRI) compared to group A (p = 0.038). The frequency of the presence of cervical cord lesions on T1-weighted MRI was higher in group C than in group A (p = 0.021)

Our research has demonstrated a complex relationship between LS and spinal cord lesion activity or contrast enhancement. Studies have shown that LS is not consistently associated with contrast enhancement on MRI, indicating its variable presentation in MS and other spinal pathologies.[35,36] This suggests that while LS is a significant clinical symptom, it does not reliably correlate with visible contrast-enhanced lesions in the spinal cord, underscoring the need for comprehensive diagnostic approaches beyond imaging alone.[35] Despite being an early indicator of neuroinflammation, it should be noted that clinical symptoms like LS may not always align with active MRI lesions.

We found that the RR type comprised the majority of patients. In 1 study from Turkey that investigated early-onset MS (<10 years of age), the RR type was found in 96.7% (n = 29) of 30 children.[37] Similar findings were also reported in previous studies.[22,38] In a Serbian POMS cohort, all the patients had the RR course of the disease.[39]

Longitudinal follow-up studies regarding POMS are scant. Mean follow-up duration was more than 10 years in our study, and we followed-up 31 patients for >10 years. Mean follow-up duration was relatively shorter in the previous studies, at about 2–4 years.[37,39,40]

We showed that cervical and thoracal spinal cord lesions were found in approx. 2/3 and 1/4 of the patients, and intracranial lesions in the vast majority of those. Similar findings were also reported.[37,40] The frequency of spinal cord lesions was lower in a previous report from Taiwan.[41] Cervical and thoracic spinal lesions were shown at a respective frequency of 33% and 43% in another study.[42] Differences in the frequency of spinal cord lesions in POMS may result from discrepancies in the manner in which systematic spinal cord imaging was obtained. We performed spinal cord MRI on all the participants. Given the high frequency of spinal cord lesions in our study, it is important to obtain a spinal cord MRI both for earlier diagnosis and follow-up. A study of the patient population revealed that more than 30% of patients had had MS for more than 10 years. This finding suggests the presence of numerous confounding factors that may contribute to the patients' current conditions and the burden of lesions as indicated by MRI. The most common symptoms associated with MS include visual disturbances, weakness

Table 3. Comparison of demographic, clinical and radiological features among the groups

Characteristics		Total (n = 92)				p-value
		Group A (n = 63)	Group B (n = 15)	Group C (n = 9)	Group D (n = 5)	
Age at onset [years], median (min/max)		14 (10/16)	14 (5/14)	14 (12/14)	14 (13/14)	0.936[k]
Age at screening [years], median (min/max)		24 (15/50)	22 (18/54)	21 (18/37)	24 (20/26)	0.780[k]
Sex (female), n (%)		43 (68.3)	10 (66.7)	7 (77.8)	3 (60)	0.889[f]
MS, n (%)	CIS	3 (4.8)	0 (0)	0 (0)	0 (0)	0.301[f]
	RR	53 (84.1)	14 (93.3)	8 (88.9)	5 (100)	
	SP	7 (11.1)	0 (0)	0 (0)	0 (0)	
	PP	0 (0)	1 (6.7)	1 (11.1)	0 (0)	
C1–4, n (%)		30 (47.6)[B,C]	12 (80)	8 (88.9)	2 (40)	0.017[f]
C5–8, n (%)		15 (23.8)	6 (40)	3 (33.3)	2 (40)	0.479[f]
Thoracal, n (%)		16 (25.4)	4 (26.7)	3 (33.3)	0 (0)	0.635[f]
Localization, n (%)	lateral	6 (9.5)	0 (0)	0 (0)	0 (0)	0.106[f]
	posterior	10 (15.9)	3 (20)	3 (33.3)	1 (20)	
	posterolateral	19 (30.2)	10 (66.7)	5 (55.6)	2 (40)	
	absent	28 (44.4)	2 (13.3)	1 (11.1)	2 (40)	
Cervical MR – T2 lesion, n (%)		35 (55.6)	13 (86.7)[A]	8 (88.9)	3 (60)	0.038[f]
Cervical MR – T1 lesion, n (%)		3 (4.8)	3 (20)	3 (33.3)[A]	0 (0)	0.021[f]
Cervical MR – gadolinium, n (%)		12 (19.1)	4 (26.7)	2 (22.2)	2 (40)	0.561[f]
Cervical MR – atrophy, n (%)		2 (3.2)	3 (20)	1 (11.1)	0 (0)	0.099[f]
Cervical MR – expansion, n (%)		10 (15.9)	3 (20)	1 (11.1)	2 (40)	0.482[f]
Intracranial lesion, n (%)		62 (98.4)	15 (100)	8 (88.9)	5 (100)	0.313[f]
Right superficial sensation, n (%)		3 (4.8)	2 (13.3)	1 (11.1)	0 (0)	0.401[f]
Left superficial sensation, n (%)		4 (6.4)	2 (13.3)	0 (0)	0 (0)	0.553[f]
Upper extremity vibration abnormality, n (%)		11 (17.5)	6 (40)	4 (44.4)	0 (0)	0.061[f]
Lower extremity vibration abnormality, n (%)		33 (52.4)	10 (66.7)	6 (66.7)	4 (80)	0.523[f]
Upper extremity position abnormality, n (%)		0 (0)	0 (0)	1 (11.1)	0 (0)	0.152[f]
Lower extremity position abnormality, n (%)		0 (0)	0 (0)	1 (11.1)	0 (0)	0.152[f]
Romberg test, n (%)		16 (25.4)	6 (40)	4 (44.4)	1 (20)	0.469[f]

[k] Kruskal–Wallis H test (Monte Carlo); [f] Fisher–Freeman–Halton test (Monte Carlo); post hoc test: Benjamin–Hochberg correction; [A] statistically significant compared with group A; [B] statistically significant compared with group B; [C] Statistically significant compared with group C; min – minimum; max – maximum; MS – multiple sclerosis; CIS – clinically isolated syndrome; RR – relapsing–remitting; SP – secondary progressive; PP – primary progressive; MR – magnetic resonance.

and gait disturbances, as well as sensory difficulties.[43] These symptoms are easily identified causes of disability. However, other common and disabling symptoms such as fatigue, cognitive decline, emotional distress, and pain are invisible.[44] Bladder and bowel dysfunction are also hidden consequences of MS, but they are among the most common and distressing symptoms.[43] This may cause delayed recognition of spinal sensory complaints, deterioration of quality of life and increased lesion burden over the years.

Histopathological studies on the relationship between the venous system and plaque have been relatively less studied for the spinal cord, unlike the brain. However, a few studies have shown that the topographic distribution of focal spinal cord lesions is related to the spinal venous system and that even the drainage areas of the spinal cord veins and the shape of the plaques are correlated.[45,46] However, visualizing the central vein sign, which is the radiological equivalent of the perivenous organization, for the spinal cord is a significant challenge. This is because the diameter of the spinal cord sulcal and radial vessels is only about 0.1–0.2 mm. On the other hand, with the developing MRI susceptibility-weighted imaging (SWI) techniques, at least 1 central vein sign can be detected in 40% of upper cord MS lesions.[47] These topographic features may also explain why LS symptoms may occur in cases involving spinal cord circulation, including cervical spinal stenosis, due to the involvement of both the venous and glymphatic systems.[48]

In a Serbian cohort including POMS, spinal cord lesions were detected in 33.3% of the patients.[39] Highly active demyelination and inflammation were observed on T1- and T2-weighted MR images in previous studies.[17,43] We found that all cervical spinal lesions were observed

Table 4. Comparison of demographic, clinical and radiological features among the groups in the patient subgroup with MS duration <10 years

Characteristics		MS duration (<10 years) (n = 61)				p-value
		Group A (n = 38)	Group B (n = 11)	Group C (n = 7)	Group D (n = 5)	
Age at onset [years], n/median (min/max)		38/13 (9/16)	11/14 (12/15)	7/14 (11/15)	5/13 (12/16)	0.437[k]
Age at screening [years], n/median (min/max)		38/21.5 (15/27)	11/21 (18/27)	7/21 (18/27)	5/24 (20/26)	0.356[k]
Sex (female), n (%)		27 (71.1)	6 (54.6)	5 (71.4)	3 (60)	0.744[f]
MS, n (%)	CIS	3 (7.9)	0 (0)	0 (0)	0 (0)	0.562[f]
	RR	32 (84.2)	11 (100)	6 (85.7)	5 (100)	
	SP	3 (7.9)	0 (0)	0 (0)	0 (0)	
	PP	0 (0)	0 (0)	1 (14.3)	0 (0)	
C1–4, n (%)		21 (55.3)	8 (72.7)	6 (85.7)	2 (40)	0.313[f]
C5–8, n (%)		9 (23.7)	3 (27.3)	2 (28.6)	2 (40)	0.899[f]
Thoracal, n (%)		14 (36.8)	2 (18.2)	2 (28.6)	0 (0)	0.380[f]
Localization, n (%)	lateral	3 (7.9)	0 (0)	0 (0)	0 (0)	0.863[f]
	posterior	6 (15.8)	3 (27.3)	2 (28.6)	1 (20)	
	posterolateral	15 (39.5)	6 (54.6)	4 (57.1)	2 (40)	
	absent	14 (36.8)	2 (18.2)	1 (14.3)	2 (40)	
Cervical MR – T2 lesion, n (%)		25 (65.8)	9 (81.8)	6 (85.7)	3 (60)	0.614[f]
Cervical MR – T1 lesion, n (%)		1 (2.6)	1 (9.1)	1 (14.3)	0 (0)	0.315[f]
Cervical MR – gadolinium, n (%)		10 (26.3)	3 (27.3)	1 (14.3)	2 (40)	0.844[f]
Cervical MR – atrophy, n (%)		1 (2.6)	1 (9.1)	0 (0)	0 (0)	0.616[f]
Cervical MR – expansion, n (%)		9 (23.7)	1 (9.1)	1 (14.3)	2 (40)	0.536[f]
Intracranial lesion, n (%)		37 (97.4)	11 (100)	6 (85.7)	5 (100)	0.390[f]
Right superficial sensation, n (%)		2 (5.3)	1 (9.1)	0 (0)	0 (0)	0.769[f]
Left superficial sensation, n (%)		2 (5.3)	1 (9.1)	0 (0)	0 (0)	0.769[f]
Upper extremity vibration abnormality, n (%)		7 (18.4)	2 (18.2)	2 (28.6)	0 (0)	0.785[f]
Lower extremity vibration abnormality, n (%)		22 (57.9)	6 (54.6)	4 (57.1)	4 (80)	0.895[f]
Upper extremity position abnormality, n (%)		0 (0)	0 (0)	1 (14.3)	0 (0)	0.192[f]
Lower extremity position abnormality, n (%)		0 (0)	0 (0)	1 (14.3)	0 (0)	0.192[f]
Romberg test, n (%)		10 (26.3)	2 (18.2)	3 (42.9)	1 (20)	0.690[f]

[k] Kruskal–Wallis H test (Monte Carlo); [f] Fisher–Freeman–Halton test (Monte Carlo); min – minimum; max – maximum. MS – multiple sclerosis; C – cervical; MR – magnetic resonance. CIS – clinically isolated syndrome; RR – relapsing remitting; SP – secondary progressive; PP – primary progressive.

on T2-weighted images, but the minority in T1-weighted images. These findings suggest that edema predominates atrophy or axonal death in our patients.

We evaluated sensory abnormalities and found that superficial tactile sensation or position sensation was abnormal in a minority of the patients. Vibration sensation was abnormal in about 1/5 of them in the upper extremities and in more than a half of them in lower extremities. Romberg test results were positive in about 1/3 of the participants. In 1 study analyzing Turkish POMS (<10 years), sensory findings were detected in 16.7% of the patients.[37] In 2 different pediatric-onset MS cohorts, sensory deficits were detected in less than half of the patients at diagnosis, and in more than a half of them during the later course of the disease.[39,49] We have identified sensory abnormalities, particularly with regard to vibration sensation. However, some previous studies did not specify sensory abnormalities or analyzed superficial sensory defects.[39,49]

The prevalence of LS could not be investigated in this study; hence, data are scant. In various studies analyzing MS patients, the prevalence of LS was shown to be about 9–41%.[26,50] One study comparing MS with neuromyelitis optica revealed a very low frequency of LS (4.5%) in MS patients.[51] To our knowledge, the present study is the first to investigate the frequency of LS and the association of it with both clinical and radiological parameters in POMS patients. We found that the frequency of LS was 31.5% in the present study and observed that LS was positive only in the past in half of the patients with LS, and it was positive in the past in the majority of those patients. This finding may suggest that paroxysmal findings may occur earlier in the course of MS. Another study suggests that LS may commonly start early in the disease course, and follow a paroxysmal pattern in some patients.[52]

We showed that the presence of LS in the past was associated with C1–4 spinal lesions in the overall patient

Table 5. Comparison of demographic, clinical and radiological features among the groups in the patient subgroup with multiple sclerosis (MS) duration >10 years

Characteristics		MS duration (>10 years) (n = 31)			p-value
		Group A (n = 25)	Group B (n = 4)	Group C – excluded (n = 2)	
Age at onset [years], n/median (min/max)		25/14 (10/16)	4/12 (5/15)	2/12 (10/14)	0.110[u]
Age at screening [years], n/median (min/max)		25/33 (24/0)	4/38 (27/54)	2/34.5 (32/37)	0.618[u]
Sex (female), n (%)		16 (64)	4 (100)	2 (100)	0.280[F]
MS, n (%)	CIS	0 (0)	0 (0)	0 (0)	0.211[F]
	RR	21 (84)	3 (75)	2 (100)	
	SP	4 (16)	0 (0)	0 (0)	
	PP	0 (0)	1 (25)	0 (0)	
C1–4, n (%)		9 (36)	4 (100)	2 (100)	**0.030**[F]
C5–8, n (%)		6 (24)	3 (75)	1 (50)	0.076[F]
Thoracal, n (%)		2 (8)	2 (50)	1 (50)	0.080[F]
Localization, n (%)	lateral	3 (12)	0 (0)	0 (0)	**0.010**[f]
	posterior	4 (16)	0 (0)	1 (50)	
	posterolateral	4 (16)	4 (100)[A]	1 (50)	
	absent	14 (56)	0 (0)[A]	0 (0)	
Cervical MR – T2 lesion, n (%)		10 (40)	4 (100)	2 (100)	**0.042**[F]
Cervical MR – T1 lesion, n (%)		2 (8)	2 (50)	2 (100)	0.080[F]
Cervical MR – gadolinium, n (%)		2 (8)	1 (25)	1 (50)	0.371[F]
Cervical MR – atrophy, n (%)		1 (4)	2 (50)	1 (50)	**0.042**[F]
Cervical MR – expansion, n (%)		1 (4)	2 (50)	0 (0)	**0.042**[F]
Intracranial lesion, n (%)		25 (100)	4 (100)	2 (100)	–
Right superficial sensation, n (%)		1 (4)	1 (25)	1 (50)	0.261[F]
Left superficial sensation, n (%)		2 (8)	1 (25)	0 (0)	0.371[F]
Upper extremity vibration abnormality, n (%)		4 (16)	4 (100)	2 (100)	**0.003**[F]
Lower extremity vibration abnormality, n (%)		11 (44)	4 (100)	2 (100)	0.100[F]
Upper extremity position abnormality, n (%)		0 (0)	0 (0)	0 (0)	–
Lower extremity position abnormality, n (%)		0 (0)	0 (0)	0 (0)	–
Romberg test, n (%)		6 (24)	4 (100)	1 (50)	**0.009**[F]

[u] Mann–Whitney U test (Monte Carlo); [f] Fisher–Freeman–Halton test (Monte Carlo); post hoc test: Benjamin– Hochberg correction; [F] Fisher's exact test (Monte Carlo); [A] statistically significant compared with group A; min – minimum; max – maximum; group D (n = 0) was not demonstrated in the table. Bold numbers indicate statistical significance. MS – multiple sclerosis; C – cervical; MR – magnetic resonance.

population. The "LS in the past" refers to LS at the disease onset. We showed that the other clinical findings of the patients with LS did not differ based on whether LS was positive in the past or later. In a study analyzing adult patients with MS, the earlier onset of the disease was associated with more positive LS.[52] Similar to our findings, the presence of LS was not associated with sex or disease pattern. They showed that female sex was associated with the presence of LS later in the disease course rather than the presence of LS at clinical onset. However, similar to our findings, the disease pattern or age at diagnosis were not associated with the time of the onset of LS in the disease course.[52] They also showed that cervical spinal lesions were associated with the presence of LS.

Lhermitte's sign is caused by a stretching of the hyperexcitable demyelinated dorsal column of the spinal cord, especially at the cervical level.[52] It is a symptom like "electric shock sensation", but may also be induced during physical examination, which makes it a sign. We analyzed both LS and symptoms, and showed a significant level of compatibility between symptom and sign. However, previous studies revealed Lhermitte's finding to be either a symptom or sign.[26,53] In a previous study, LS was defined as a symptom and grouped as a probable, definite or possible LS.[26] They also showed that limb movements, laughing, sneezing, or coughing, other than neck flexion, may precipitate LS. Lhermitte's sign is not a sensitive or specific sign, which may occur in a number of conditions such as MS, Behçet's disease, systemic lupus erythematosus, herpes zoster, spinal cord compression, vitamin B12 deficiency, radiculopathy, or spondylitis.[54] In a previous study, LS was shown to be more common in MS or subacute combined degeneration.[26] It may suggest that demyelination with axonal preservation could be valuable for spontaneous

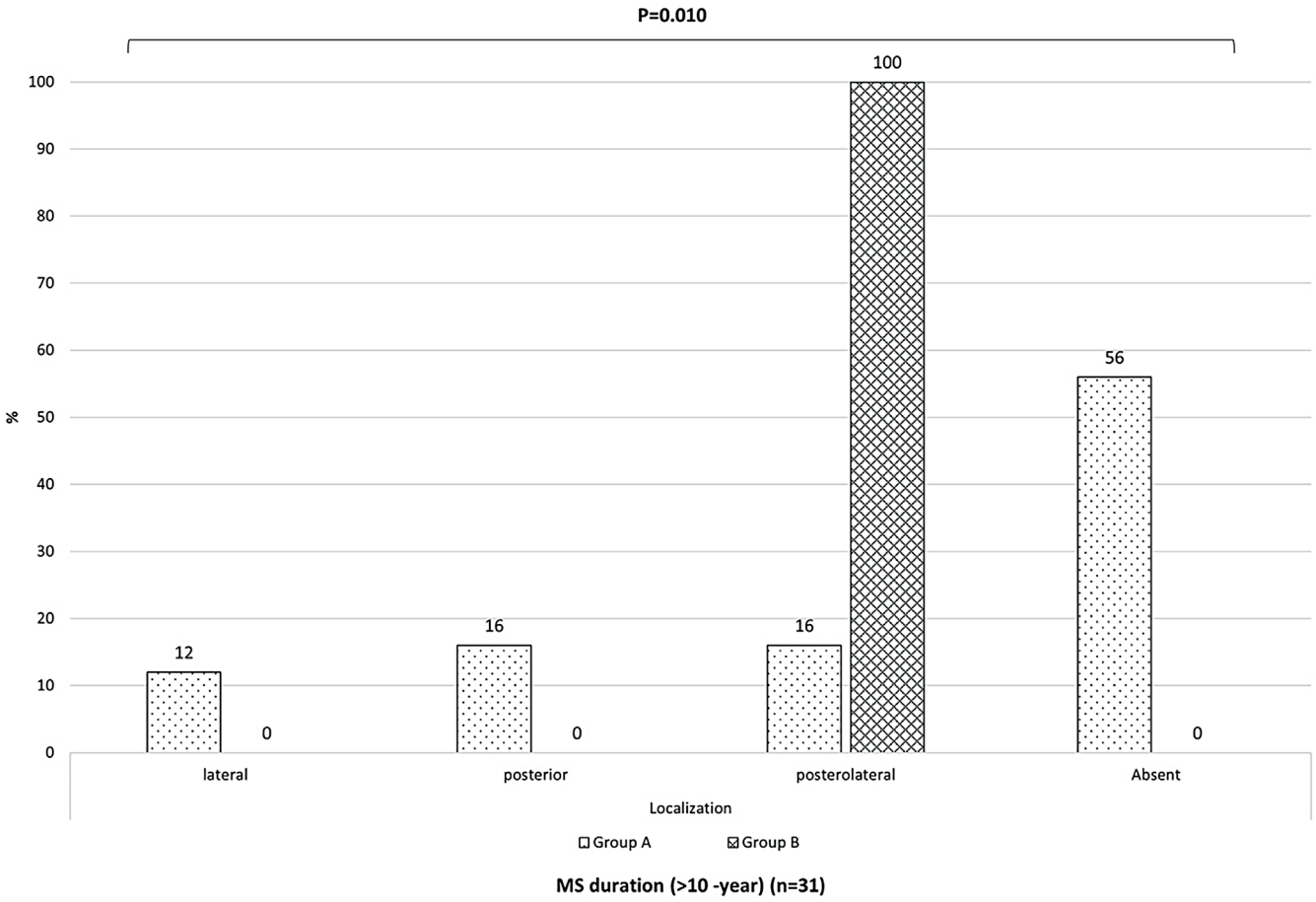

Fig. 3. Comparison of localization features among the groups in the patient subgroup with multiple sclerosis (MS) duration >10 years

activity to emerge. We excluded vitamin B12 deficiency, SLE, spinal cord compression, spondylitis, radiculopathy, and cervical disc herniation.

In our study, in the subgroup of patients with a follow-up duration >10 years, the presence of LS in the past was associated with C1–4 and posterolateral spinal lesions, spinal lesions on T2-weighted MRI, atrophy or expansion on MRI, and abnormality of vibration sensation on the upper extremities. An unexpected finding was that there was no correlation between contrast enhancement and the presence of LS. This can be explained by the relatively low number of patients included in the study. In addition, the intricate relationship between LS and spinal cord lesion activity or contrast enhancement reveals that LS does not consistently correlate with contrast enhancement on MRI, thus demonstrating its variable manifestation in MS and other spinal conditions. Romberg positivity was also associated with LS. Given the known mechanism of LS, such findings can be expected. The spine is known to be most mobile in the neck, which may suggest the association of cervical spinal MS lesions with LS. This association was also observed in a previous study.[27] One study did not show any correlation of clinical symptoms with MRI findings.[55] We showed that clinical features were not associated with the presence of LS in the subgroup of those patients with a follow-up duration <10 years. Hence, localization of spinal lesions was associated with the presence of LS only later in the disease course.

Harmony in brain–body communication is the basis of wellbeing. The interaction between cognitive and behavioral wellbeing and brain and body functioning has become a central area of study for neurologists and neuroscientists in clinical and non-clinical contexts. Brain–body axis dysfunctions occur in many psychiatric and neurological diseases.[56]

Chronic immunological dysregulations in MS may reflect a long-term stress response to homeostatic dysregulation in the CNS, ultimately leading to neurodegeneration. Increasing evidence has shown that similar processes occur in conditions such as ischemic stroke and that inflammation is important in neurodegeneration beyond primary vascular changes.[57]

Glucocorticoid receptors are one of the most important components of the stress response. In neuroscience studies related to fear extinction, evaluation of functional brain regions and connections with functional MRI helps to find the organ underlying fear regulation and the potential therapeutic target by providing valuable information, especially about neuropharmacology, neurotransmitters and protection systems.[58] Integrating these mechanisms with

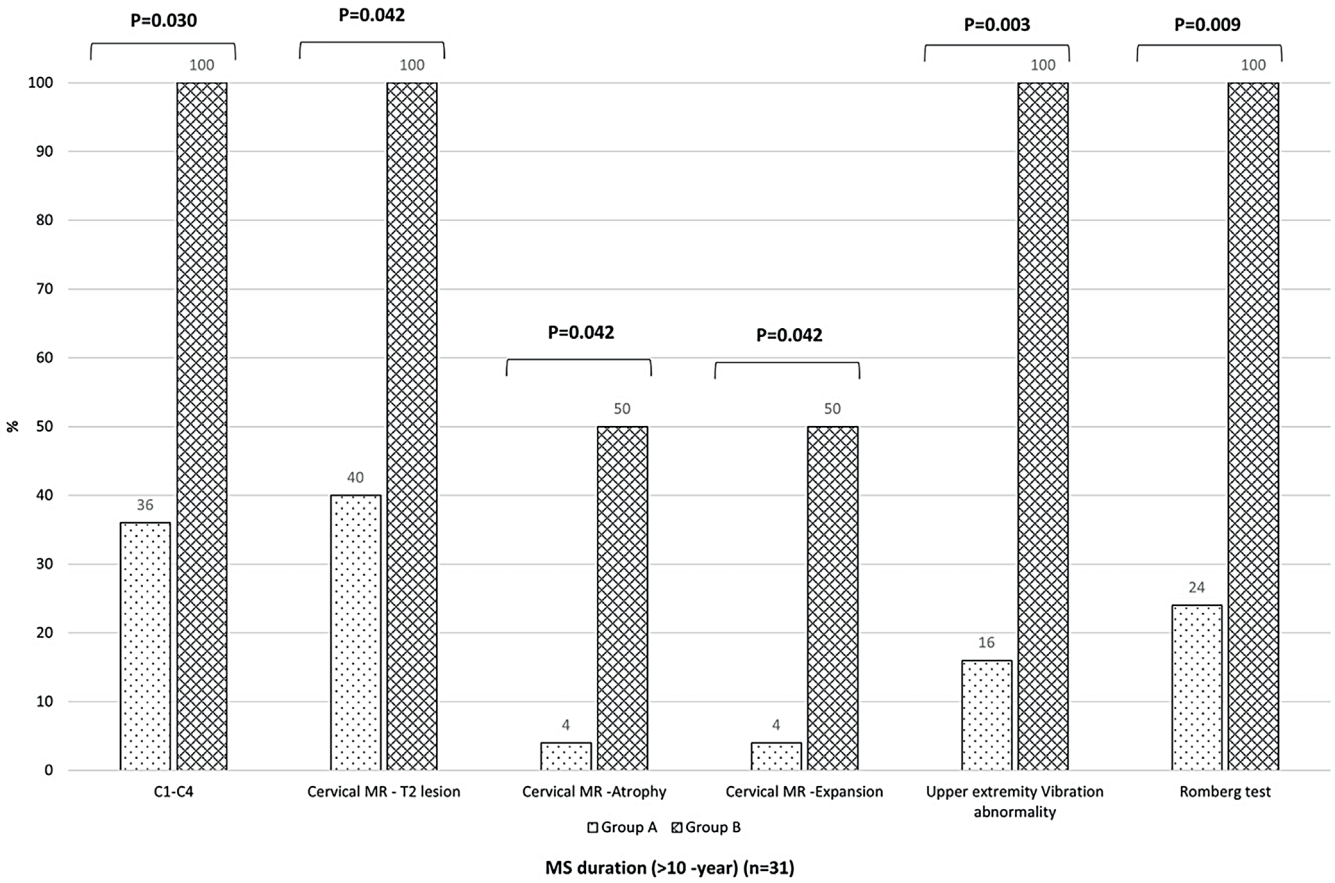

Fig. 4. Detailed comparison of clinical and radiological features among subgroups of multiple sclerosis (MS) patients with a disease duration exceeding 10 years, labeled as group A and B. The analysis encompasses a total of 31 patients. C1–4 lesions were more frequent in group B than in group A (p = 0.030). The frequency of the presence of cervical cord lesions on T2-weighted magnetic resonance imaging (MRI), and atrophy and expansion on cervical MRI were higher in group B when compared to those in group A (p = 0.042, p = 0.042 and p = 0.042; respectively). Vibration abnormality in the upper extremity was found to be significantly more pronounced in group B compared to group A (p = 0.003). A significant disparity was noted in the Romberg test, with 100% of group B exhibiting abnormalities compared to 24% in group A (p = 0.009)

abnormal oscillatory patterns in electroencephalography (EEG) may provide a better understanding of the disease-initiating processes in POMS.[58]

Both MRI and some electrophysiological and specific laboratory tests are used for early indicators of chronic inflammation.[59,60] Different markers from brain and body functioning can be integrated into these tests to improve diagnostic and prognostic accuracy. An integrative brain–body assessment approach can provide a more comprehensive understanding of neurobiological mechanisms and help guide treatment strategies, such as selecting appropriate medications or implementing targeted interventions for personalized medical approaches. Recent studies have shown that EEG biomarkers such as abnormal oscillatory patterns or decreased connectivity may indicate early signs of cognitive decline, while cardiac measurements may reflect autonomic dysfunction associated with neurodegenerative processes.[61,62] By integrating these measurements with AI-based MRI techniques, it is possible to increase diagnostic accuracy and follow the progression of the disease, facilitating the development of personalized treatment plans.

Limitations

We investigated POMS and LS in a relatively large population, within a long follow-up period, and revealed the association of clinical and radiological findings with LS. For the first time, the frequency of LS in a POMS sample was investigated. We conducted routine spinal MRI examinations in the whole group, but could not analyze either somatosensorial-evoked potential or visual-evoked potential. In a study reporting a strong relationship between the presence of abnormalities seen on MRI of the cervical spinal cord and the LS in MS, it was stated that the posterior column was particularly affected.[27] In another study, delayed somatosensory evoked potentials (SSEP) conduction was detected in 92% of patients who were identified as having LS.[63] Therefore, we think that SSEP examination can be performed for patients who define LS but do not have a cervical cord lesion, and that the clinical benefit of SSEP can be evaluated with larger patient groups in patients who define LS and have cervical cord abnormalities. Additionally, the absence of data regarding other clinical characteristics, such as the Expanded Disability Status Scale (EDSS),

relapse rate, and time from recent relapse at screening, as well as the history of treatment with disease-modifying therapies, represents another limitation of the study.

Conclusions

We included a relatively large sample of POMS patients with a long follow-up and found LS in 1/3 of the patients. The presence of LS seems to be associated with cervical spinal lesions, but not with sex or disease pattern. Our study is the first study to analyze the frequency and association of LS with other clinical and radiological findings in a POMS cohort. Studies on early diagnosis and prognosis of MS using multidemensional data with computer-aided diagnosis and DL/ML methods have focused mostly on brain imaging. In order to make AI-based spinal lesion definition in the radiological differentiation of conditions such as MOGAD, NMOSD, ADEM, and CIS with POMS, the characteristics of these lesions must be recognized correctly. We recommend routine spine imaging upon a diagnosis of MS. Further multicenter studies should be conducted to more specifically identify LS in POMS patients. Based on the results of our study, we recommend considering advances in AI and ML to analyze multimodal data for future research directions.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.14188165. The package includes the following files:

Supplementary Table 1. Normality assumption table.

Supplementary Table 2. Analysis of test statistics of Table 3,4,5.

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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Diagnostic and prediction value of synthetic magnetic resonance imaging in acute ischemic stroke patients

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Abstract

Background. Current knowledge regarding synthetic magnetic resonance imaging in ischemic stroke (MAGiC) is inadequate.

Objectives. The study aimed to investigate the diagnostic and prognostic prediction value of MAGiC in acute ischemic stroke (AIS) patients.

Materials and methods. This prospective observational study enrolled 197 AIS patients between January 2022 and May 2023. All patients underwent routine magnetic resonance imaging (MRI), computed tomography (CT) scans, doppler ultrasound, MAGiC, and dynamic contrast-enhanced (DCE)-MRI. The levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-ch), low-density lipoprotein cholesterol (LDL-ch), C-reactive protein (CRP), and procalcitonin (PCT) were also measured, and the National Institutes of Health Stroke Scale (NIHSS) was used to evaluate stroke severity.

Results. T2 and proton density (PD) values were markedly lower in severe patients than in mild-to-moderate patients, and the DCE-MRI K^{trans} value was substantially higher in severe patients compared to mild-to-moderate patients. Furthermore, T2 and PD correlated negatively, while K^{trans} correlated positively with CRP. Receiver operating characteristic (ROC) showed T2 and K^{trans} to have the best diagnostic potential as MAGiC and DCE-MRI parameters, respectively. As such, combining T2 and K^{trans} could improve severe stroke diagnosis accuracy. Moreover, TG, LDL-ch, CRP, T2, and K^{trans} were independent risk factors for severe stroke.

Conclusions. T2 and PD MAGiC parameters and the DCE-MRI K^{trans} parameter could be used as indices to predict severe stroke, while combining T2 and K^{trans} might provide better diagnostic accuracy.

Key words: MAGiC, ischemic stroke, DCE-MRI, diagnosis, prognosis

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Background

Ischemic stroke accounts for more than 85% of all strokes and is the leading global cause of disability and mortality, especially in non-high-income countries.[1,2] However, less than 5% of ischemic stroke patients receive intravenous thrombolysis.[3,4] Generally, timely diagnosis is the critical factor for patient treatment and prognosis,[5] and delay beyond the therapeutic time window may lead to longer treatment duration and worse prognosis.[6]

Synthetic magnetic resonance imaging (MRI), a recently developed method, can provide quantitative and qualitative T1 and T2 maps and images.[7] A typical synthetic MRI method, magnetic resonance imaging compilation (MAGiC) from GE Healthcare (GEHC; Chicago, USA), uses multi-dynamic multi-echo (MDME).[8] In recent years, synthetic MRI modalities such as MAGiC have been gradually applied to the diagnosis of various diseases, including Alzheimer's disease, brain cancer, multiple sclerosis, and spondyloarthritis.[9–12] However, knowledge of MAGiC in ischemic stroke is still inadequate.

Objectives

The present study aimed to investigate the diagnostic and prognostic prediction value of MAGiC in acute ischemic stroke (AIS) patients to provide more clinical evidence and experience for MAGiC in stroke.

Materials and material

Patients

This prospective observational study enrolled 197 AIS patients admitted to our hospital between January 2022 and May 2023. The inclusion criteria were: 1) ischemic stroke diagnosed with imaging, including MRI, computed tomography (CT) and Doppler ultrasound, 2) hospital admission within 48 h of stroke, and 3) no anticoagulant treatment within 3 months of the study. The exclusion criteria were: 1) hemorrhagic stroke, 2) receiving prior anticoagulant therapy, and 3) having severe liver, cardiovascular or renal dysfunction. All patients underwent clinical tests, including imaging and laboratory evaluations on admission, and no interventions were made to the treatment. All patients provided written informed consent. Study approval was obtained from the ethical committee of the Fourth Hospital of Changsha, China (approval No. CSSDSYY-LLSC-KYXM-2019-20).

Imaging strategy

All patients received routine MRI, 3-dimensional time-of-flight (3D-TOF) magnetic resonance angiography (MRA) for evaluation of cerebral vascular condition, CT scan, Doppler ultrasound, MAGiC, and dynamic contrast-enhanced (DCE)-MRI when admitted. All data were independently reviewed and analyzed by 2 radiologists with over 5 years of experience using the dedicated Advantage Windows AW 4.7 workstation (GE Healthcare). When discrepancies arose between the 2 radiologists, a 3rd independent radiologist was involved for re-analysis, with a consensus reached through discussion and evaluation.

For MAGiC, a SIGNA Architect 3.0T whole-body scanner (GE Healthcare) was used, and patients received routine axial (AX), T2-weighted and T1-weighted imaging with repetition time (TR) of 4,000 ms, echo time (TE) of 20/99.8 ms, echo chain length of 12, field of view (FOV) of 24 × 24, matrix of 240 × 240, section thickness of 4 mm, and bandwidth of 25.0 Hz/pixel. The data were imported into the AW 4.7 workstation to generate T1, T2 and proton density (PD) maps. The diffusion-weighted imaging (DWI) data were also imported to the AW 4.7 workstation and analyzed using the READYView package. Regions displaying a high signal on DWI and a low apparent diffusion coefficient (ADC) value were identified as infarct lesions. Subsequently, the corresponding infarct lesion on the T2 fluid-attenuated inversion recovery (T2-FLAIR) map was located, and a region of interest (ROI) was delineated to encompass the lesion. The ROI was set to 10 mm^2. The parameters of T1, T2 and PD within the ROI were measured twice, and the mean values were recorded.

For DCE-MRI, patients received LAVA-T1WI (flip angle = 90°, TR = 2,000 ms, TE = 45 ms, FOV = 24 × 24 cm, layer thickness = 5 mm, and layer spacing = 1 mm), LAVA-T1WI dynamic enhanced scanning with 35 phases in 1 min and 10 s (flip angle = 90°, TR = 2000 ms, TE = 45 ms, FOV = 24 × 24 cm, layer thickness = 5 mm, and layer spacing = 1 mm), following injection of gadolinium diamine (0.2 mL/kg, 3 mL/s) through the elbow vein and 15 mL normal saline (3 mL/s). The data were imported into the GenIQ package of AW 4.7 workstation, and the parameter maps of contrast agent transfer rate between blood and tissue (K^{trans}), contrast agent back-flux rate constant (K_{ep}) and extravascular extracellular fractional volume (V_e) were generated. The diffusion-weighted imaging data were also imported into the AW 4.7 workstation and analyzed using the READYView package. The infarct lesion was defined as a region exhibiting a distinctly high signal on DWI and a low ADC value. Subsequently, the DWI and aforementioned K^{trans}, K_{ep} and V_e maps were merged. The ROI was determined using the same method as before, with an area of 10 mm^2 to encompass the infarct lesion. The mean values of duplicate K^{trans}, K_{ep} and V_e were recorded.

Clinical characteristics

Characteristics, including age, sex, body mass index (BMI), and medical history were recorded. The levels of total cholesterol (TC), triglyceride (TG), high-density

lipoprotein cholesterol (HDL-ch), low-density lipoprotein cholesterol (LDL-ch), C-reactive protein (CRP), and procalcitonin (PCT) were evaluated on admission using an AU5800 Beckman automatic biochemical analyzer (Beckman Coulter, Brea, USA). The National Institutes of Health Stroke Scale (NIHSS) was employed to measure stroke severity as mild (<6), moderate (6–16) or severe (≥16).[13]

Statistical analyses

The Kolmogorov–Smirnov test assessed data normality, with non-normally distributed data expressed as median (range and interquartile range (IQR)) and normally distributed data expressed as mean ± standard deviation (SD). Mann–Whitney U tests and t-tests compared normally or non-normally distributed data, respectively. The variance homogeneity was determined with Levene's analysis in the t-tests. Rates were compared using χ^2 tests, correlation analysis used Spearman's test, receiver operating characteristic (ROC) curves assessed diagnostic value, logistic regression was conducted using (enter method), goodness-of-fit analysis used Nagelkerke R^2, and analysis of the linear relationship between independent variables and log-odds employed a Box–Tidwell test with Bonferroni correction. The variance inflation factor (VIF) determined multicollinearity using linear. All calculations employed IBM SPSS v. 22.0 (IBM Corp., Armonk, USA) or GraphPad Prism v. 6.0 (GraphPad Software, Inc., San Diego, USA), with $p < 0.05$ indicating a significant difference.

Results

Basic characteristics of all patients

As shown in Table 1, among all 197 ischemic stroke patients, 110 had mild-to-moderate stroke and 87 had severe stroke. The basic characteristics of the different patient groups were analyzed and compared. Levene's analysis showed TG ($p = 0.121$) and LDL-ch ($p = 0.198$) had homogeneity of variance (Supplementary Table 1). The NIHSS scores and CRP levels were significantly higher, while HDL-ch was substantially decreased in severe patients compared to the mild-to-moderate patients (all $p < 0.05$). No significant differences were found for other indices.

Comparison of MAGiC and DCE-MRI parameters for stroke patients with different severity

Typical MAGiC and DCE-MRI images are shown in Fig. 1A,B. Comparing the MAGiC and DCE-MRI parameters between mild-to-moderate and severe stroke patients showed that the T2 and PD values were remarkably lower in severe patients than in mild-to-moderate patients (both $p < 0.001$; Table 2). No significant difference was found for T1. For DCE-MRI parameters, only K^{trans} was markedly elevated in severe patients compared to the mild-to-moderate patients ($p < 0.001$), while no significant difference was found for K_{ep} or V_e.

Table 1. Basic characteristics of all patients

Variables	Mild-to-moderate patients (n = 110)	Severe patients (n = 87)	Z, t or χ^2 value*	p-value
Age [years]	61 (36–85, 27.25)	63 (35–84, 26.00)	–0.108	0.914[a]
Sex (% female)	49 (45.79)	45 (51.72)	0.704	0.480[c]
BMI [kg/m^2]	27.15 (19.41–33.98, 8.08)	26.66 (19.14–33.72, 7.53)	–1.077	0.281[a]
Risk factors, n (%)	–	–	1.023	0.600[c]
Hypertension	34 (31.78)	21 (24.14)	–	–
Diabetes	30 (28.04)	27 (31.03)	–	–
Smoking	49 (45.79)	38 (43.68)	–	–
NIHSS	8 (1–15, 8.25)	23 (16–35, 9.00)	–12.050	<0.001[a]
TC [mmol/L]	4.41 (3.26–5.38, 1.17)	4.20 (3.25–5.33, 1.10)	–1.653	0.098[a]
TG [mmol/L]	1.47 ±0.30	1.53 ±0.33	–1.376	0.170[b]
HDL-ch [mmol/L]	1.09 (0.96–1.25, 0.14)	1.06 (0.95–1.24, 0.14)	–1.997	0.046[a]
LDL-ch [mmol/L]	2.97 ±0.41	3.09 ±0.46	–1.917	0.057[b]
CRP [mg/L]	10.20 (1.16–16.89, 7.71)	19.40 (5.64–34.66, 14.32)	–8.444	<0.001[a]
PCT [pg/mL]	30.30 (7.79–49.89, 21.46)	28.51 (6.27–49.50, 19.97)	–0.929	0.353[a]

*Z, t or χ^2 values were calculated with Mann–Whitney U test, t-test or χ^2 test, respectively. [a]For non-normally distributed continuous data were expressed with median (range, interquartile range (IQR)), and p-value was calculated with Mann–Whitney U test. [b]For non-normally distributed data expressed with (mean ± stadard deviation (SD)), p-value was calculated using t-test. [c] χ^2 test was used to analyze the rates; BMI – body mass index; NIHSS – National Institutes of Health Stroke Scale; TC – total cholesterol; TG – triglyceride; HDL-ch – high-density lipoprotein cholesterol; LDL-ch – low-density lipoprotein cholesterol; CRP – C-reactive protein; PCT – procalcitonin.

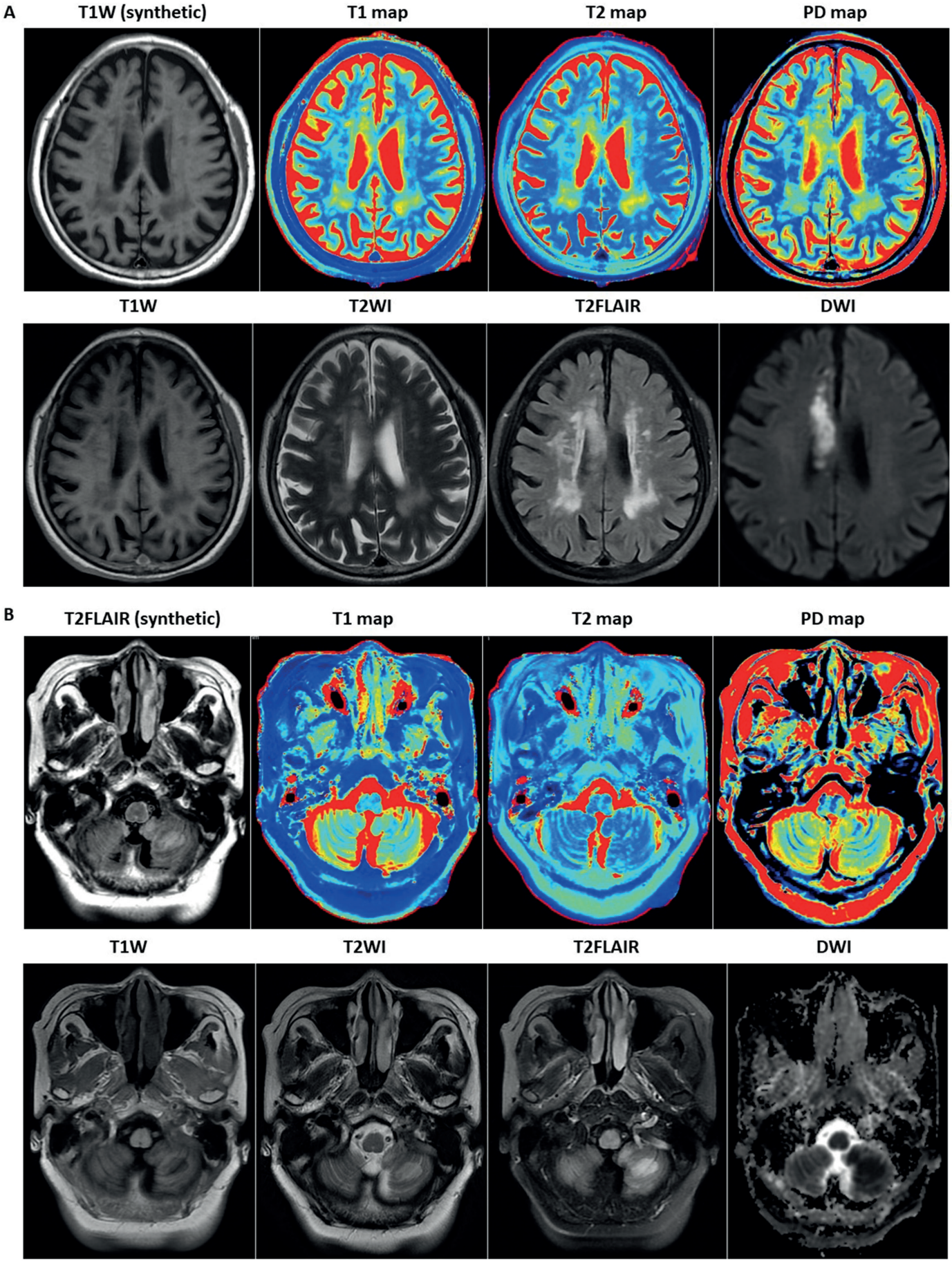

Fig. 1. A. Typical images for MAGiC and DCE-MRI of an 84-year-old female stroke patient with acute cerebral infarction in the right frontoparietal lobe. The images are synthetic T1WI, T1 map, T2 map, PD map, T1W, T2W, T2-FLAIR, and DWI; B. Typical images for MAGiC and DCE-MRI of a 63-year-old female stroke patient with multiple acute phase cerebral infarctions in the left posterior medulla oblongata and left cerebellar hemisphere. The images are listed as T2-FLAIR (synthetic), T1map, T2map, PD map, T1W, T2WI, T2-FLAIR, and DWI

MAGiC – resonance imaging in ischemic stroke; DCE-MRI – contrast-enhanced MRI; PD – proton density; DWI – diffusion-weighted imaging; FLAIR – fluid-attenuated inversion recovery.

Table 2. Comparison of MAGiC and DCE-MRI parameters for stroke patients with different severity

Variables	Mild-to-moderate patients (n = 110)	Severe patients (n = 87)	Z value*	p-value#
T1 [ms]	1948.95 (1503.30–2297.41, 374.68)	1894.65 (1503.26–2299.90, 488.63)	–0.435	0.663
T2 [ms]	85.46 (75.18–94.85, 11.43)	76.02 (65.47–84.94, 10.19)	–8.239	<0.001
PD [pu]	74.07 (60.28–94.75, 17.10)	68.12 (60.31–79.99, 8.14)	–4.625	<0.001
K^{trans} [min^{-1}]	0.08 (0.05–0.12, 0.05)	0.11 (0.08–0.15, 0.04)	–7.162	<0.001
K_{ep} [min^{-1}]	1.07 (0.10–1.90, 1.05)	1.03 (0.11–1.90, 0.92)	–0.267	–0.790
V_e [%]	7.47 (1.08–14.81, 8.01)	7.12 (1.01–14.87, 6.75)	–1.263	0.206

*Z or t values were calculated using Mann–Whitney U test. #All data in this table are non-normally distributed data expressed with median (range, IQR), and all p-values were calculated using Mann–Whitney U test; PD – proton density; K^{trans} – volume transfer constant; K_{ep} – rate constant; V_e – extracellular extravascular volume fraction.

Correlation between MAGiC and DCE-MRI parameters and laboratory indices

Spearman's test analyzed the correlation between MAGiC and DCE-MRI parameters and the laboratory indices, with the data shown in Supplementary Table 2. T2 and PD values were negatively correlated with CRP, while K^{trans} correlated positively. No other significant relationships were observed.

Diagnostic value of MAGiC and DCE-MRI parameters for severe stroke patients

Receiver operating characteristic curves were drawn to evaluate the diagnostic value of MAGiC and DCE-MRI parameters for severe stroke patients. As shown in Fig. 2, T2 had the best diagnostic potential in MAGiC parameters, with an area under the curve (AUC) = 0.842, sensitivity = 80.46%, specificity = 64.55%, and a cutoff value <81.78 ms. For DCE-MRI parameters, K^{trans} showed the best diagnostic potential, with AUC = 0.794, sensitivity = 60.92%, specificity = 73.64%, and a cutoff value >0.105. We used the T2 and K^{trans} cutoff values to predict severe stroke and found that T2 had sensitivity of 64.22%, specificity of 80.68% and accuracy of 55.33%, while K^{trans} showed sensitivity of 64.63%, specificity of 70.43% and accuracy of 41.62% (Table 3). Combining both achieved sensitivity = 57.86%, specificity = 89.47% and accuracy = 71.07%.

Logistic regression for severe stroke patients

Univariate and multivariate binary logistic regression was conducted for the severe stroke risk factors. Univariate

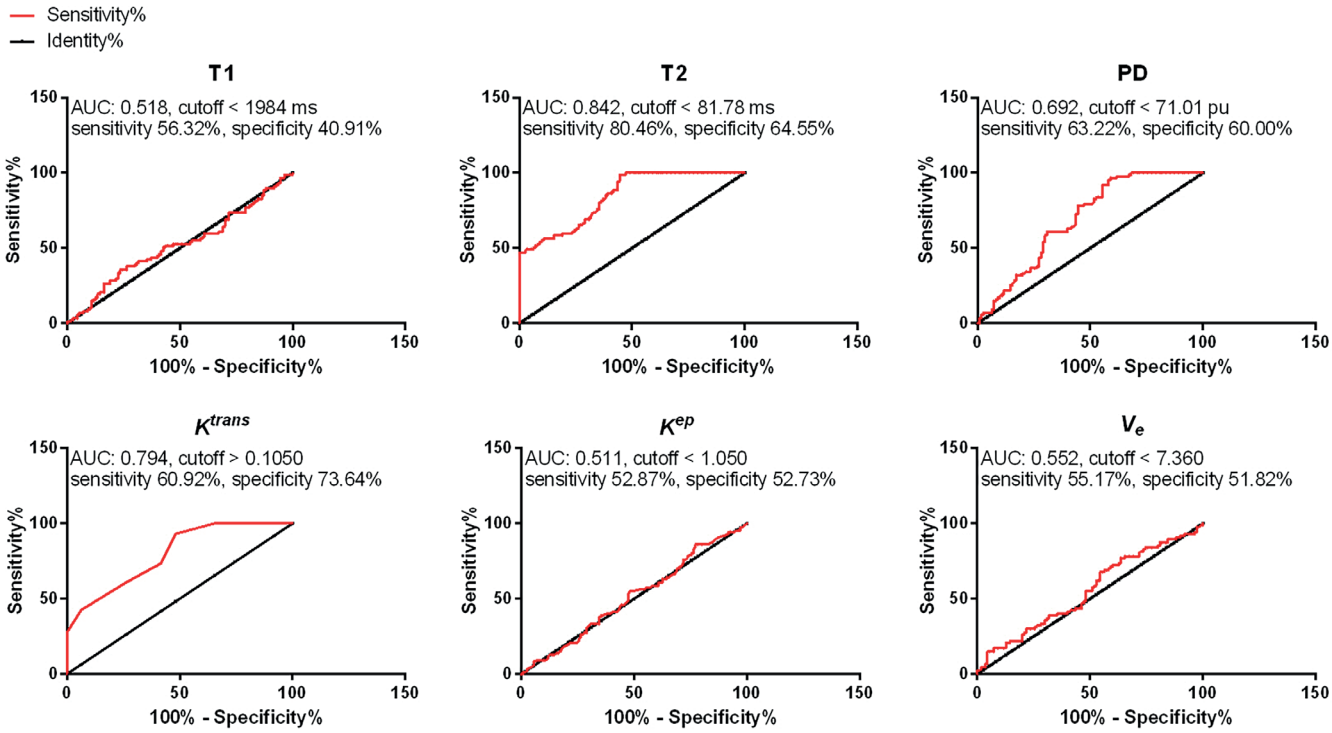

Fig. 2. Receiver operating characteristic (ROC) curves of T1 (A), T2 (B), PD (C), K^{trans} (D), K_{ep} (E), and V_e (F) for diagnosis of severe stroke

PD – proton density; K^{trans} – volume transfer constant; K_{ep} – rate constant; V_e – extracellular extravascular volume fraction.

Table 3. Sensitivity, specificity and accuracy for T2 and K^{trans} to predict severe stroke

Methods	True positive	False positive	True negative	False negative	Sensitivity%	Specificity%	Accuracy%
T2	70	39	71	17	64.22	80.68	55.33
K^{trans}	53	29	81	34	64.63	70.43	41.62
T2+ K^{trans}	81	59	51	6	57.86	89.47	71.07

* Sensitivity = true positive/(true positive + false negative) × 100%; specificity = true negative/(true negative + false positive) × 100%; accuracy = (true positive + true negative)/(true positive + false negative + false positive + true negative) × 100%; K^{trans} – volume transfer constant.

Table 4. Univariate and multivariate binary logistic regression for severe stroke patients

Variables	Univariate			Multivariate		
	OR	95% CI	p-value	OR	95% CI	p-value
Age	1.002	0.983–1.022	0.805	1.004	0.975–1.033	0.808
Sex	0.757	0.430–1.330	0.333	1.024	0.434–2.415	0.956
BMI	0.965	0.904–1.029	0.277	0.980	0.885–1.085	0.697
Hypertension	0.711	0.376–1.344	0.294	1.236	0.454–3.366	0.679
Diabetes	1.200	0.647–2.227	0.563	0.646	0.248–1.683	0.371
Smoking	0.965	0.548–1.701	0.903	0.904	0.395–2.067	0.810
TC	0.696	0.446–1.086	0.110	0.710	0.374–1.351	0.297
TG	1.881	0.763–4.640	0.170	9.297	2.117–40.817	0.003
HDL-ch	0.021	0.001–0.780	0.036	0.236	0.002–36.594	0.575
LDL-ch	1.902	0.979–3.694	0.058	3.251	1.162–8.091	0.025
CRP	1.295	1.202–1.396	<0.001	0.155	0.064–0.371	<0.001
PCT	0.990	0.968–1.012	0.365	0.975	0.943–1.008	0.135
T1	1.000	0.998–1.001	0.571	0.999	0.997–1.000	0.137
T2	0.783	0.730–0.839	<0.001	11.983	4.547–31.577	<0.001
PD	0.901	0.865–0.939	<0.001	2.040	0.884–4.710	0.095
K^{trans}	2.681	1.984–3.624	<0.001	0.122	0.048–0.312	<0.001
K_{ep}	0.947	0.558–1.606	0.840	0.535	0.237–1.206	0.131
V_e	0.953	0.890–1.021	0.174	1.001	0.901–1.112	0.980

95% CI – 95% confidence interval; OR – odds ratio; BMI – body mass index; TC – total cholesterol; TG – triglyceride; HDL-ch – high-density lipoprotein cholesterol; LDL-ch – low-density lipoprotein cholesterol; CRP – C-reactive protein; PCT – procalcitonin; K^{trans} – volume transfer constant; K_{ep} – rate constant; V_e – extracellular extravascular volume fraction.

analysis found that HDL-ch, CRP, T2, PD, and K^{trans} were risk factors for severe stroke, and multivariate analysis showed that the Nagelkerke R^2 for multivariate logistic regression was 0.598. The results of the Box–Tidwell test and the VIF values are presented in Supplementary Tables 3,4. Unfortunately, the Box–Tidwell test found that CRP, T2, PD, and K^{trans} did not meet the criteria for a linear relationship. Consequently, we converted these data into categorical variables. For T2, PD and K^{trans}, we utilized the cutoff values from the previous diagnostic analysis to categorize them into low and high groups (T2 < or >81.78, PD < or >71.01 and K^{trans} < or > 0.105). For CRP, we used its median value as the threshold to divide the data into low and high groups (≤ or >12.79). No variables exhibited signs of multicollinearity (VIF > 10 or tolerance <0.1). In multivariate analysis, TG, LDL-ch, CRP, T2, and K^{trans} were independent risk factors for severe stroke (Table 4).

Discussion

Despite the development of diagnostic methods, new approaches for accurate and timely diagnosis of ischemic stroke patients are always needed. In this research, we conducted a prospective observational study to investigate the application of MAGiC combined with DCE-MRI to the diagnosis of ischemic stroke patients with different severity. We found that T2, PD and K^{trans} could be used as indices to predict severe stroke, and combining T2 and K^{trans} might provide better diagnostic accuracy.

Several studies noted the potential use of MAGiC in neurological diseases, including stroke, with a recent study showing that MAGiC PSIR Vessel had a higher AUC value for vascular stenosis >50% in stroke patients than time-of-flight (TOF) magnetic resonance angiography (MRA).[14] Although evidence for MAGiC in stroke patients is still inadequate, the modality is used in neurodegenerative

diseases such as Alzheimer's disease and other cognitive impairments. In Alzheimer's disease, MAGiC T1 and T2 values in the right insula cortex and left hippocampus were markedly increased compared to controls.[15] Another study used synthetic MRI to detect white matter hyperintensities (WMHs) and found a significant association between myelin loss and WMHs in cognitively impaired patients.[16] Additionally, a recent study demonstrated that patients with primary insomnia showed a negative correlation between cerebral blood flow and MAGiC T2 values.[17]

The current study showed that T2 and PD values were lower in severe stroke patients, with AUC of 0.842 and 0.692, respectively, and could be used as a diagnostic marker. In addition, we found that T2 and PD values correlated negatively with CRP levels and identified T2 and CRP values as independent risk factors for severe stroke. Given that severe stroke is often associated with an activated inflammatory response, we hypothesize a potential connection between T2 values and inflammatory conditions. However, it is premature to draw definitive conclusions without further investigation.

DCE-MRI is widely used in stroke diagnosis, with early studies showing that it could measure atherosclerotic plaques, blood–brain barrier function, and vascular and hemodynamic features.[18–20] A recent study reported convolutional neural networks, which provided better DCE-MRI efficacy in stroke diagnosis.[21] In an animal model of stroke, DCE-MRI measured post-stroke outcome, angiogenesis and vascular function.[22] In our investigation, we observed that K^{trans} was markedly increased in severe stroke patients and that combining T2 and K^{trans} showed better diagnostic accuracy for severe stroke. Furthermore, K^{trans} correlated positively with CRP levels. Similar to the T2 value, we speculate that K^{trans} might partially reflect the inflammatory condition in stroke patients. However, this hypothesis requires more comprehensive studies to gain deeper insights.

Limitations

Study limitations include 1) enrolling 197 patients who were from a single center and 2) the focus on short-term clinical outcomes of the ischemic stroke patients. As such, whether MAGiC could provide indications for patients' long-term condition is unclear.

Conclusions

Magnetic resonance imaging in ischemic stroke can be used to predict ischemic stroke severity, with T2 and PD showing potential as prediction markers. The combination of T2 and K^{trans} may provide a new assessment method for the diagnosis of severe stroke patients. This study may provide more clinical evidence for the application of MAGiC in the diagnosis of ischemic stroke.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10838478. The package includes the following files:

Supplementary Table 1. Original results of t-test and Levene analysis for TG and LDL-ch in t-test of Table 1.

Supplementary Table 2. Spearman's correlation among MAGiC and DCE-MRI parameters and the laboratory indices.

Supplementary Table 3. Box–Tidwell test for all variables in logistic regression analysis.

Supplementary Table 4. Box–Tidwell test for all variables in logistic regression analysis.

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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Serum fibroblast growth factor 19 level correlates inversely with clinical and endoscopic activity of inflammatory bowel disease

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Conflict of interest
None declared

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Abstract

Background. Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic condition with relapsing–remitting course. Diarrhea and abdominal pain are the most common IBD symptoms. Fibroblast growth factor 19 (FGF19) is an endocrine factor that inhibits hepatic bile acid production and may be used as a diagnostic marker for bile acid malabsorption.

Objectives. To assess serum FGF19 levels in active and inactive phases of IBD and find a potential correlation between FGF19 and disease activity.

Materials and methods. Fasting serum FGF19 levels were measured in 105 IBD patients (47 UC patients, 41 CD patients without previous ileocecal resection (NR-CD), 17 CD patients after ileocecal resection (IR-CD), and 17 control subjects). The disease activity was assessed using clinical, laboratory and endoscopic criteria.

Results. Inverse correlations were found between FGF19 level and intensity of diarrhea (in UC), abdominal pain intensity (in UC and IR-CD) and inflammatory markers (in UC and IR-CD). Moreover, FGF19 concentration was inversely correlated with clinical and endoscopic activity indices in UC and CD.

Conclusions. Fluctuations in FGF19 level related to clinical and endoscopic activity of UC and CD revealed a clear pattern of higher values in remission than in active disease phases. Fibroblast growth factor 19 may serve as a potential diagnostic biomarker and constitute a new therapeutic target in IBD.

Key words: disease activity, fibroblast growth factor 19, inflammatory bowel disease

Cite as
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Background

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic condition with relapsing–remitting course. The not fully understood pathogenesis of IBD encompasses genetic predisposition, immune system response disturbances and environmental factors.[1,2]

Bile acids (BAs) play a crucial role in regulating various gastrointestinal functions, including secretion, motility, immune response, intestinal mucosa integrity, and visceral sensitivity.[3] During absorption in the terminal ileum, BAs activate farnesoid X receptor (FXR) and promote the transcription of fibroblast growth factor 19 (FGF19),[4] a human factor with endocrine properties. Fibroblast growth factor 19 is secreted into the enterohepatic circulation, and after reaching the liver, it inhibits hepatic BA production.[5] Bile acid malabsorption (BAM) leads to a disturbance in FGF19 expression, resulting in elevated BA synthesis. Therefore, lower FGF19 level has been suggested as a diagnostic marker for BAM.[6]

Due to decreased FGF19 expression, BA production in the liver is enhanced, and more unabsorbed BAs enter the colon lumen, leading to BA diarrhea and abdominal pain deterioration.[7–10] In IBD patients, ileal resection is not the only factor leading to impaired FGF19 secretion. Recent research indicates that inflammatory cytokines decrease FXR expression, affecting FGF19 production. Moreover, FXR agonists exhibit anti-inflammatory effects.[11] Given the critical role of FGF19 in modulating gastrointestinal function via BA synthesis regulation and its interactions with FXR exerting an immunomodulatory effect, this factor is emerging as a potential new diagnostic and therapeutic target in IBD.

Objectives

This study aimed to assess fasting serum FGF19 levels during active and inactive phases of IBD and explore the correlations between FGF19 level and intensity of the main IBD symptoms, indices of IBD activity and inflammatory markers.

Materials and methods

Study participants

In total, 113 IBD patients were enrolled in the study, with an IBD diagnosis confirmed using histological examination. The exclusion criteria were: other bowel diseases, chronic liver diseases (except single cysts and steatosis), diabetes, body mass index (BMI) >30 kg/m^2, hyperlipidemia treatment, malignancies, alcohol dependence syndrome, and a history of abdominal surgical procedures (except appendectomy and procedures related to IBD, such as ileocecal resection). The control group consisted of 17 healthy volunteers, including 9 men and 8 women, with a median age of 28 (27–30) years and without any gastroenterological symptoms.

Eight patients were excluded from the final analyses (2 due to missing data, 2 due to outlier test results and 4 due to discrepancies between the clinical status and the results of additional tests). Among the 105 patients involved in the final analyses, 3 subgroups were distinguished: UC (n = 47), CD without ileocecal surgery in the past (NR-CD) (n = 41) and CD after ileocecal resection (IR-CD) (n = 17).

The study was approved by the Ethics Committee of Wroclaw Medical University, Poland (approval No. KB-700/2020). Written informed consent was obtained from all participants before study enrollment.

Assessment of disease activity

All enrolled patients underwent a detailed clinical interview based on a questionnaire to assess their symptoms. Stool type was assessed using the Bristol Stool Form Scale (BSFS), diarrhea was defined as having a minimum of 3 bowel movements per day, with loose or watery stools, and pain intensity was assessed using the visual analogue scale (VAS).

Standard laboratory tests of blood, urine and stool were also conducted, though colonoscopy and enterography were only undertaken in subjects with clinical indications for such examinations. The clinical and endoscopic activity of IBD was assessed based on validated scales and indices, with the Rachmilewitz index and the Mayo Endoscopic Score used in UC patients, and Crohn's disease activity index (CDAI) and the simple endoscopic score for CD (SES-CD) applied in CD patients. Patients were classified as having an inactive phase of the disease based on fecal calprotectin level lower than 200 μg/g, 0–4 points in the Rachmilewitz index and 0–2 points in the Mayo Endoscopic Score in UC patients, and CDAI score lower than 200 points and SES-CD score lower than 7 points in CD patients. All other subjects were assigned to the active subgroup. Crohn's disease patients with active changes in enterography were automatically assigned to the active phase subgroup.

In the control group, a detailed interview identified exclusion criteria, and fasting serum FGF19 and fecal calprotectin levels were tested.

Quantitative evaluation of serum FGF19 and fecal calprotectin levels

Participants provided a fasting blood sample, taken before 9 AM, and a stool sample, which were stored at –80°C until analysis. The quantitative evaluation of serum FGF19 [pg/mL] and fecal calprotectin [μg/g] levels used immunoenzymatic methods: human FGF-19 ELISA (BioVendor, Laboratorni Medicina a.s., Brno, Czech Republic) and EK-CAL (Bühlmann Laboratories, Schönenbuch, Switzerland), respectively.

Statistical analyses

Individual values were presented as numbers with percentages, mean with standard deviation (±SD), or median with 1st and 3rd quartiles (Q1–Q3). When the number of observations was below 10, the normality of the data distribution was not checked and was considered non-normal. When the number of observations was between 10 and 50, the normality of the data distribution was determined using the Shapiro–Wilk test. The Kolmogorov–Smirnov test with Lilliefors correction was used when the number of observations was equal to or greater than 50.

To compare categorical variables, the assumption of expected frequencies was assessed for values <5 in a maximum of 20% of cell fields for the χ^2 test. Pearson's χ^2 test of independence without Yates's continuity correction was used if the assumption was met. Otherwise, Fisher's exact test was used.

Quantitative variables with a normal distribution were assessed for homogeneity of variance using Levene's test. Student's t-test was then employed if there were no significant differences. Otherwise, a t-test with independent variances was conducted. The Mann–Whitney U test (MWU) was used to compare quantitative variables with non-normal distribution. Kruskal–Wallis test followed by Dunn's multiple comparisons were employed to compare quantitative variables in more than 2 groups.

Spearman's rank correlation (r) or Kendall's Tau-c correlation (Tau-c) were calculated to test associations between variables. The assumptions of normal distribution and multicollinearity required for Pearson's linear correlation were unmet. Monotonicity, the strength of monotonicity, function return, and, whenever possible, the sign of the derivative for Spearman's correlation were assessed using the original data. The involvement of tied ranks was examined, defined as the proportion of observations sharing the same rank, regardless of the number of unrelated ranks. When the involvement of tied ranks was significant (20% of tied ranks), Kendall's correlation was employed. The Bonferroni correction was incorporated into the family of hypotheses.

Results

Group characteristics

The detailed characteristics of the patient subgroups are presented in Table 1. There were no significant differences between the subgroups with respect to sex, age, BMI, and disease duration. The whole group consisted of 68 men (65%) and 37 women (35%) with a median age of 33 (27–41) years and disease duration of 63 (13–132) months.

The clinical and endoscopic disease activity indices and data on disease localization in CD patients are given in Table 1. As expected, patients with active UC had diarrhea more frequently than those with inactive UC. Patients with active CD also experienced diarrhea more often, though the difference was not statistically significant. During flares, patients with CD and UC reported higher levels of abdominal pain, though this was only statistically significant in NR-CD. Comparing the frequency of medications used in active and inactive phases, only steroids were administered more frequently during UC exacerbation (Table 1).

To verify whether other variables affected the concentration of FGF19, its levels were compared with respect to the duration of the disease and used medications. No statistically significant differences were found (Table 2,3).

Active UC patients exhibited significantly higher inflammatory parameters, lower hemoglobin levels, and decreased total cholesterol and albumin levels than inactive UC patients. Such alterations were not observed in CD subgroups. In patients with NR-CD, the only difference between the exacerbation and remission phases concerned fecal calprotectin levels. All patients also underwent testing for serum BA levels. However, no significant differences were found between active and inactive phases of the disease in particular subgroups with respect to that parameter (Table 4).

Serum FGF19 level in particular subgroups

Lenicek et al.[12] established FGF19 cutoff value below 60 pg/mL to identify patients with BAM. Therefore, the same cutoff was used in this study. Fibroblast growth factor 19 values below 60 pg/mL occurred in 16 patients, including 7 with active UC, 1 with active NR-CD, 2 with inactive NR-CD, and 6 with active IR-CD. An increased FGF19 level was found in 11 patients, including 1 with active UC, 4 with inactive UC, 4 with active NR-CD, and 2 with inactive NR-CD.

Fluctuations in FGF19 level related to the type of disease and its activity were observed. The median FGF19 level in active UC was significantly lower compared to inactive UC. However, there was no significant difference in FGF19 level between active UC and controls. Moreover, the median FGF19 level in inactive UC was higher than in the control group (Fig. 1).

No statistically significant differences in FGF19 level were observed between active and inactive NR-CD subgroups or the controls. Patients with active IR-CD had significantly lower FGF19 than those with inactive IR-CD. However, there was no difference between the subgroups and the control group. Moreover, median FGF19 in active IR-CD was significantly lower than in active and inactive NR-CD (Fig. 2).

The median serum FGF19 levels in CD with respect to disease localization and activity are presented in Table 5.

Table 1. Detailed characteristics of the studied patients' subgroups

Variable	Active UC (Group 1)	Inactive UC (Group 2)	Active NR-CD (Group 3)	Inactive NR-CD (Group 4)	Active IR-CD (Group 5)	Inactive IR-CD (Group 6)	Statistical test 1 vs 2	Statistical test 3 vs 4	Statistical test 5 vs 6
Group characteristics									
n	31	16	25	16	11	6	–	–	–
Men, n (%)	23 (74.2)	9 (56.3)	16 (64.0)	9 (56.3)	7 (63.6)	4 (66.7)	χ^2 test = 1.56 df = 1 p = 0.211	χ^2 test = 0.25 df = 1 p = 0.619	p = 1.000*
Age, median (Q1–Q3)	36 (26–40)	34 (24–46)	31 (27–34)	30 (26–40)	33 (27–45)	41 (35–47)	z = –0.06 p = 0.955**	z = –0.20 p = 0.841**	z = –1.31 p = 0.191**
Duration of the disease [months], median (Q1–Q3)	18 (2–60)	58 (13–108)	53 (12–96)	108 (27–159)	132 (108–204)	186 (120–216)	z = –1.65 p = 0.099**	z = –1.55 p = 0.121**	z = –0.76 p = 0.449**
BMI, mean ±SD	21.79 ±3.58	22.24 ±4.20	21.87 ±4.01	22.51 ±3.09	21.99 ±4.23	23.91 ±2.08	t = –0.38 df = 45 p = 0.704#	t = –0.54 df = 39 p = 0.592#	z = –1.06 p = 0.291**
Disease localization (ileitis/ileocolitis/colitis)	–	–	5/13/6	5/8/3	2/9/0	1/5/0	–	–	–
Disease activity index									
CDAI, median (Q1–Q3)	–	–	231.10 (164.66–415.60)	86.43 (42.89–110.03)	337.24 (196.29–364.96)	66.75 (31.85–199.26)	–	z = 4.26 p < 0.001**	z = 2.46 p = 0.014**
SES-CD, median (Q1–Q3)	–	–	77 (3–11)	33 (2–6)	88 (4–11)	55 (2–9)	–	t = 2.47, df = 20 p = 0.023##	z = 1.22 p = 0.221**
Rachmilewitz index, median (Q1–Q3)	9.0 (5–12)	1.5 (0–4)	–	–	–	–	z = 4.16 p < 0.001**	–	–
Mayo Encoscopic Score, median (Q1–Q3)	3 (2–3)	0 (0–1)	–	–	–	–	z = 4.47 p < 0.001**	–	–
Symptoms									
Diarrhea, n (%)	19 (61.3)	2 (12.5)	10 (40.0)	3 (18.9)	7 (63.6)	1 (16.7)	p = 0.002*	p = 0.187*	p = 0.131*
BSFS, median (Q1–Q3)	6 (5–7)	4 (3–5)	4 (4–6)	5 (4–6)	6 (5–7)	6 (4–6)	z = 3.59 p < 0.001**	t = –0.53 df = 39 p = 0.596#	z = 0.79 p = 0.427**
Number of liquid stools per week, median (Q1–Q3)	35 (3–72)	0 (0–6)	7 (1–28)	23 (1–13)	10 (6–20)	7 (5–11)	z = 3.80 p < 0.001**	z = 0.90 p = 0.367**	z = 0.51 p = 0.608**
Abdominal pain in VAS per week, median (Q1–Q3)	4.2 (1.7–5.5)	0.4 (0–3.5)	4.6 (2.6–7.3)	0 (0–0)	6.8 (4.8–7.7)	0 (0–1.2)	z = 2.56 p = 0.010**	z = 4.97 p < 0.001**	z = 2.09 p = 0.037**
Abdominal bloating, n (%)	15 (48.4)	11 (68.8)	17 (68.0)	10 (62.5)	8 (72.7)	5 (83.3)	χ^2 test = 1.77 df = 1 p = 0.813	χ^2 test = 0.13 df = 1 p = 0.717	p = 1.000*
Treatment									
Mesalamine, n (%)	31 (100)	15 (93.8)	17 (68.0)	11 (68.8)	6 (54.5)	3 (50.0)	p = 0.340*	χ^2 test = 0.00 df = 1, p = 0.959	p = 1.000*
Steroids, n (%)	23 (77.4)	3 (18.8)	12 (48.0)	3 (18.8)	5 (45.5)	0 (0)	p < 0.001*	p = 0.097*	p = 0.102*
Azathioprine, n (%)	9 (29.0)	3 (18.8)	11 (44.0)	5 (31.3)	2 (18.2)	0 (0)	p = 0.505*	χ^2 test = 0.67 df = 1, p = 0.414	p = 0.515*
Biological treatment, n (%)	1 (3.2)	2 (12.5)	2 (8.0)	3 (18.8)	0 (0)	0 (0)	p = 0.264*	p = 0.362*	–
Antibiotics, n (%)	8 (25.8)	3 (18.8)	8 (32.0)	1 (6.3)	4 (36.4)	0 (0)	p = 0.725*	p = 0.066*	p = 0.237*
Probiotics, n (%)	6 (19.4)	2 (12.5)	3 (12.0)	2 (12.5)	3 (27.3)	2 (33.3)	p = 0.697*	p = 1.000*	p = 1.000*

UC – ulcerative colitis; NR-CD – Crohn's disease without ileocecal surgery in the past; IR-CD – Crohn's disease after ileocecal resection in the past; BMI – body mass index; CDAI – Crohn's disease activity index; SES-CD – simple endoscopic score for Crohn's disease; BSFS – Bristol Stool Form Scale; VAS – visual analogue scale; Q1 – 1st quartile; Q3 – 3rd quartile; SD – standard deviation; χ^2 test – Pearson's χ^2 test of independence without Yates's continuity correction; df – degrees of freedom; *Fisher's exact test; z – value of the Mann–Whitney U test statistic; **Mann–Whitney U test; t – value of the test statistic; #Student's t-test; ##t-test with independent variance estimation. The Bonferroni correction was incorporated into the family of hypotheses. The statistical significance level was set in group characteristics part at p < 0.013, in disease activity index part at p < 0.025, in symptoms part at p < 0.010, and in treatment part as p < 0.008.

Table 2. Median serum FGF19 level depending on disease duration

Median serum FGF19 [pg/mL] level depending on disease duration			
<2 years	2–10 years	>10 years	p-value (Kruskal–Wallis test)
160.70 (101.00–223.55)	125.12 (70.50–175.50)	145.30 (81.10–181.85)	p = 0.136, H = 3.9, df = 2

FGF19 – fibroblast growth factor 19; H – value of the test statistic of the Kruskal–Wallis test; df – degrees of freedom.

Table 3. Median serum FGF19 level depending on used medications

Median serum FGF19 [pg/mL] level depending on medication taken		
Medication		p-value (Mann–Whitney U-test)
With steroids 120.30 (66.84–163.65)	Without steroids 164.68 (97.56–200.59)	z = –2.44, p = 0.015
With antibiotics 107.80 (52.80–199.80)	Without antibiotics 159.96 (91.41–187.90)	z = –1.63, p = 0.103
With probiotics 133.80 (63.69–181.85)	Without probiotics 158.58 (88.45–191.28)	z = –1.28, p = 0.199
With vitamin D 147.50 (108.00–181.85)	Without vitamin D 145.90 (81.10–187.90)	z = 0.42, p = 0.677

FGF19 – fibroblast growth factor 19; z – value of the Mann–Whitney U test. The Bonferroni correction was incorporated into the family of hypotheses. The statistical significance level was set at $p < 0.013$.

Table 4. Laboratory test results of the studied patients' subgroups

Variable	Active UC (Group 1)	Inactive UC (Group 2)	Active NR-CD (Group 3)	Inactive NR-CD (Group 4)	Active IR-CD (Group 5)	Inactive IR-CD (Group 6)	Statistical test 1 vs 2	Statistical test 3 vs 4	Statistical test 5 vs 6
Hemoglobin [g/dL], mean ±SD	11.6 ±1.6	14.1 ±1.9	12.3 ±2.5	13.2 ±2.2	12.5 ±1.9	14.8 ±1.4	t = –4.73 df = 45 p < 0.001#	t = –1.25 df = 39 p = 0.219#	z = –2.26 p = 0.024*
CRP [mg/L], median (Q1–Q3)	16.6 (5.2–46.8)	1.8 (0.9–6.9)	8.0 (4.8–35.0)	4.2 (1.3–9.5)	78.0 (2.3–22.2)	1.2 (0.8–1.9)	z = 3.54 p < 0.001*	z = 2.07 p = 0.038*	z = 2.76 p = 0.006*
Fecal calprotectin [µg/g], mean ±SD	1528.7 ±673.5	78.9 ±61.2	1499.7 ±678.7	78.4 ±48.5	730.8 ±575.6	68.1 ±29.1	t = 10.86 df = 26 p < 0.001##	t = 9.97 df = 23 p < 0.001##	z = 1.91 p = 0.056*
Total cholesterol [mg/dL], median (Q1–Q3)	135.0 (122.0–168.0)	192.5 (169.0–228.5)	150.0 (139.0–187.0)	171.0 (138.5–197.0)	120.0 (99.0–163.0)	172.5 (159.0–198.0)	z = 3.45 p < 0.001*	t = –0.76 df = 39 p = 0.452#	z = –2.26 p = 0.024*
LDL [mg/dL], mean ±SD	85.1 ±27.7	115.4 ±36.3	86.4 ±30.5	97.6 ±40.3	62.4 ±24.5	97.0 ±23.9	t = –3.16 df = 44 p = 0.003#	t = –1.01 df = 39 p = 0.318#	z = –2.46 p = 0.014*
HDL [mg/dL], mean ±SD	41.3 ±13.9	53.6 ±15.0	51.9 ±13.5	55.4 ±16.9	43.1 ±12.7	54.0 ±11.9	t = –2.69 df = 44 p = 0.009#	t = –0.73 df = 39 p = 0.469#	z = –1.46 p = 0.144*
Triglycerides [mg/dL], median (Q1–Q3)	104.5 (68.0–125.0)	88.5 (65.0–122.0)	88.0 (70.5–105.5)	80.0 (66.5–115.5)	99.0 (62.0–108.0)	110.0 (108.0–136.0)	z = 1.02 p = 0.310*	z = 0.08 p = 0.934*	z = –1.71 p = 0.087*
Albumin [mg/dL], mean ±SD	3.5 ±0.6	4.3 ±0.5	3.9 ±0.5	4.1 ±0.5	3.6 ±0.5	4.3 ±0.2	t = –4.75 df = 44 p < 0.001#	t = –1.49 df = 39 p = 0.143#	z = –2.67 p = 0.008*
Bile acids [µmol/L], median (Q1–Q3)	1.3 (1.0–2.7)	1.7 (1.0–2.8)	1.6 (1.2–2.5)	1.8 (1.3–3.1)	2.5 (1.4–4.7)	2.6 (1.9–3.6)	z = –0.45 p = 0.659*	z = –0.58 p = 0.566*	z = 0.00 p = 1.000*

UC – ulcerative colitis; NR-CD – Crohn's disease without ileocecal surgery in the past; IR-CD – Crohn's disease after ileocecal resection in the past; CRP – C-reactive protein; LDL – low-density lipoprotein; HDL – high-density lipoprotein; SD – standard deviation; Q1 – 1st quartile; Q3 – 3rd quartile; z – value of the Mann–Whitney U test statistic; *Mann–Whitney U test; t – value of the test statistic; df – degrees of freedom; #Student's t-test; ##t-test with independent variance estimation. The Bonferroni correction was incorporated into the family of hypotheses. The statistical significance level was set at $p < 0.006$.

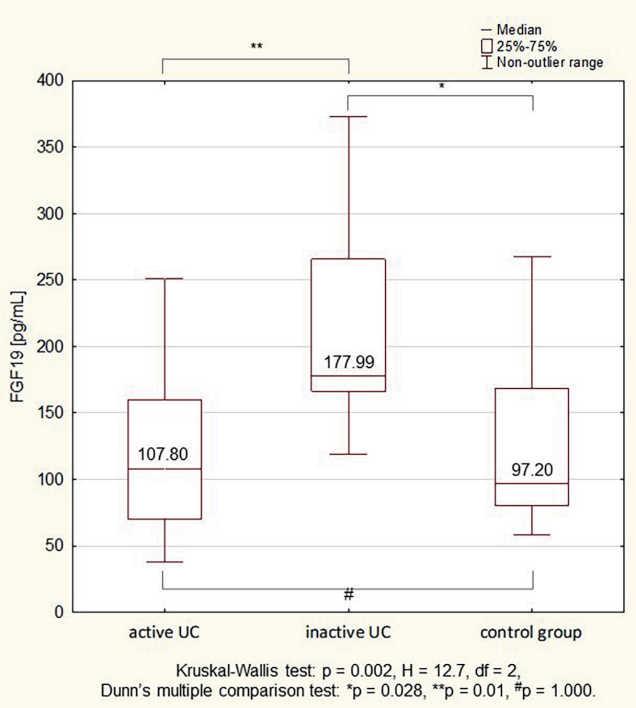

Fig. 1. Median fibroblast growth factor 19 (FGF19) levels in patients with ulcerative colitis (UC)

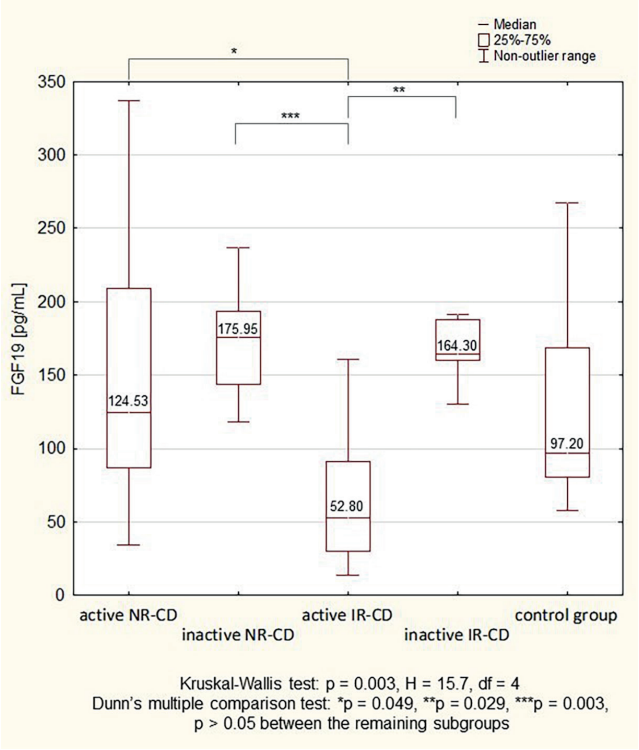

Fig. 2. Median fibroblast growth factor 19 (FGF19) levels in patients with Crohn's disease (CD)

Table 5. Median serum FGF19 level in Crohn's disease patients with respect to disease localization of the disease and activity

Crohn's disease localization and activity	n	FGF19 [pg/mL] median (Q1–Q3)
Active ileitis	8	76.64 (32.35–164.70)
Inactive ileitis	6	136.44 (110.80–175.50)
Active ileocolitis	21	140.81 (78.10–165.30)
Inactive ileocolitis	13	164.05 (97.56–186.23)
Active colitis	6	114.94 (83.70–394.80)
Inactive colitis	3	336.80 (83.90–510.40)

FGF19 – fibroblast growth factor 19; Q1 – 1st quartile; Q3 – 3rd quartile.

Correlations of FGF19 level with specific variables

A strong inverse correlation was observed between FGF19 and C-reactive protein (CRP) levels in the IR-CD patient subgroup, though not in the NR-CD nor UC subgroups. Regarding fecal calprotectin, FGF19 level was inversely correlated with that marker in UC patients, while no correlation was identified in CD patients (Table 6).

Inverse correlations between fasting serum FGF19 and measures of diarrhea severity, including BSFS and the number of liquid stools per week, were found in the UC patients. However, no correlation between FGF19 level and diarrhea severity score was demonstrated in CD patients. Interestingly, abdominal pain intensity correlated inversely with FGF19 level in UC and IR-CD patients (Table 6).

Inverse correlations were found between FGF19 and clinical and endoscopic activity indices, including the Rachmilewitz activity index (Fig. 3), the Mayo Endoscopic Score (Fig. 4), CDAI (Fig. 5), and SES-CD (Fig. 6, Table 7).

Discussion

The results of the current study demonstrate fluctuations in FGF19 levels related to the clinical and endoscopic activity of UC and CD, with a clear pattern of lower values in the active phase than in remission. Previous studies have reported that in UC patients serum FGF19 level was elevated[13] or in a range[12] comparable to healthy controls. Another study showed similar fluctuations in FGF19 level based on UC activity, with higher FGF19 in remission and lower in active disease, but the differences were not statistically significant.[14] The larger sample size in the current study probably contributed to achieving statistical significance.

Decreased FGF19 in active UC correlated inversely with fecal calprotectin as a marker of intestinal inflammation. Pro-inflammatory cytokines, secreted during flares, disrupt the regulation of the FXR-FGF19 axis through inhibition of the expression of gene encoding apical sodium-dependent bile acid transporter (ASBT), which is responsible for the active BA absorption.[15] A previous study

Table 6. Correlation of serum FGF19 level with individual variables

Variables	UC patients		NR-CD patients		IR-CD patients	
	correlation coefficient	p-value	correlation coefficient	p-value	correlation coefficient	p-value
FGF19 and BSFS	Tau-c = –0.24	0.022	Tau-c = –0.03	0.759	Tau-c = –0.15	0.392
FGF19 and number of liquid stools per week	Tau-c = –0.23	0.026	Tau-c = –0.03	0.711	Tau-c = –0.22	0.230
FGF19 and abdominal pain intensity	Tau-c = –0.22	0.043	Tau-c = –0.08	0.462	Tau-c = –0.48	0.008
FGF19 and CRP	r = –0.25	0.098	r = –0.22	0.166	r = –0.59	0.012
FGF19 and fecal calprotectin	r = –0.34	0.046	Tau-c = –0.17	0.176	r = –0.47	0.088

Involvement of tied ranks in UC patients: FGF19 (0%), BSFS (98%), number of liquid stools per week (60%), abdominal pain intensity (47%), CRP (0%), fecal calprotectin (19%); in NR-CD patients: FGF19 (0%), BSFS (100%), number of liquid stools per week (61%), abdominal pain intensity (41%), CRP (0%), fecal calprotectin (42%); in IR-CD patients: FGF19 (0%), BSFS (88%), number of liquid stools per week (56%), abdominal pain intensity (41%), CRP (0%), fecal calprotectin (0%). UC – ulcerative colitis; NR-CD – Crohn's disease without ileal surgery in the past; IR-CD – Crohn's disease after ileocecal resection in the past; FGF19 – fibroblast growth factor 19; BSFS – Bristol Stool Form Scale, CRP – C-reactive protein; r – Spearman's rank correlation coefficient; Tau-c – Kendall Tau-c correlation coefficient.

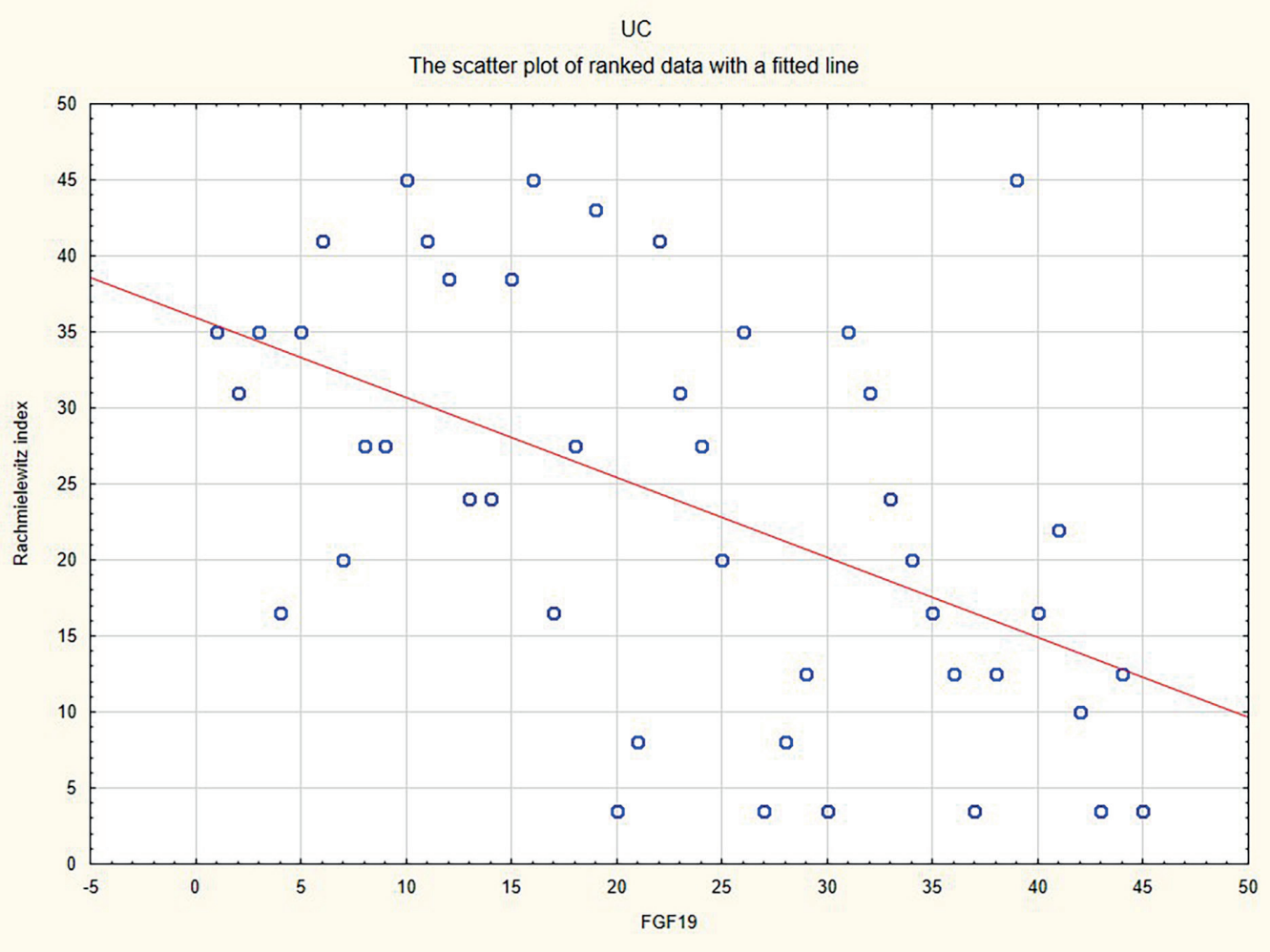

Fig. 3. An inverse correlation between serum fibroblast growth factor 19 (FGF19) and Rachmilewitz index in ulcerative colitis (UC) patients; Tau-c = –0.37, $p < 0.001$

Tau-c – Kendall Tau-c correlation coefficient.

demonstrated reduced ASBT expression in descending colon tissue samples from patients with active UC compared to healthy individuals.[16] Moreover, inflammatory cytokines may directly diminish FXR activation, subsequently decreasing FGF19 expression.[11]

Another factor affecting FGF19 fluctuations may be related to IBD treatment, in particular steroids used for flares, with available data suggesting that steroid therapy is associated with decreased FXR activation.[11] Potentially, the enhancement of FGF19 production in IBD remission

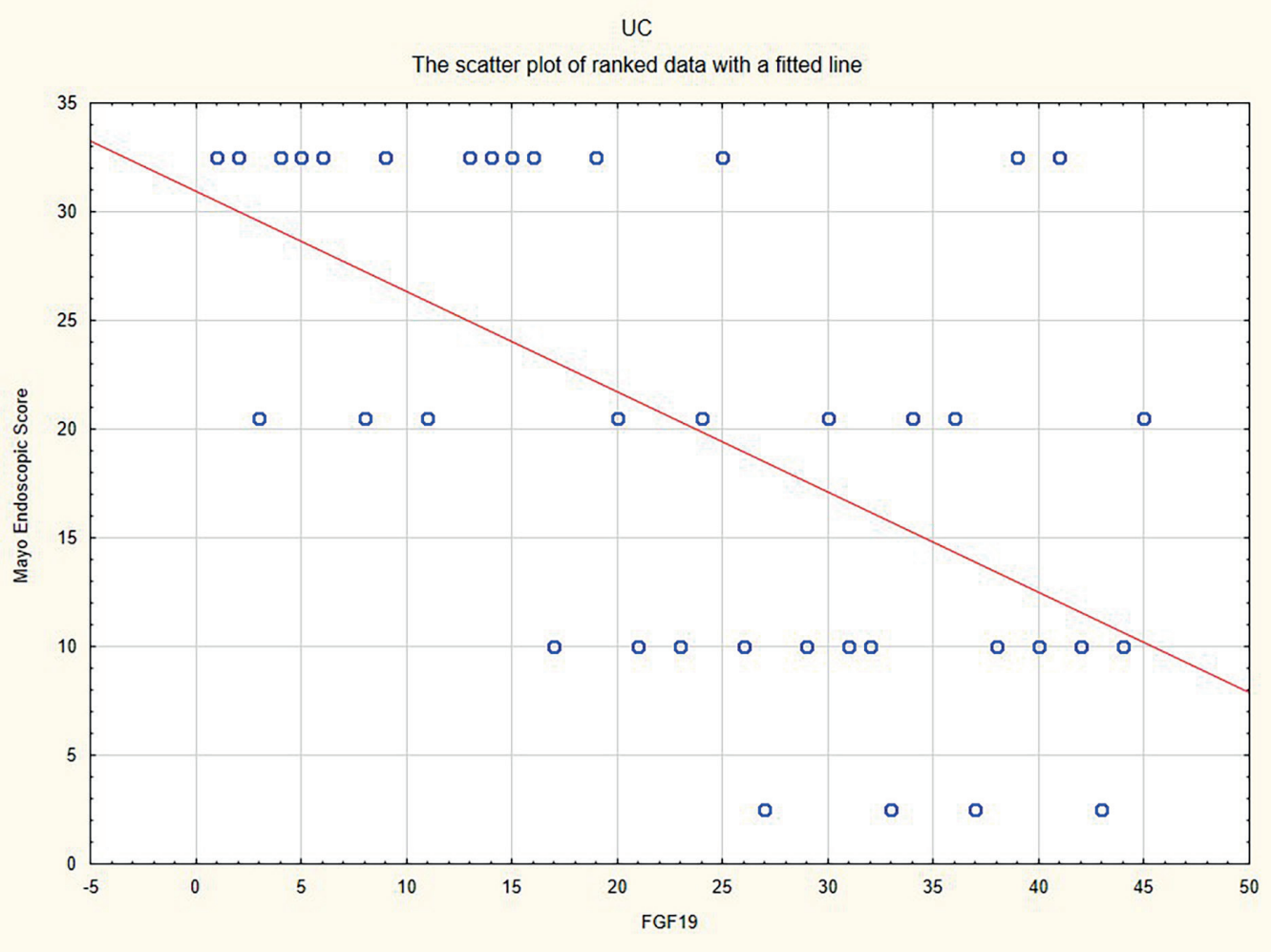

Fig. 4. An inverse correlation between serum fibroblast growth factor 19 (FGF19) and Mayo Endoscopic Score in ulcerative colitis (UC) patients; Tau-c = –0.43, $p < 0.001$

Tau-c – Kendall Tau-c correlation coefficient.

may be influenced by a post-steroid therapy effect since corticosteroids, besides their anti-inflammatory effect, enhance BA absorption and stimulate ASBT expression.[17] However, steroid use was not associated with lower FGF19 concentrations in the current study.

Furthermore, deficiency of nutritional components and vitamins in the diet can lead to reduced activation of receptors other than FXR that participate in the regulation of FGF19 expression, such as vitamin D receptor, retinoid X receptor and pregnane X receptor.[18] In addition, the shorter transit time during flares and the consequent malabsorption may also contribute to BAM.[14]

In this study, 26.7% of patients with active UC had FGF19 level below 60 pg/mL, corresponding to BAM. Lower FGF19 level can lead to enhanced BA production and symptom aggravation.[19] Indeed, we demonstrated a negative correlation between FGF19 level, BSFS, number of liquid stools, and abdominal pain intensity.

The analysis of FGF19 in CD patients was more complex due to the different disease localizations and anatomical alterations caused by ileocecal resection.

In patients with NR-CD, we did not observe fluctuations in FGF19 level related to disease activity. Moreover, this subgroup did not demonstrate any correlation between FGF19 level and inflammatory parameters or the intensity of the main symptoms. Most NR-CD patients had ileocolonic localization of the disease, as in the study by Wilson et al.,[20] who also reported no significant difference in FGF19 level between active and inactive NR-CD subjects.

Based on previous reports that CD patients with colitis only had comparable FGF19 level with healthy controls and showed no fluctuations in FGF19 level depending on disease activity,[7,12] colonic involvement in patients with NR-CD could affect the results.

When analyzing the FGF19 level in relation to disease localization, the lowest concentrations were observed in ileitis, with a trend towards lower values during the active phase. Previously, reduced ASBT expression was shown in ileal tissue samples from CD patients compared to healthy controls.[16] Earlier reports also found that CD patients with ileitis or after ileocecal resection were

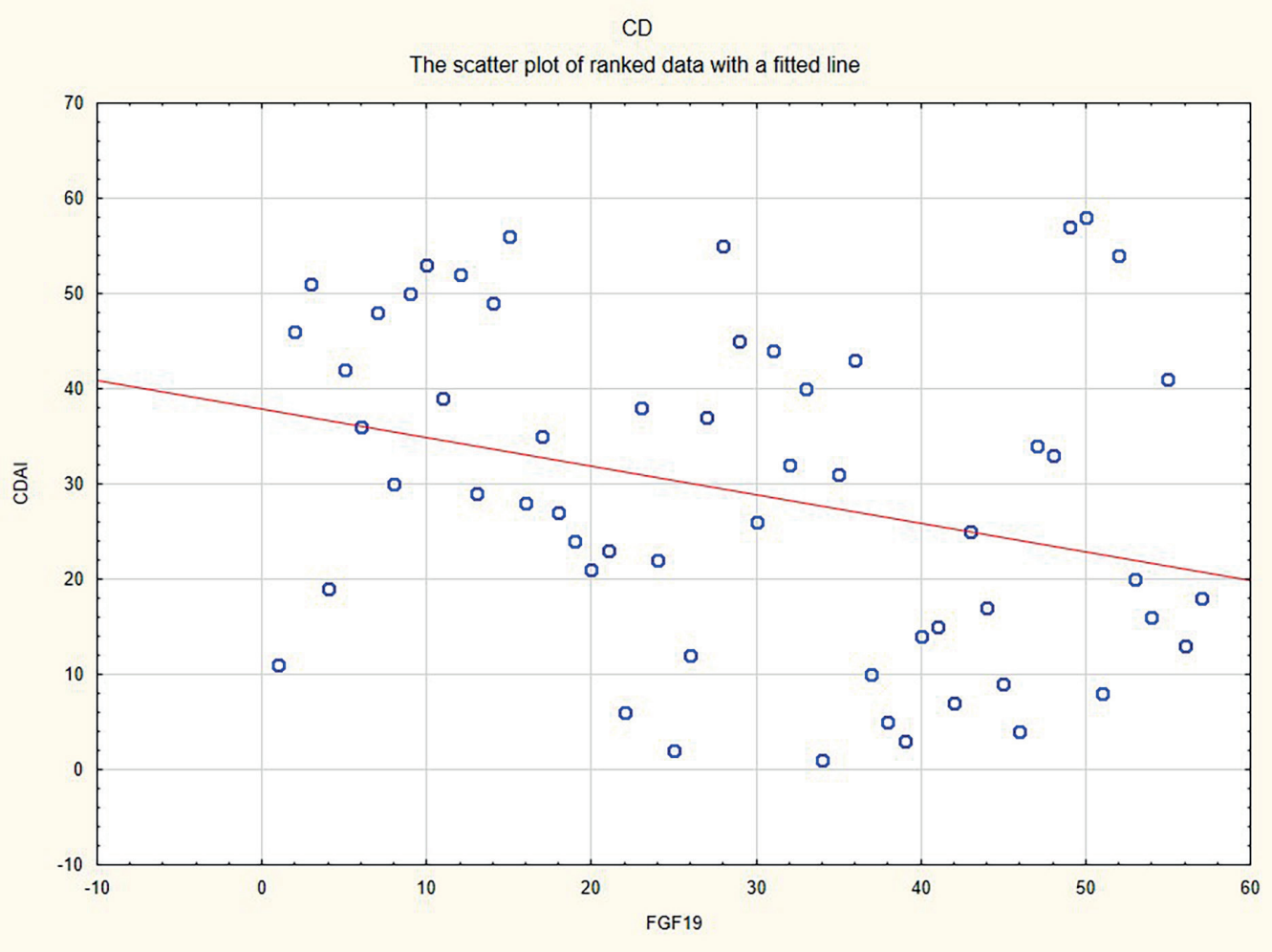

Fig. 5. An inverse correlation between serum fibroblast growth factor 19 (FGF19) and Crohn's disease (CD) activity index in CD patients; r = –0.29, p = 0.026
r – Spearman's rank correlation coefficient.

characterized by lower FGF19 levels,[12,13,21] consistent with our results.

In the current study, the lowest FGF19 levels were observed in active IR-CD patients, but patients with inactive IR-CD were characterized by higher FGF19 levels. This finding suggests that ileocecal resection is not the only determinant of reduced FGF19 level. The process of active inflammation seems to be important in this case. In that subgroup, we observed a strong negative correlation of FGF19 with CRP. Lyutakov et al.[22] did not confirm a correlation between FGF19 and CRP in IBD patients. However, Nolan et al.[7] found a negative correlation between serum FGF19 and CRP in NR-CD patients with ileitis. The evidence confirms that patients with active IBD are more likely to have BAM than those in remission.[23] In this study, 54.5% of patients with active IR-CD had FGF19 values pointing to BAM.

Previous studies have shown inverse correlations between FGF19, BSFS and stool frequency among patients with CD.[7,22] However, such a correlation was not found in the current study, probably due to the limited sample size. Although FGF19 level was related to disease activity in the examined IR-CD patients, the intensity of diarrhea in those with active and inactive disease was not significantly different. Notably, an inverse correlation was also shown between FGF19 level and abdominal pain intensity.

Alongside the fluctuations in FGF19 level associated with IBD activity, we also demonstrated inverse correlations between FGF19 level and clinical and endoscopic activity indicators. No previous studies have confirmed an association between FGF19 level and disease activity using validated scales, including the Mayo Endoscopic Score, Rachmilewitz index[14,22] and CDAI.[22]

Fibroblast growth factor 19, as a product of FXR activation, can serve as a marker of FXR activity,[13] with the results of recent studies pointing to an immunomodulatory effect of FXR activation.[24–26] The association of FGF19 level with disease activity, inflammatory markers and primary symptoms may suggest potential new diagnostic markers and a novel therapeutic target of FGF19 analogs or FXR agonists. The results of preliminary studies with 2 FGF19 analogs, aldafermin and M52, are encouraging.[25,27–31]

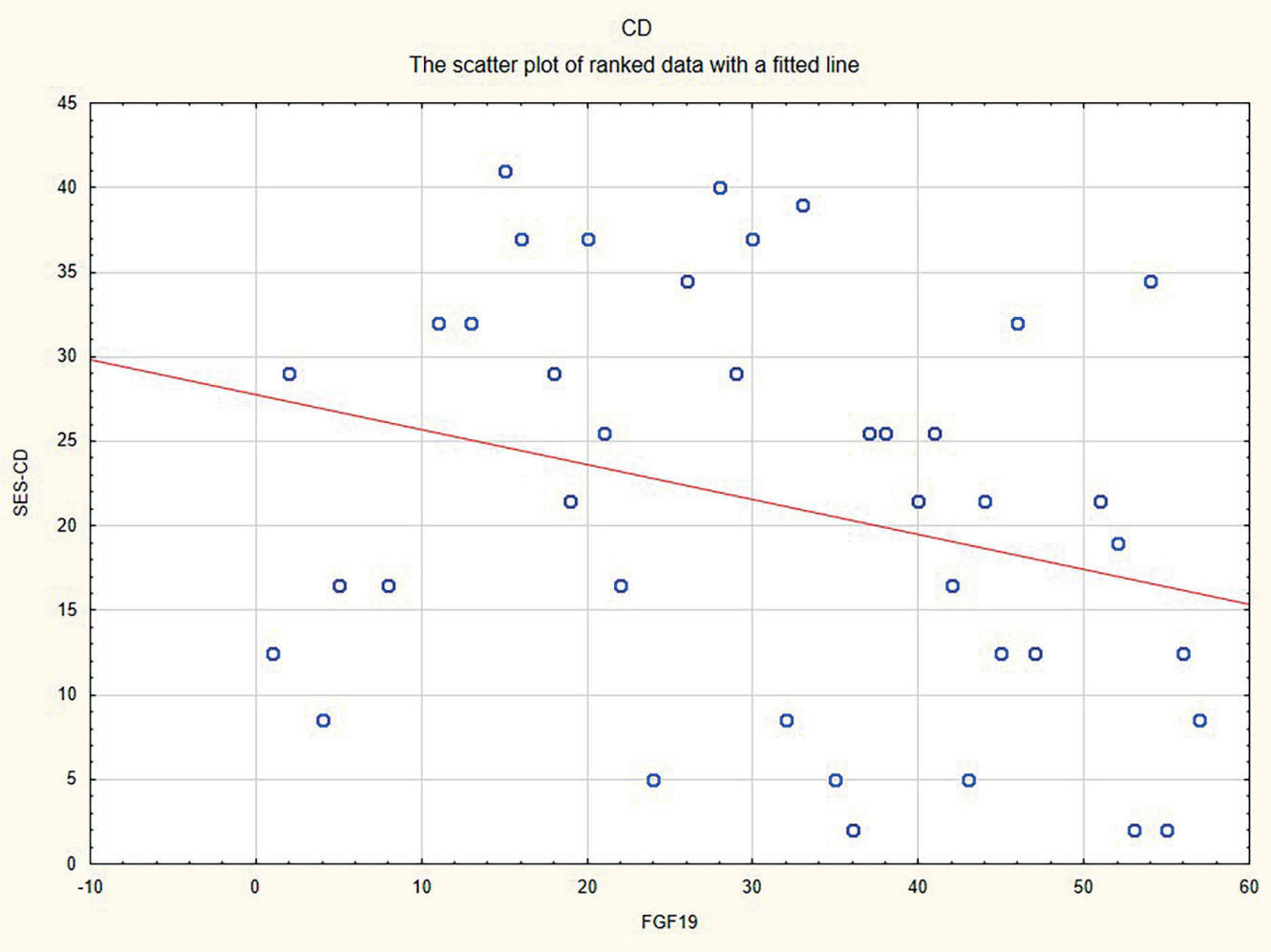

Fig. 6. An inverse correlation between serum fibroblast growth factor 19 (FGF19) and simple endoscopic score for Crohn's disease (CD) in CD patients; Tau-c = –0.23, p = 0.038

Tau-c – Kendall Tau-c correlation coefficient.

Table 7. Correlation of serum FGF19 level with clinical and endoscopic activity indices

Variables	Correlation coefficient	p-value
FGF19 and Rachmilewitz index	Tau-c = –0.37	<0.001
FGF19 and Mayo Endoscopic Score	Tau-c = –0.43	<0.001
FGF19 and CDAI	r = –0.29	0.026
FGF19 and SES-CD	Tau-c = –0.23	0.038

Involvement of tied ranks in patients with ulcerative colitis: FGF19 (0%), Rachmilewitz index (82%), Mayo Endoscopic Score (100%); in patients with Crohn's disease: FGF19 (0%), CDAI (0%), SES-CD (88%); CDAI – Crohn's disease activity index; SES-CD – simple endoscopic score for Crohn's disease; r – Spearman's rank correlation coefficient; Tau-c – Kendall Tau-c correlation coefficient.

Limitations

Among the limitations of the current study was the relatively small sample size of particular subgroups, especially patients with CD after ileocecal resection. However, the FGF19 level in this group was analyzed separately in patients in remission and flare for the first time. Hence, we indicated that the disease activity is an important factor affecting FGF19 expression apart from ileocecal resection. Moreover, a detailed evaluation of the exact length of resected ileum was unavailable. The number of subjects in the remaining subgroups was comparable to other studies.[7,13,14,20] Another limitation of this study was its cross-sectional design.

A substantial drawback of the study is that no additional tests were performed to confirm the existence of suspected BAM. However, while interpreting the current results, we relied on the recently reported cutoff value of FGF19 below 60 pg/mL to identify BAM in IBD patients (test sensitivity and specificity of 80% and 65%, respectively).[12] The designated cutoff was confirmed by Lyutkaov et al.[32] Regarding the novelty of the study, this is the first report on the inverse correlation between FGF19 level and validated scales of IBD activity.

Conclusions

The current results confirm an association between clinical and endoscopic IBD activity and serum FGF19 level. Local intestine inflammation in UC, reflected by fecal calprotectin level, significantly impacted FGF19 expression. Lower values of FGF19 were associated with the severity of diarrhea and abdominal pain. Regarding CD, FGF19 fluctuations related to disease activity were only observed in IR-CD patients. Higher FGF19 values in inactive compared to active IR-CD indicate that inflammation is a critical factor influencing FGF19 level. Assessing FGF19 can help identify patients who could benefit from sequestrant therapy as a treatment for BAM and a marker of disease activity. Furthermore, novel medications targeting the FXR-FGF19 pathway could have potential anti-inflammatory effects. Further studies exploring the efficacy and safety of FXR agonists and FGF19 analogs in intestinal inflammation are awaited.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10690921. The package includes the following files:

Supplementary Data 1. Median FGF19 levels in subgroups of UC. Description of statistical analysis.

Supplementary Data 2. Median FGF19 levels in patients with CD). Description of statistical analysis.

Supplementary Data 3. Detailed characteristics of the patient subgroups. Description of statistical analysis.

Supplementary Data 4. Median serum FGF19 levels depending on disease duration and medications used. Description of statistical analysis.

Supplementary Data 5. Laboratory test results of the patient subgroups. Description of statistical analysis.

Supplementary Data 6. Correlation between serum FGF19 level and individual variables. Scatter plots.

Supplementary Data 7. Correlation between serum FGF19 level and clinical and endoscopic activity indices. Scatter plots.

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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Anti-inflammatory effect of echinacoside in collagen-induced arthritis via Nrf2/Drp1 pathway

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Conflict of interest
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Abstract

Background. Oxidative damage plays an important role in the progression of rheumatoid arthritis (RA). Emerging research evidence suggests that natural antioxidants may effectively ameliorate this disease.

Objectives. To investigate the therapeutic effect of echinacoside (ECH) in a collagen-induced arthritis (CIA) mouse model and thus elucidate the underlying molecular mechanism in RA.

Materials and methods. Collagen-induced arthritis mice were intraperitoneally administered 1% dimethyl sulfoxide (DMSO) (control) or 0.6 mg of ECH every other day for 1 month. Arthritis scores and the number of affected paws were assessed. On day 60, mice were euthanized, synovial tissue specimens were obtained, and serum interleukin (IL)-6 and IL-1β expression levels were measured. Mitochondrial morphologies, reactive oxygen species (ROS) content, expression of dynamin-related protein 1 (Drp1), IL-6, nod-like receptor protein 3 (NLRP3), kelch-like ECH-associated protein 1 (Keap1), and nuclear factor-erythroid-2-related factor 2 (Nrf2) contents in synovium were analyzed and compared between DMSO- and ECH-treated CIA mice.

Results. Following ECH treatment, mitochondria of CIA-induced mice were found to be elongated, while their arthritis scores, inflammation and the number of affected paws, and the expression levels of Drp1, NLRP3, IL-6, ROS, and Keap1 were all found to be significantly reduced. Conversely, the level of antioxidant factor Nrf2 was found to be elevated. Further, mitochondrial fission was found to be inhibited in synovial tissues.

Conclusions. Our findings validate the therapeutic efficacy of ECH in the CIA mouse model. Echinacoside may suppress oxidative stress and inhibit inflammation by regulating the Nrf2/Drp1 pathway, thus supporting its utility in the treatment of RA.

Key words: rheumatoid arthritis, antioxidant effect, Drp1 protein, echinacoside, mitochondrial fission

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and bone deformities in multiple joints.[1] Despite significant advances in RA therapy, the functional disability rate remains high and continues to pose a significant threat to human health. Studies have shown that increased oxidative damage in synovial tissue plays a pivotal role in the progression of RA,[2] and increasing evidence suggests that natural antioxidant supplementation may have the potential to inhibit the progression of synovitis, and thereby serve as an effective therapeutic intervention in RA.

Oxidative damage in synovial tissues, induced by the excessive production of reactive oxygen species (ROS), plays a critical role in the progression of RA. An increased ROS content activates the release of inflammatory mediators in joints, which subsequently exacerbates the local proliferation of synovial tissues, thus eliciting an inflammatory response.[3] Being highly dynamic organelles, mitochondria are not only the major sources of ROS production but also the prime targets of their deleterious effects. Mitochondria constantly cycle through fusion and fission events and form net-like structures to balance their morphology and functions.[4,5] Studies have shown that excessive mitochondrial fission leads to the destruction of net-like structures, which in turn increases ROS production, resulting in oxidative damage. Moreover, increased mitochondrial fission has been implicated in a multitude of diseases.[6,7] A previous study has demonstrated increased fragmentation, an abnormal mitochondrial crest, and a damaged mitochondrial network structure in RA synovium.[8,9] Furthermore, our previous study established that dynamin-related protein 1 (Drp1 or DNM1L) is crucial for mitochondrial fission and plays an important role in the pathogenesis of RA. Finally, previous research also demonstrated that disease progression in RA could be significantly suppressed by inhibiting Drp1-mediated oxidative stress in synovium.[9]

In recent years, an increasing number of studies focused on the antioxidant role of nuclear factor erythroid 2 related factor 2 (Nrf2) in cellular defense against mitochondrial oxidative stress.[10–13] Studies have shown that Nrf2 activation, in response to increased oxidative stress, decreases Drp1-mediated mitochondrial fission and contributes to mitochondrial hyperfusion.[14] Moreover, knocking out the *Nrf2* gene resulted in reduced activity of mitochondrial respiratory chain complex I and mitochondrial fission.[15–17] Interestingly, mitoquinone (MitoQ), a mitochondria-targeted antioxidant, was shown to exert a protective effect by inhibiting Nrf2/Drp1-mediated mitochondrial fission.[11] Taken together, these reports highlight the quintessential role of Nrf2 in the maintenance of mitochondrial fission/fusion balance and the cytoprotective response against oxidative stress.

Echinacoside (ECH), a naturally occurring phenylethanoid glycoside (PhG), is the main component of the herb cistanche *(Cistanches Herba)*. Echinacoside has also been shown to possess different pharmacological activities, including neuroprotection, anti-tumor, antioxidant, anti-inflammatory, and immunomodulatory effects.[18–20] Furthermore, it was shown to have therapeutic effects in various cell cultures and disease models of Parkinson's disease and cancer.[18,21–26] A recent study demonstrated that ECH significantly inhibited Drp1 translocation into mitochondria, and regulated mitochondrial dynamics, which in turn reduced ROS production, resulting in the alleviation of neuroinflammation in rats with chronic cervical spinal cord compression.[27] However, the therapeutic effects of ECH in RA have not been completely explained yet.

Objectives

In this study, we examined the effects of ECH in a collagen-induced arthritis (CIA) mouse model and elucidated the underlying molecular mechanism in the pathogenesis of RA.

Materials and methods

Reagents

Bovine type II collagen (cat. No. 20022) and incomplete Freund's adjuvant (IFA; cat. No. 7002) were procured from Chondrex, Inc. (Woodinville, USA). Freund's complete adjuvant (FCA) (cat. No. F5881) and dimethyl sulfoxide (DMSO) (cat. No. D2650) were purchased from Sigma-Aldrich (St. Louis, USA). Echinacoside (cat. No. B115837) was purchased from Aladdin (Shanghai, China). The specific antibodies anti-kelch-like ECH-associated protein 1 (Keap1) monoclonal antibody (cat. No. 60027-1-Ig) and anti-Nrf2 polyclonal antibody (cat. No. 16396-1-AP) were procured from Proteintech Group, Inc. (Rosemont, USA). Anti-Drp1 rabbit polyclonal antibody (cat. No. GB11855), anti-NLRP3 (nod-like receptor protein 3) rabbit polyclonal antibody (cat. No. GB11300), anti-interleukin (IL)-6 rabbit polyclonal antibody (cat. No. GB11117) were purchased from Servicebio Technology Co., Ltd. (Wuhan, China). IL-6 Mouse Uncoated enzyme-linked immunosorbent assay (ELISA) Kit (cat. No. 88-7064) and IL-1β ELISA kit (cat. No. 88-7013) were procured from Invitrogen (Waltham, USA). ROS Fluorescent Probe-Dihydroethidium (DHE) was purchased from Vigorous Co., Ltd. (Beijing, China). The BCA protein assay kit (cat. No. P0010S) was purchased from Beyotime Biotechnology (Shanghai, China).

Study design and sample size

Based on experience, the CIA mouse model was established and then randomly divided into control (n = 8) and ECH treatment (n = 6) groups. The sample size was

determined based on our previous experience. Arthritis scores, histological changes, changes in blood and local joint inflammatory factors, and changes in local joint mitochondrial morphology and expression of antioxidant factors were observed in both groups.

Inclusion and exclusion criteria

Seven-week-old male DBA/1 mice, purchased from Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China), were housed in a specific pathogen-free facility with free access to food and water, as described previously.[9] Initially, the mice were immunized with an intradermal injection of 200 μg/mouse bovine type II collagen (emulsified with an equal volume of FCA) at the tail base. On day 21, mouse immunization was boosted at a different location with an intradermal injection of 200 μg/mouse bovine type II collagen (emulsified with an equal volume of IFA). From day 22, the mice began to develop symptoms, which presented as swollen joints and slow movement, and by day 27, mice with successful model induction were included in the experiment, and mice with failed induction were excluded.

Randomization, blinding and outcome measures

On day 28, the mice were randomly assigned to intraperitoneal injections of DMSO (vehicle, n = 8) or ECH (0.6 mg/mouse, n = 6) once every other day for 1 month. The clinical score was assessed by 2 independent, blinded observers. On day 60, the mice were sacrificed, and whole knee joints and blood samples were collected. The animal study was approved by the Ethics Committee of Shaanxi University of Chinese Medicine (Xianyang, China; approval No. SUCMDL20210308017).

Clinical and histological assessment of arthritis

The clinical scoring system, "arthritis score" (each paw was ranging from 0 to 4), was employed to assess the severity of arthritis as described previously, with a maximum score for each mouse of 16.[9] On day 60, the mice were euthanized, and their whole knee joint tissue sample was collected. Subsequently, the tissue specimens were fixed in 10% paraformaldehyde overnight and embedded in paraffin. Then, they were stained using hematoxylin and eosin (H&E) and toluidine blue. Histopathological analyses were performed to assess cartilage damage and bone erosion.

Transmission electron microscopy analysis

Synovial tissue specimens were fixed in glutaraldehyde at 4°C for 2 h. Then, the specimens were post-fixed with 1% citric acid-0.1M phosphate buffer (PB) for 2 h at room temperature. Before embedding, the tissue specimens were dehydrated and cleared. Ultra-thin sections (70 nm) double stained with uranylacetate and lead citrate were directly examined using transmission electron microscopy (TEM) (TEM HT7700; Hitachi High Technologies America, Inc., Schaumburg, USA).

Immunohistochemical (IHC) analysis

Synovial tissue sections were fixed in 10% paraformaldehyde overnight, embedded in paraffin and sectioned. Then, samples were incubated with 3% H_2O_2 for 10 min and heated with antigen retrieval solution. After blocking non-specific binding sites with 5% bovine serum albumin (BSA) (cat. No. AR0004; Boster Biological Engineering Co., Ltd., Wuhan, China) at 37°C for 10 min, the sections were probed using anti-Drp1 antibody (1:200), anti-IL-6 antibody (1:200) and anti-NLRP3 antibody (1:100) at 4°C overnight. Subsequently, the sections were incubated with the secondary antibody, horseradish peroxidase (HRP) goat anti-rabbit IgG antibody (1:2000) (cat. No. AS014; ABclonal Inc., Woburn, USA) for 1 h at room temperature, and visualized using diaminobenzidine. Images were obtained under a light microscope (Olympus Corp., Tokyo, Japan).

Enzyme-linked immunosorbent assay

The blood was obtained when the mice were sacrificed on day 60 and clotted at room temperature for 2 h to obtain serum. IL-6 and IL-1β in the serum were measured according to the manufacturer's protocol (cat. No. 88-7013, 88-7064; Invitrogen). Finally, the absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, USA).

Reactive oxygen species analysis

Another portion of synovial tissue was placed in liquid nitrogen and then cut into frozen sections. The cellular ROS content in crystalline synovial membrane sections was determined with probe-DHE according to the manufacturer's protocol. In mitochondria, when DHE gets oxidized by superoxide, it emits bright red fluorescence. Briefly, DHE was diluted to a final concentration of 20 μM with a serum-free medium. Then, the cell suspension was incubated with DHE at 37°C in the dark for 30 min, and nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI). The images were acquired using an inverted fluorescence microscope (model IX-53: Olympus Corp., Tokyo, Japan).

Immunofluorescence staining

Nuclear factor-erythroid-2-related factor 2 accumulation and Keap1 expression were analyzed with immunofluorescence. Briefly, synovial sections were fixed

with 10% paraformaldehyde overnight and embedded in paraffin before sectioning. Then, samples were incubated in a blocking solution (containing Triton X-100 and 5% BSA) for 1 h at room temperature. After washing, the cells were incubated with anti-Nrf2 antibody (1:50) and anti-Keap1 antibody (1:50) at 4°C overnight. They were then incubated with Cy3-conjugated secondary antibody (1:500) for 2 h and counter-stained with DAPI. The fluorescence signals were evaluated using an standing fluorescence microscope (Nikon Eclipse C1; Nikon Corp., Tokyo, Japan).

Statistical analyses

All statistical analyses were conducted using R software (v. 4.3.2; R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS v. 29.0 (IBM Corp., Armonk, USA). The normality of data was assessed using the Shapiro–Wilk test. In this study, all data exhibited non-normal distribution and were presented as median (1st quartile (Q1), 3rd quartile (Q3)). Mixed analysis of variance (ANOVA) was employed for the analysis of repeated measurement data with independent treatments. This analysis was conducted using the 'ezANOVA' function from the 'ez' package in R. We performed Mauchly's test for sphericity and $p > 0.05$, which indicates variants are independent. Mixed ANOVA allows for more accurate modeling of the data by accounting for both within-subject (time) and between-subject (group) effects, which is essential for the robustness and validity of the results. The nonparametric 2-way ANOVA was used for repeated measures between independent treatments. The test for repeated measures is used when the assumptions of a standard ANOVA (such as normality of residuals) are not met. This analysis was conducted using the 'Anova' function from the 'car' package in R. We performed multiple comparisons using the glht() function in the R package 'multcomp'. 'glht' stands for general linear hypothesis tests and is used to perform multiple pairwise-comparisons for parametric models. Differences between 2 independent treatment groups were compared using the Wilcoxon rank sum test. A significance level of $p < 0.05$ was considered statistically significant.

Results

ECH ameliorates disease progression of CIA in mice

Echinacoside has been shown to have therapeutic effects in various cell cultures and disease models of Parkinson's disease and cancer. To investigate whether ECH treatment has a therapeutic effect on RA, we used the CIA mouse model. Collagen-induced arthritis mice were randomized and treated with ECH (0.6 mg/mouse) or DMSO (control) every other day for 1 month. Notably, we observed that administration of ECH in CIA mice significantly decreased the arthritis scores from day 32 (Fig. 1A, Table 1,2 and

Table 1. Comparison of arthritis scores at different time between the 2 groups

Days post immunization	Clinical parameters (arthritis scores)	
	DMSO-treated mice (n = 8)	ECH-treated mice (n = 6)
28	2.00 (1.25, 2.75)	2.00 (1.00, 2.25)
30	2.00 (1.25, 3.50)	1.50 (1.00, 2.25)
32	3.50 (1.25, 4.00)	2.00 (1.00, 2.25)*
34	3.00 (2.00, 4.75)	2.00 (1.00, 3.00)*
36	3.50 (2.00, 4.00)	2.00 (0.75, 3.25)**
38	4.00 (2.25, 4.75)	2.50 (2.00,4.00)
40	4.00 (3.00, 4.00)	3.00 (1.75, 4.00)*
42	5.00 (3.50, 5.75)	3.00 (1.75, 3.25)***
44	6.00 (4.25, 6.00)	3.50 (1.75, 4.00)***
46	6.00 (4.25, 6.75)	3.00 (1.75, 3.50)***
48	6.00 (5.25, 7.00)	3.50 (2.00, 5.00)***
50	6.00 (6.00, 6.75)	3.50 (2.00, 5.25)***
52	6.50 (6.00, 8.00)	4.00 (2.00, 5.25)***
54	7.50 (6.00, 9.75)	5.00 (3.50, 6.50)***
56	7.50 (6.25, 10.75)	4.50 (3.50, 6.50)***

DMSO – dimethyl sulfoxide; ECH – echinacoside. The normality of measurement data was assessed using the Shapiro–Wilk test. In this study, the data exhibited non-normal distribution and were presented as mean (1st quartile (Q1), 3rd quartile (Q3)). The results were analyzed with mixed analysis of variance (ANOVA) test; *$p < 0.05$ vs the control; **$p < 0.01$ vs the control, ***$p < 0.001$ vs the control. The Shapiro–Wilk test was shown in Supplementary Table 1.

Table 2. Comparison of arthritis scores at different time between the 2 groups (mixed ANOVA test)

Effect	Dfn	Dfd	SSn	SSd	F-value	p-value
Intercept	1	12	3,538.305	240.756	176.36	<0.001
Group	1	12	208.006	240.756	10.368	0.007
Time	14	168	451.695	170.578	31.776	<0.001
Group:time	14	168	44.66	170.578	3.142	<0.001

Dfn – degrees of freedom in the numerator; Dfd – degrees of freedom in the denominator; SSn – sum of squares in the numerator; SSd – sum of squares in the denominator. The results were analyzed with mixed analysis of variance (ANOVA) test. The difference of arthritis score between the 2 groups was statistically significant ($F = 10.368$, $p = 0.007$). The difference of arthritis scores at different time points was statistically significant ($F = 31.776$, $p < 0.001$). The interaction of group and time point was statistically significant ($F = 3.142$, $p < 0.001$). The post hoc between-group analysis was shown in Supplementary Table 2.

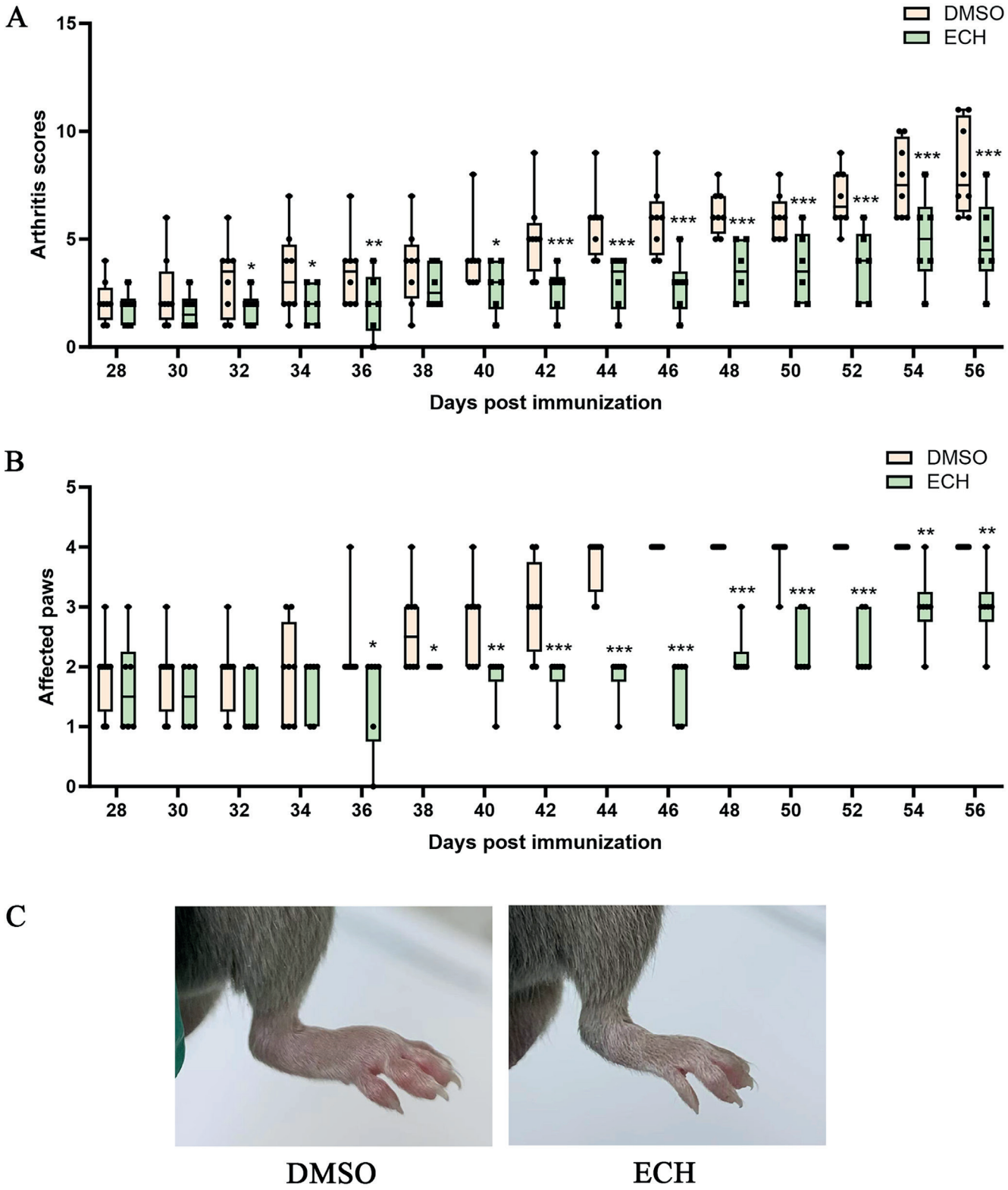

Fig. 1. Echinacoside (ECH) ameliorates disease progression of collagen-induced arthritis (CIA) in mice. Following the induction of CIA, mice were randomized and treated intraperitoneally with dimethyl sulfoxide (DMSO) or 0.6 mg of echinacoside (ECH) every other day for 1 month. Arthritis scores and number of affected paws in CIA mice were compared between the DMSO and ECH groups (A, B). Mixed analysis of variance (ANOVA) was employed for the analysis of repeated measurement data with independent treatments. The non-parametric two-way ANOVA was used for repeatedly measured counted data between independent treatments. Data are expressed as mean (1st quartile (Q1), 3rd quartile (Q3)). In a box plot, the top whiskers contain 25% high-value data, the box contains 50% intermediate data, the middle line of the box indicates the median, and the bottom whiskers contain 25% low-value data. Data points represent each piece of data. Representative paw images of CIA mice following DMSO and ECH administrations (C)

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 3. Comparison of affected paws at different time between the 2 groups

Days post immunization	Clinical parameters (affected paws)	
	DMSO-treated mice (n = 8)	ECH-treated mice (n = 6)
28	2.00 (1.25, 2.00)	1.50 (1.00, 2.25)
30	2.00 (1.25, 2.00)	1.50 (1.00, 2.00)
32	2.00 (1.25, 2.00)	1.00 (1.00, 2.00)
34	2.00 (1.00, 2.75)	2.00 (1.00, 2.00)
36	2.00 (2.00, 2.00)	2.00 (0.75, 2.00)*
38	2.50 (2.00, 3.00)	2.00 (2.00, 2.00)*
40	3.00 (2.00, 3.00)	2.00 (1.75, 2.00)**
42	3.00 (2.25, 3.75)	2.00 (1.75, 2.00)***
44	4.00 (4.00, 4.00)	2.00 (1.75, 2.00)***
46	4.00 (4.00, 4.00)	2.00 (1.00, 2.00)***
48	4.00 (4.00, 4.00)	2.00 (2.00, 2.25)***
50	4.00 (4.00, 4.00)	2.00 (2.00, 3.00)***
52	4.00 (4.00, 4.00)	2.00 (2.00, 3.00)***
54	4.00 (4.00, 4.00)	3.00 (2.75, 3.25)**
56	4.00 (4.00, 4.00)	3.00 (2.75, 3.25)**

DMSO – dimethyl sulfoxide; ECH – echinacoside. The normality of measurement data was assessed using the Shapiro–Wilk test. In this study, the data exhibited non-normal distribution and were presented as mean (1st quartile (Q1), 3rd quartile (Q3)). The results were analyzed with non-parametric two-way analysis of variance (ANOVA) analysis. *$p < 0.05$ vs the control; **$p < 0.01$ vs the control; ***$p < 0.001$ vs the control. The Shapiro–Wilk test was shown in Supplementary Table 3.

Supplementary Table 1,2) and number of affected paws from day 36 (Fig. 1B, Table 3,4 and Supplementary Table 3,4), thus ameliorating arthritis symptoms compared with the control group (Fig. 1C). Therefore, our findings suggest that ECH could have a potential therapeutic effect in CIA mice.

ECH inhibits mitochondrial fission in the synovium of CIA mice

Next, we examined the effect of ECH administration on the synovial membrane in CIA mice. Histological analysis revealed that ECH-treated CIA mice exhibited intact joint architecture and showed a significant reduction in synovial inflammation (Fig. 2D,F) compared to controls (Fig. 2C,E). As suggested by our previous study, disease progression in RA is correlated with abnormal mitochondrial fission.[9] Therefore, we compared the mitochondrial morphologies of synovium between the DMSO- and ECH-treated CIA mice by TEM. Interestingly, the mitochondria of synovial tissue specimens from ECH-treated CIA mice were found to be elongated (Fig. 2B) compared with that of control (Fig. 2A). Therefore, our results propose that ECH may ameliorate RA symptoms by inhibiting mitochondrial fission in the synovium of CIA mice.

ECH suppresses inflammation in synovium by attenuating Drp1-mediated mitochondrial fission

Our previous studies found that by inducing abnormal mitochondrial division, Drp1 mediates oxidative stress in synovium and the release of inflammatory factors, aggravating synovial inflammation in RA.[9] To further examine the role of ECH on inflammation, we analyzed the expressions of Drp1, NLRP3 and IL-6 in synovium of CIA mice by performing IHC analysis. Notably, in synovial tissue specimens of CIA mice, the expression levels of Drp1, NLRP3 and IL-6 were found to be significantly downregulated compared to controls (Fig. 3A–C). Furthermore, we performed ELISA to analyze the expressions of IL-6 and IL-1β in peripheral serum samples of CIA mice. Our analysis revealed that, in ECH-treated CIA mice, the expression levels of IL-6 were significantly lower, in contrast to IL-1β expression levels and compared with that of controls (Fig. 3D, Table 5,6 and Supplementary Table 5). Our findings indicate that ECH may, therefore, suppress inflammation in synovium by attenuating Drp1-mediated mitochondrial fission in CIA mice.

ECH inhibits oxidative stress-mediated inflammation via Keap1-Nrf2 signaling pathway

Oxidative damage in synovial tissues, induced by excessive production of ROS, plays a critical role in the progression of RA. Therefore, we examined the impact of ECH treatment on oxidative stress response in the synovial tissues of CIA mice. To achieve this, we first determined the intracellular ROS levels by DHE staining. Expectedly, treatment with ECH slightly reduced the levels of ROS in synovial tissue

Table 4. Comparison of affected paws at different time between the 2 groups (nonparametric two-way ANOVA analysis)

Effect	Dfn	Sum Sq	F	p-value
Intercept	1	1291.43	4126.478	<0.001
Group	1	57.3	183.0938	<0.001
Time	14	86.09	19.6484	<0.001
Group:time	14	19.08	4.3545	<0.001
Residuals	180	56.33		

Dfn – degrees of freedom in the numerator; Sum Sq – sum of squares. The results were analyzed with nonparametric analysis of variance (ANOVA) analysis. The difference of affected paws between the 2 groups was statistically significant ($F = 183.0938$, $p < 0.001$). The difference of affected paws at different time points was statistically significant ($F = 19.6484$, $p < 0.001$). The interaction of group and time point was statistically significant ($F = 4.3545$, $p < 0.001$). The post hoc between-group analysis was shown in Supplementary Table 4.

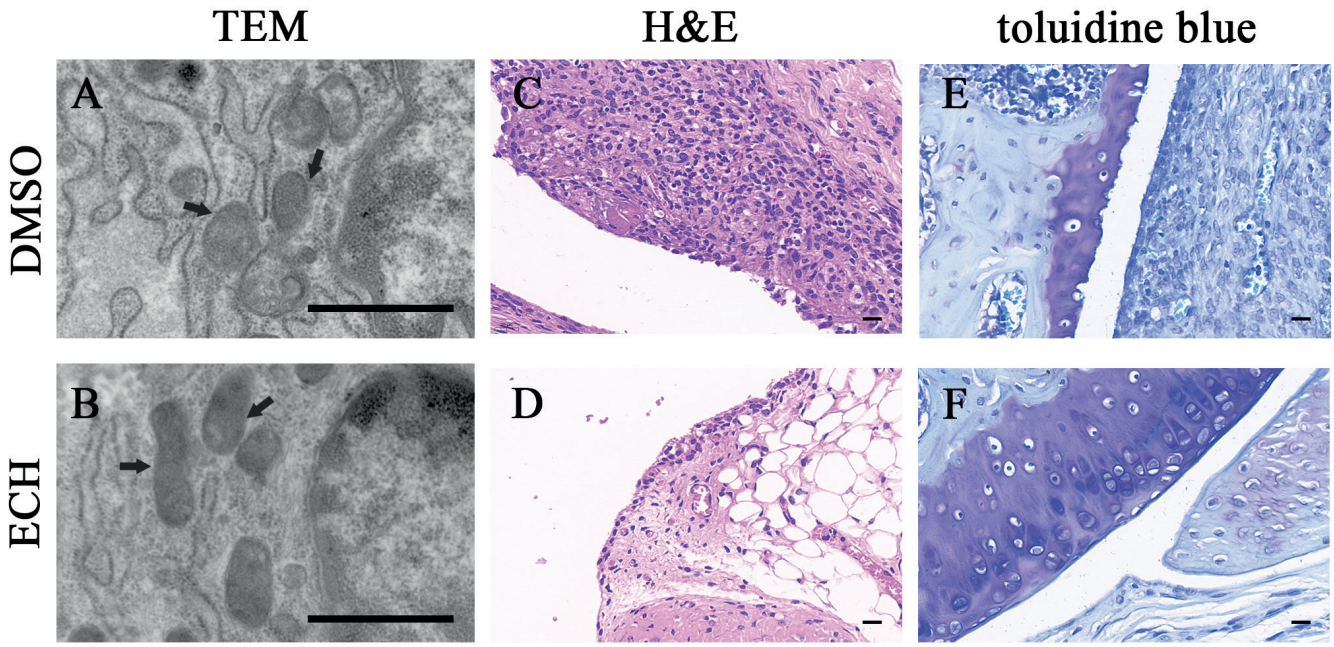

Fig. 2. Echinacoside (ECH) inhibits mitochondrial fission in the synovium of collagen-induced arthritis (CIA) mice. Representative transmission electron microscopy (TEM) images of mitochondrial morphology, scale bar = 1 μm (A, B). Representative images of hematoxylin & eosin (H&E) staining, magnification = × 40, scale bar = 20 μm (C, D). Representative images of toluidine blue staining, magnification = ×40, scale bar = 20 μm (E, F)

Table 5. Comparison of cytokines in blood serum between the 2 groups

Cytokines	Cytokines in blood serum	
	DMSO-treated mice (n = 8)	ECH-treated mice (n = 6)
IL-6 [pg/mL]	41.55 (19.30, 71.13)	10.41 (7.86, 31.70)*
IL-1β [pg/mL]	32.39 (26.77, 34.29)	35.64 (29.01, 38.93)

IL – interleukin. The normality of measurement data was assessed using the Shapiro–Wilk test. In this study, the data exhibited non-normal distribution and were presented as mean (1st quartile (Q1), 3rd quartile (Q3)). The results were analyzed with Wilcoxon rank sum test; *p < 0.05 vs the control. The Shapiro–Wilk test was shown in Supplementary Table 5.

Table 6. Comparison of cytokines in blood serum between the 2 groups (Wilcoxon rank sum test statisticsa))

Test statistics	IL-6 expression	IL-1β expression
Mann–Whitney U	7.000	12.500
Wilcoxon W	28.000	48.500
Z	–2.197	–1.495
Asymp. Sig. (2-tailed)	0.028	0.135
Exact Sig. [2*(1-tailed Sig.)]	0.029[b]	0.142[b]

IL – interleukin. [a] grouping variable: group. [b] Not corrected for ties. The difference of IL-6 expression between the 2 groups was statistically significant (Z = –2.197, p = 0.028). The difference of IL-1β expression between the 2 groups was not statistically significant (Z = –1.495, p = 0.135).

specimens of CIA mice compared with that of controls (Fig. 4A). Moreover, Nrf2 has an important role in the maintenance of mitochondrial fission/fusion balance and the cytoprotective response against oxidative stress. Finally, to investigate the effect of ECH on the Keap1-Nrf2 signaling pathway, we detected the expression levels of Keap1 and Nrf2 using immunofluorescent assay. Notably, we observed that ECH treatment significantly increased Nrf2 nuclear translocation and decreased Keap1 levels in the synovial cytoplasm of CIA mice (Fig. 4B,C). Our findings suggest that ECH might attenuate oxidative stress response in synovium via the Keap1-Nrf2 signaling pathway.

Discussion

In the present study, we found that ECH has a protective antioxidant role in CIA mice. Such findings support the anti-oxidative effect of ECH in different diseases, including osteoarthritis, ethanol-induced liver injury and inflammatory disease.[27–30] To the best of our knowledge, this study is the first of its kind to validate the therapeutic efficacy of ECH in the CIA mouse model. A key finding of this study is that ECH might suppress oxidative stress-mediated inflammation via the Nrf2/Drp1 pathway.

Oxidative stress plays an important role in the progression of RA, and treatment with antioxidant drugs can effectively ameliorate the clinical symptoms of the disease.[2,3,13] Previous studies have shown that ECH exerts several pharmacological effects, including antioxidant and anti-inflammatory ones.[18,19] According to a report from 2021, ECH alleviates ethanol-induced oxidative stress and liver steatosis by reducing ROS levels and increasing antioxidant oxidase levels.[29] Further strengthening the results, we found that ROS levels declined sharply following ECH treatment. Consistent with previous observations,[29–32] we revealed that ECH administration significantly decreased arthritis scores and the number of affected paws in CIA mice. Meanwhile, it was also visible that in mice treated with ECH, malondialdehyde (MDA) expression levels were moderately downregulated in contrast

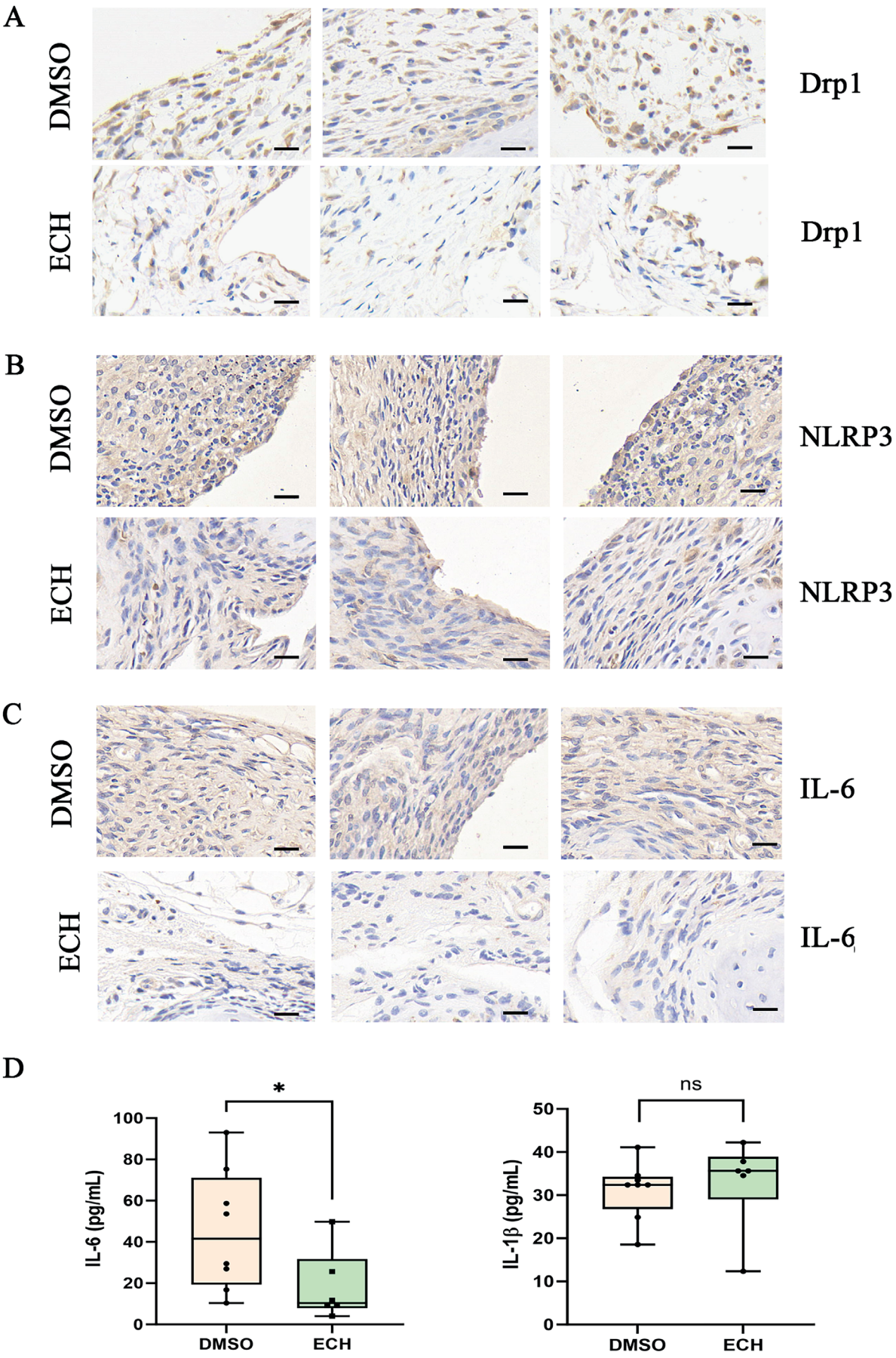

Fig. 3. Echinacoside (ECH) suppresses inflammation in synovium by attenuating dynamin-related protein 1 (Drp1)-mediated mitochondrial fission. Representative immunohistochemical (IHC) images showing the expressions of Drp1, NLRP3 and interleukin (IL)-6 in the synovium of collagen-induced arthritis (CIA) mice (A). Relative expression levels of IL-6 and IL-1β in the peripheral serum of dimethyl sulfoxide (DMSO)- and ECH-treated CIA mice, as determined using enzyme-linked immunosorbent assay (ELISA) (B). The results were analyzed using the Wilcoxon rank sum test. Data are expressed as mean (1st quartile (Q1), 3rd quartile (Q3)). In a box plot, the top whiskers contain 25% high-value data, the box contains 50% intermediate data, the middle line of the box indicates the median, and the bottom whiskers contain 25% low-value data. Data points represent each piece of data

*$p < 0.05$; ns $p > 0.05$.

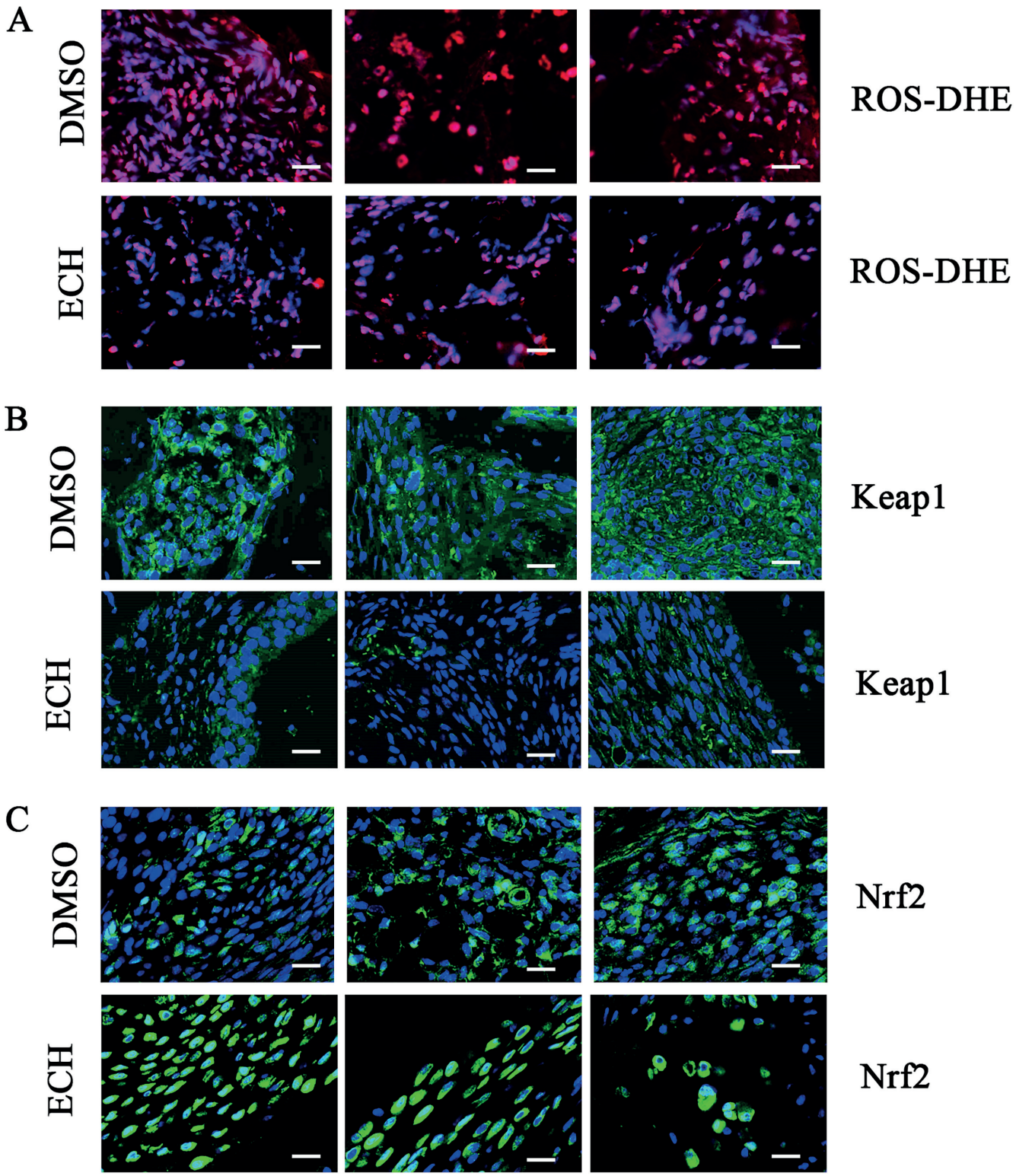

Fig. 4. Echinacoside (ECH) inhibits oxidative stress-mediated inflammation via the Keap1–Nrf2 signaling pathway. Representative immunofluorescence images (magnification = ×40, scale bar = 20 μm) illustrating the expression levels of reactive oxygen species (ROS), intracellular Keap1 and Nrf2 (A, B, C) in the synovium of dimethyl sulfoxide (DMSO)- and ECH-treated collagen-induced arthritis (CIA) mice

Keap1 – kelch-like ECH-associated protein 1; Nrf2 – nuclear factor-erythroid-2-related factor 2.

to glutathione (GSH) levels when compared to controls (data not shown). Our findings demonstrated that ECH significantly ameliorates inflammation in CIA mice, thus supporting its therapeutic utility in RA.

It is well known that increased mitochondrial fission directly promotes ROS production[33] and our previous report found that increased mitochondrial fission plays a crucial role in the progression of the disease.[9] In this

study, we observed that mitochondria of synovial tissue specimens from ECH-treated CIA mice were found to be elongated, thus indicating a significant reduction in mitochondrial fragmentation compared to DMSO-treated mice. In addition, the expression of ROS mediators was found to be downregulated after ECH administration. Supporting these results, our histological analysis revealed that ECH-treated CIA mice exhibited intact joint architecture and showed a significant reduction in synovial inflammation and bone erosion compared to controls. Collectively, these findings suggest that ECH can inhibit mitochondrial fission in the synovium via downregulation of ROS, which ultimately leads to the amelioration of clinical RA symptoms. Given that mitochondrial fusion and fission are essential for regulating mitochondrial morphology and governing its functions,[6] and several studies have shown that ECH may affect mitochondrial functions,[31,32] it is possible that ECH may modulate mitochondrial autophagy and apoptosis to affect CIA-mediated inflammation.

Dynamin-related protein 1 is one of the key regulators of mitochondrial fission.[5] According to a report from 2020, ECH was shown to attenuate neuroinflammation by regulating Drp1-dependent mitochondrial fission in rats subjected to chronic cervical cord compression.[27] Moreover, our previous study demonstrated elevated expression of Drp1 in RA, and that Drp1-targeted therapy may yield clinical benefits and improve prognosis in RA.[9] In line with this finding, we found that ECH treatment downregulated Drp1 expression and reduced synovial inflammation in CIA mice. Further strengthening these results, the serum expression level of the proinflammatory cytokines IL-6 were found to be significantly lower in ECH-treated CIA mice compared to the control mice. Collectively, these findings suggest that ECH might suppress inflammation in synovium by attenuating Drp1-mediated mitochondrial fission, and thus Drp1-targeted ECH therapy may improve the prognosis for patients with RA.

It has been well established that Nrf2 is closely linked to the regulation of mitochondrial biogenesis, dynamics and mitophagy, and is involved in antioxidant defense.[15,34,35] Studies have shown that Nrf2 activation could decrease mitochondrial fission through the degradation of Drp1, thus unraveling a novel mechanism by which Nrf2 mitigates oxidative stress.[14] According to a report from 2022, ECH alleviates osteoarthritis in rats by activating the Nrf2-HO-1 signaling pathway.[28] Another report from 2021 indicated that ECH targets Nrf2 to regulate oxidative stress and improve alcohol-induced liver injury.[29] In the current study, we found that ECH treatment significantly increased Nrf2 nuclear translocation and decreased Keap1 levels in the synovial cytoplasm of CIA mice. Interestingly, we also observed that ECH treatment significantly reduced NLRP3 expression. Hennig et al. have shown that ROS regulates NLRP3 inflammasome activation, and further demonstrated a correlation between Nrf2 activation and NLRP3 inflammasome inhibition in many different disease models related to inflammation.[36] Therefore, in line with previous reports, the results of this study indicate that ECH might activate the Nrf2 transcription factor and increase its nuclear translocation, in turn leading to the inhibition of NLRP3 and the alleviation of synovial inflammation associated with RA. Collectively, our findings suggest that ECH attenuates oxidative stress response in synovium via the Nrf2/Drp1 pathway.

Limitations

The primary limitation of this study is that the results may not be clinically relevant to humans with RA. Further, mice were given tap water and solid food ad libitum. Weight loss may have resulted in bone erosion. In addition, tests involving gene knockout mice are needed to further study the interactions of ECH with Drp1 and Nrf2. Finally, we did not perform safety tests and did not examine whether there are any gender differences. Hence, further investigations are warranted.

Conclusions

The results of this study highlight that ECH treatment led to a decline in Drp1 expression levels, which altered mitochondrial morphology and reduced ROS production. Therefore, our findings validate the therapeutic efficacy of ECH in the CIA mouse model. In conclusion, ECH may suppress oxidative stress and inhibit inflammation by regulating the Nrf2/Drp1 pathway, thus supporting its utility in the treatment of RA.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10665399. The package includes the following files:

Supplementary Table 1. Comparison of arthritis scores at different time between the 2 groups (Shapiro–Wilk test).

Supplementary Table 2. Comparison of arthritis scores at different time between the 2 groups (post hoc between-group analysis).

Supplementary Table 3. Comparison of affected paws at different time between the 2 groups (Shapiro–Wilk test).

Supplementary Table 4. Comparison of affected paws at different time between the 2 groups (post hoc between-group analysis).

Supplementary Table 5. Comparison of cytokines in blood serum between the 2 groups (Shapiro–Wilk test).

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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Ethoxyquin mediates lung fibrosis and cellular immunity in BLM-CIA mice by inhibiting HSP90

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. Patients with rheumatoid arthritis-associated interstitial lung disease (RA-ILD) are characterized by severe pulmonary fibrosis and immune dysregulation. Heat shock protein 90 (HSP90) is involved in the progression of pulmonary fibrosis and the immune response.

Objectives. This study aimed to explore whether HSP90 regulates the development of RA-ILD and its underlying mechanism.

Materials and methods. In vivo, collagen-induced arthritis (CIA)-mice were treated with bleomycin (BLM) to establish an arthritic mouse model of pulmonary fibrosis. In vitro, human lung fibroblast 1 (HLF1) was exposed to transforming growth factor beta 1 (TGF-β1) to simulate an RA-ILD model. The RA-ILD models were treated with the HSP90 inhibitor ethoxyquin (EQ) to explore the potential mechanism of HSP90 in RA-ILD. Histopathological analysis was performed, and pulmonary fibrosis was evaluated. The differentiation of M1/M2 macrophages and Th1/Th17/Treg cells was assessed. The role of the TGF-β/Smad2/3 pathway in EQ-mediated RA-ILD progression was also explored.

Results. HSP90α and HSP90β were upregulated in the RA-ILD models. Ethoxyquin mitigated arthritis in BLM-CIA mice, and reduced the expression of alpha-smooth muscle actin (α-SMA), collagen I (Col-1) and fibronectin (FN), as well as hydroxyproline content, thereby relieving pulmonary fibrosis. In addition, EQ increased M1 macrophages and inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) levels; conversely, EQ decreased M2 macrophages and vascular endothelial growth factor (VEGF)-A and TGF-β1 contents. It also decreased Th17 (interleukin (IL)-17) while increasing Th1 (interferon gamma (IFN-γ)) and Treg (Foxp3), and restricted the expression of transforming growth factor beta type receptor I and II (TGF-βRI and TGF-βRII) and the phosphorylation of Smad2 and Smad3.

Conclusions. This study revealed that EQ regulated pulmonary fibrosis and cellular immunity by inhibiting HSP90, appearing to act through the TGF-β/Smad2/3 pathway. These findings suggest that EQ holds potential as a therapeutic agent for treating RA-ILD.

Key words: pulmonary fibrosis, HSP90, cellular immunity, rheumatoid arthritis-associated interstitial lung disease, ethoxyquin

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Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by joint damage and inflammation.[1] It often also leads to the involvement of extra-articular organs, and one of the most common manifestations is interstitial lung disease (ILD), which affects more than 60% of RA patients. Unfortunately, the median survival time for patients with RA-associated ILD (RA-ILD) is only 3–7 years.[2,3] In addition to the dysregulated immune response, patients with RA-ILD develop irreversible lung fibrosis that resembles idiopathic pulmonary fibrosis.[4,5] While certain anti-fibrotic medications such as nintedanib and pirfenidone have shown promise as potential treatments for RA-ILD, some patients may require additional immunomodulatory therapy.[6,7] As a result, there is a pressing need to develop new treatment approaches that specifically target RA-ILD. This would significantly contribute to improving patients' overall health and quality of life.

T cell activation plays a crucial role in the pathology of pulmonary fibrosis.[8] T cells, which can be further categorized based on surface markers and cell functions, include natural killer T cells, CD8 cytotoxic T lymphocytes, γδ T cells, Treg (T regulatory, Foxp3) cells, and T helper cells. T helper cells can be further divided into different subsets such as Th1 (interferon gamma (IFN-γ)), Th2 (interleukin (IL)-4), Th17 (IL-17), and Tfh (T follicular helper). Studies have shown that nintedanib, for instance, can regulate T cell activation and promote the release of IFN-γ.[9] In the lungs of patients with RA-ILD, IL-17A is upregulated. This elevation of IL-17A stimulates the proliferation of fibroblast-like synoviocytes and the production of extracellular matrix (ECM) proteins.[10]

Macrophages are also present in the lungs of individuals with pulmonary fibrosis. Inflammatory responses typically lead to the activation of M1 macrophages by Th1 cells, while the Th2 cytokine IL-4 induces alternative activation of M2 macrophages (anti-inflammatory, pro-fibrotic).[11,12] Macrophages, acting as antigen-presenting cells, participate in T cell-mediated immune responses, and the activation of M1 or M2 macrophages can affect the occurrence of T cell responses.[13] Inhibition of M2 macrophage polarization has been reported to ameliorate the fibrotic phenotype of RA-ILD.[14] However, there is still a need to uncover more details about the various cellular immune states that exist in RA-ILD.

Heat shock protein 90 (HSP90) is a type of molecular chaperone protein involved in regulating protein balance, adaptive immune response, and cell differentiation and development.[15] In patients with pulmonary fibrosis, HSP90 has been reported to modulate collagen deposition and wound healing, increasing interest in the potential beneficial effect of HSP90 inhibition in pulmonary fibrosis.[16] Previous evidence has shown that citrullinated HSP90 (citHSP90) plays a significant role in immune response in RA-ILD. The citHSP90 stimulates T cells in RA-ILD to produce IFN-γ in response to a Th1 response.[17,18] However, further confirmation is needed to understand the precise role of HSP90 in the fibrotic and immune responses observed in RA-ILD.

Ethoxyquin (EQ) has long been used as an additive in animal feed to improve growth performance and disease resistance.[19,20] Due to its anti-inflammatory and antioxidant properties, EQ has shown potential in preventing acute liver injury and cancer.[21,22] Previous studies have illustrated the ability of EQ to decrease HSP90 activity, thereby alleviating peripheral axonal injury induced by chemotherapy and providing neuroprotection,[23,24] and suggesting the emergence of EQ as a potential inhibitor of HSP90. However, whether EQ can modulate RA-ILD progression by regulating HSP90 activity still needs to be elucidated.

The TGF-β/Smad2/3 signaling pathway is widely recognized to mediate the process of pulmonary fibrosis.[25,26] transforming growth factor-beta (TGF-β) drives the epithelial–mesenchymal transition (EMT) by activating the transcription factor involved in EMT (EMT-TF). The EMT is a crucial pathway for the formation of myofibroblasts, which are the central cells in pathological fibrosis.[27,28] Studies have found that microRNA-18-5p limited TGF-β/Smad2/3 signaling and prevented the EMT of pleural mesothelial cells induced by bleomycin (BLM), ultimately alleviating subpleural lung fibrosis.[29] Vitamin D deficiency, on the other hand, leads to activation of the TGF-β/Smad2/3 signaling and collagen deposition in the lungs, accelerating BLM-induced pulmonary fibrosis.[26] However, whether HSP90 mediates the TGF-β/Smad2/3 signaling pathway to affect the progression of RA-ILD remains to be elucidated.

The establishment of the preventative models has helped to understand the role of EQ in RA-ILD. Arthritis and pulmonary fibrosis are 2 key pathological features of RA-ILD.[30] As an in vivo model, the collagen II (Col-2)-induced arthritis (CIA) model has been widely chosen for studying the pathogenesis of RA, as it is cost-effective and shares immunological and pathological features relatively similar to human RA.[31,32] Bleomycin is further used to induce animal pulmonary fibrosis and lung injury.[33] Additionally, TGF-β1 is activated in the process of pulmonary fibrosis. TGF-β1 predominantly drives lung fibroblast differentiation into myofibroblasts and stimulates excessive secretion of ECM proteins by myofibroblasts, leading to ECM deposition and fibrosis.[34,35] Therefore, the BLM-CIA mouse model and TGF-β1-induced human lung fibroblast 1 (HLF1) cell model were chosen to assess the effect of EQ on pulmonary fibrosis.

Objectives

This study aims to generate a mouse model of RA-ILD, establish a TGF-β1-induced HLF1 cell model, and investigate the mechanism through which EQ affects the physiological and pathological phenotypes and immune cell characteristics associated with RA-ILD. These investigations will provide new insights for treating RA-ILD.

Materials and methods

Animal model

Male C57BL/6 mice (6–8 weeks old) were ordered from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Mice were randomly divided into 3 groups (n = 6 in each group): sham, CIA+BLM and CIA+BLM+EQ. A brief flowchart of the animal model procedure is shown in Fig. 1. The CIA mice were treated with BLM to mimic the RA-ILD model as described previously.[5,36] The CIA was induced by Col-2 emulsified in Freund's complete adjuvant. On day 0, a subcutaneous injection of 100 µL of emulsion containing 100 µg of Col-2 and 200 µg of *Mycobacterium tuberculosis* (Mtb) was administered at the base of the tail. On day 21, booster immunization was performed following the same procedure. On day 25, mice in the CIA+BLM and CIA+BLM+EQ groups were subjected to intrabronchial injections of 5 mg/kg BLM. On the same day, 8 h after BLM induction, mice in the CIA+BLM+EQ group were intraperitoneally injected with extra 0.36 mg/mouse EQ (E8260; Sigma Aldrich, St. Louis, USA).[22] From day 25 to day 45, mice received EQ 3 times a week for 3 weeks. The mice in the sham group received an equivalent dose of normal saline.

Airway hyperresponsiveness analysis

A lung function challenge test was carried out the day following the final treatment administration. After the mouse was anesthetized and fixed, the skin of the neck was incised, and the trachea was bluntly dissected. After the trachea was exposed, sutures were used to pass through the trachea, the T-shaped entrance was opened in the trachea, and the endotracheal tube was inserted and then ligated and fixed. The airway responsiveness of the mice in each group was measured using a closed plethysmography system. The mice were sealed in a body scanning box, and after the baseline was stabilized, a bronchial challenge was performed with 0.5, 1, 2, 4, and 6 mmol/L methacholine (Mch) solutions (A2126; Sigma-Aldrich). At the end of each excitation, the 2nd excitation and detection were performed after the baseline stabilized. Changes in airway resistance parameters at different Mch concentrations were observed and calculated. Percentage reduction of lung compliance was calculated as

(lung compliance value before challenge – lung compliance value after challenge)/ lung compliance value before challenge × 100%.

Tissue staining

Histopathological analysis was performed using hematoxylin and eosin (H&E) and Sirius red staining. Lung tissues were fixed in 4% paraformaldehyde. Joint tissues were fixed in 10% formalin and decalcified with ethylenediaminetetraacetic acid (EDTA). Paraffin embedding was performed on both lung and joint tissues, followed by sectioning into thin slices measuring 4–5 µm. The extent of the damage in lung and synovial tissues was assessed using H&E staining. Collagen deposition in the synovium was assessed with Sirius red staining. Sections were deparaffinized with xylene and hydrated with alcohols of varying concentrations. Subsequently, sections were stained with H&E and Sirius red dye (Abiowell, Changsha, China). After dehydration, the sections were observed under an optical microscope (BA210T; Motic, Xiamen, China), and randomly selected fields of view were photographed.

Immunohistochemistry staining

After deparaffinization and hydration, sections of the left lungs were heated in 0.01 mol/L citrate buffer for thermal antigen retrieval. To eliminate endogenous enzymes, 1% periodate was added. After washing with phosphate-buffered saline (PBS), the sections were incubated overnight at 4°C with alpha-smooth muscle actin (α-SMA) antibody (1:300, BM0002; Boster, Wuhan, China). The next day, horseradish peroxidase (HRP)-labeled mouse antibody (1:100, AWS0003; Abiowell) was added. Briefly, 4′,6-diamidino-2-phenylindole (DAPI) was chosen to stain nuclei, and hematoxylin was used to counterstain tissues. Finally, images were acquired under an optical microscope (BA210T; Motic) (×100 and ×400 magnification) and analyzed with IPP (Image-Pro-Plus; Media Cybernetics, Rockville, USA).

Flow cytometry

To assess the immune status of RA-ILD, peripheral blood and bronchoalveolar lavage fluid (BALF) were collected from the mice, and the proportion of immune cells was detected.

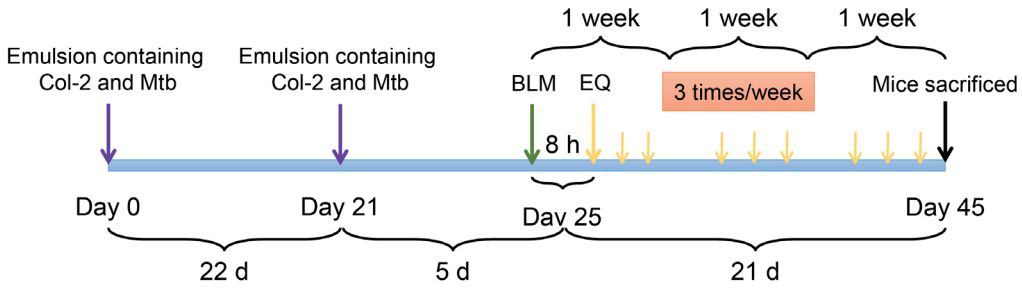

Fig. 1. Flowchart of the animal model procedure

Red cell lysate was added to fresh blood. After centrifugation, the cell pellet was suspended in PBS. Cells (1×10^5 cells/100 µL) were washed with 0.01 M PBS (pH 7.4) and resuspended in the culture medium. Then, F4/80-FITC (11-4801-82; eBioscience, San Diego, USA) and CD11c-PE (12-0114-82; eBioscience) or F4/80-FITC and CD206-PE (12-2061-82; eBioscience) were added, and the cells were incubated in the dark for 30 min and washed with PBS. Subsequently, cells were analyzed for M1/M2 macrophage ratio with flow cytometry (A00-1-1102; Beckman Coulter, Fullerton, USA).

For Th1/Th17 detection, a cell stimulation cocktail was added to suspend cells. Cells were stimulated at 37°C for 4 h. After centrifugation, 0.5% bovine serum albumin (BSA)-PBS was added to wash the cells. Cells were suspended with intracellular fixation buffer and fixed at room temperature. Subsequently, cells were suspended with permeabilization buffer. CD4-FITC (11-0041-82; eBioscience) and IFNγ-PE (12-7311-82; eBioscience) or CD4-FITC and IL17-PE (12-7177-81; eBioscience) were added and incubated for 30 min in the dark. Cells were washed with 0.5% BSA-PBS and analyzed using flow cytometry.

For Treg detection, cells were fixed and permeabilized. Subsequently, CD4-FITC, CD25-APC (17-0251-82; eBioscience) and Foxp3-PE (12-5773-82; eBioscience) were added, and the cells were incubated in the dark for 30 min. Cells were washed with 0.5% BSA-PBS and analyzed with flow cytometry.

Enzyme-linked immunosorbent assay

Vascular endothelial growth factor (VEGF)-A, inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), TGF-β, IFN-γ, IL-17, and Foxp3 levels in mouse serum and BALF were detected according to the kit instructions. VEGF-A (CSB-E04756m), iNOS (CSB-E08326m), TNF-α (CSB-E04741m), TGF-β (CSB-E04726m), and IFN-γ (CSB-E04578m) detection kits were ordered from Cusabio (Wuhan, China). The Foxp3 detection kit (YJ037859) was purchased from Yuanju Biological Co., Ltd. (Shanghai, China).

Hydroxyproline detection

To quantify collagen metabolism, a hydroxyproline assay was performed according to the kit manual (A030-2-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The hydrolysate was added, and the tissue was hydrolyzed by heating in a water bath. The pH was adjusted to 6–6.8. The hydrolysate containing activated carbon was added and mixed well. The supernatant was collected after centrifugation, and the absorbance value of each tube was measured at 550 nm. The data show hydroxyproline content (µg per mg) of tissue.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cell lysates and tissues using TRIzol (15596026CN; Thermo Fisher Scientific, Waltham, USA). RNA was then converted into cDNA using the HiFiScript cDNA Synthesis Kit (CW2569; CWBIO, Taizhou, China). The UltraSYBR mix kit (CW2601; CWBIO) was used to perform quantitative real-time polymerase chain reaction (qPCR) with the PCR system. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative level of the target after *β-actin* standardization. The primer sequence is shown in Table 1.

Western blot

Radioimmunoprecipitation assay (RIPA; AWB0136; Abiowell) was used to extract total protein from cell lysates or tissues, and then the protein concentration was quantified with a bicinchoninic acid (BCA) kit (AWB0104; Abiowell). Then, the total protein was separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose (NC) membrane. The membrane was mixed with 5% skim milk powder and incubated for 90 min to prevent nonspecific binding. Subsequently, the membrane was incubated with

Table 1. Primer sequences used in the study

Targets	F (5′-3′)	R (5′-3′)
M-TGF-βR	AATTCCTCGAGACAGGCCATTT	CCAGCTGACTGCTTTTCTGTAG
M-Smad2	AATCATTGCAACAAGAGGCAGT	ATTCCCGTCCCCATCATCCT
M-Smad3	CCCTAGTCAAGCCCAGTCCCT	AGCCTCCTAAACAAGAGTCCACACC
M-IFN-γ	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT
M-IL-17	AGACTACCTCAACCGTTCCAC	CACCAGCATCTTCTCGACCC
M-Foxp3	CTCCAATCCCTGCCCTTGACC	ACATCATCGCCCGGTTTCCA
M-β-actin	ACATCCGTAAAGACCTCTATGCC	TACTCCTGCTTGCTGATCCAC
H-ACTA2 (α-SMA)	CTATGAGGGCTATGCCTTGCC	GCTCAGCAGTAGTAACGAAGGA
H-COL1A1 (Col-1)	GCAAGAACCCCGCCCGCACC	GCTCTCGCCGAACCAGACATGCC
H-FN	ATTCACCTACAATGGCAGGACGTT	GCACCAAAGATGTCCGTCCTGT
H-β-actin	ACCCTGAAGTACCCCATCGAG	AGCACAGCCTGGATAGCAAC

Table 2. Antibodies used in the study

Indicator	Dilution	Origin	Cat. No. and manufacturer
HSP90α	1: 1,000	mouse	ab128483; Abcam
HSP90β	1: 5,000	rabbit	ab203085; Abcam
α-SMA	1: 2,000	rabbit	55135-1-AP; Proteintech
Col-1	1: 2,000	rabbit	14695-1-AP; Proteintech
FN	1: 5,000	rabbit	15613-1-AP; Proteintech
TGF-βRI	1: 1,000	rabbit	ab235578; Abcam
TGF-βRII	1: 1,000	rabbit	ab259360; Abcam
p-Smad2	1: 5,000	rabbit	ab188334; Abcam
Smad2	1: 6,000	rabbit	12570-1-AP; Proteintech
p-Smad3	1: 2,000	rabbit	ab52903; Abcam
Smad3	1: 3,000	mouse	66516-1-Ig; Proteintech
β-actin	1: 5,000	mouse	66009-1-Ig; Proteintech
HRP goat anti-mouse IgG	1: 5,000	mouse	SA00001-1; Proteintech
HRP goat anti-rabbit IgG	1: 6,000	rabbit	SA00001-2; Proteintech

TGF-βRI and TGF-βR II – transforming growth factor beta type receptor I and II; FN – fibronectin; HRP – horseradish peroxidase; IgG – immunoglobulin G; Col-1 – collagen 1; α-SMA – alpha-smooth muscle actin.

primary antibody at 4°C overnight. After washing with Tris-buffered saline with Tween (TBST), the membrane was mixed with the secondary antibody for 90 min. Finally, the membrane was exposed to Enhanced Chemiluminescence (ECL) Plus (AWB0005; Abiowell), and the protein bands were visualized using a gel imaging system (ChemiScope6100; Clinx, Shanghai, China). Antibody information is shown in Table 2.

Cell culture

Human lung fibroblast 1 (HLF1) was ordered from Pricella (Wuhan, China). Cells were maintained in Ham's F-12K medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. To explore the effects of EQ in vitro, 3 groups were set up (n = 6 in each group): control, TGF-β1 and TGF-β1+EQ. Cells were exposed to 10 ng/mL TGF-β1 for 48 h,[37] and treated with different concentrations (0, 1, 2, 4, 6, 8, and 10 μg/mL) of EQ for 48 h.

Cell counting kit

The toxic response of EQ to HLF1 cells was tested using a Cell Counting Kit-8 (CCK-8) assay. 5×10^3 cells were seeded in 96-well plates. After the cells adhered to the wall, 10 μL of CCK-8 solution was added to each well. After incubation at 37°C for 4 h, the absorbance of the samples was measured at 450 nm.

Immunofluorescence

Expression of α-SMA in HLF1 cells was evaluated using an immunofluorescence (IF) assay. Cells were fixed with 4% paraformaldehyde and washed with PBS, incubated with 0.3% Triton X-100 for 30 min, washed with PBS, then thoroughly mixed with 5% BSA for 1 h and washed with PBS. Antibody α-SMA (1:50, BM0002; Boster) was added and incubated overnight at 4°C. Then, the anti-mouse secondary antibody (1:200, AWS0004b; Abiowell) was added and incubated at 37°C for 90 min. DAPI (4′,6-diamidino-2-phenylindole) was applied to stain the nucleus for 10 min. Finally, images were acquired (×400 magnification) using fluorescence microscopy (BA410E; Motic) and photographed.

Statistical analyses

GraphPad Prism v. 9 (GraphPad Software, San Diego, USA) was used for statistical analysis. Data were expressed as the mean with a 95% confidence interval (95% CI). Normal distribution was assessed using the Shapiro–Wilk test, and the Brown–Forsythe test was used to confirm variance homogeneity (Supplementary Tables 1–6). One-way analysis of variance (ANOVA) and two-way ANOVA were used to compare groups. Tukey's post hoc test was adopted. All experiments consisted of 6 biological replicates, each representing the average of 3 technical replicates. The threshold for statistical significance was set at $p < 0.05$.

Results

Effects of EQ on disease manifestations in BLM-CIA mice

A mouse model of BLM-CIA was established to investigate the impact of EQ on RA-ILD. All the mice in the sham group survived during modeling. The CIA+BLM group

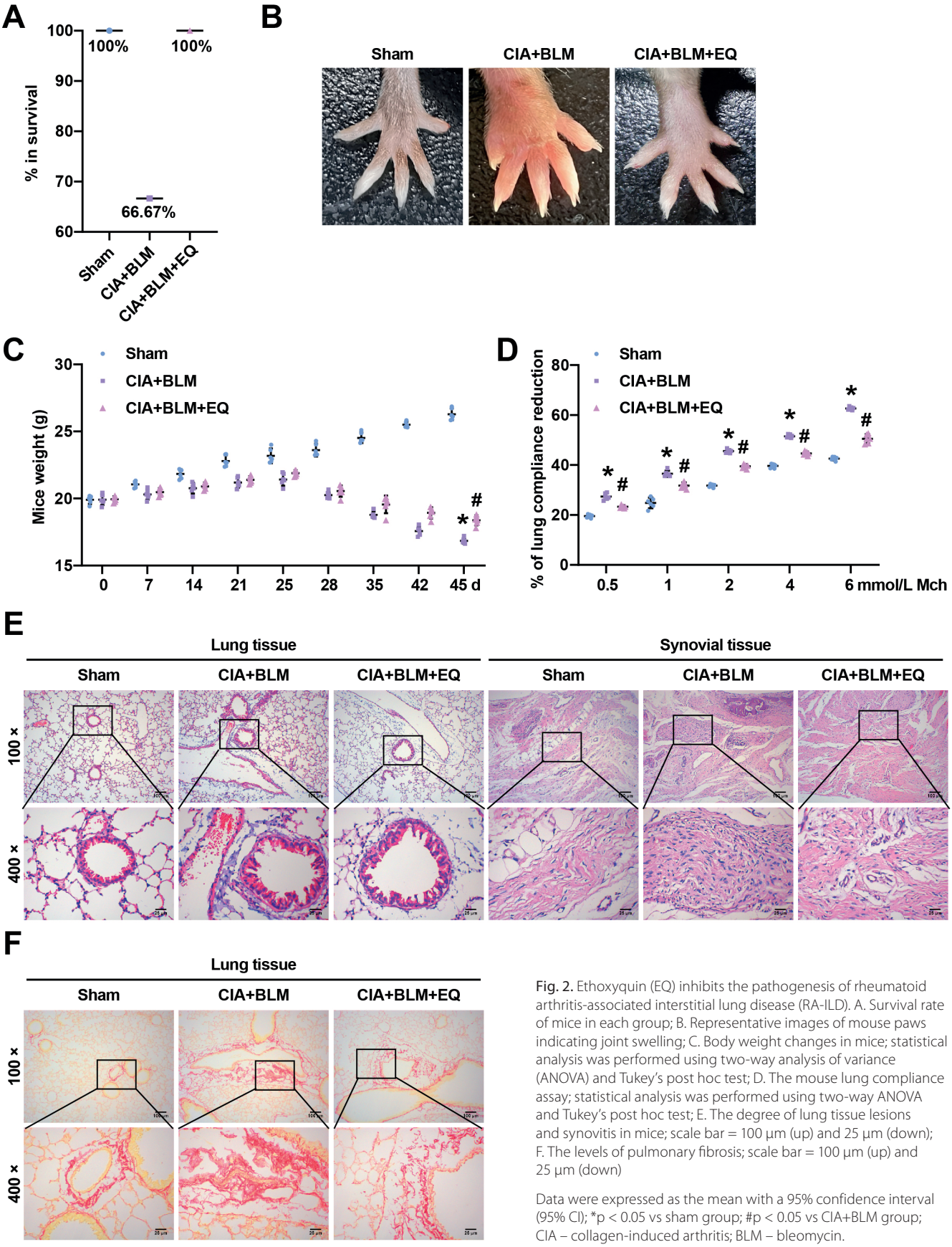

Fig. 2. Ethoxyquin (EQ) inhibits the pathogenesis of rheumatoid arthritis-associated interstitial lung disease (RA-ILD). A. Survival rate of mice in each group; B. Representative images of mouse paws indicating joint swelling; C. Body weight changes in mice; statistical analysis was performed using two-way analysis of variance (ANOVA) and Tukey's post hoc test; D. The mouse lung compliance assay; statistical analysis was performed using two-way ANOVA and Tukey's post hoc test; E. The degree of lung tissue lesions and synovitis in mice; scale bar = 100 µm (up) and 25 µm (down); F. The levels of pulmonary fibrosis; scale bar = 100 µm (up) and 25 µm (down)

Data were expressed as the mean with a 95% confidence interval (95% CI); *p < 0.05 vs sham group; #p < 0.05 vs CIA+BLM group; CIA – collagen-induced arthritis; BLM – bleomycin.

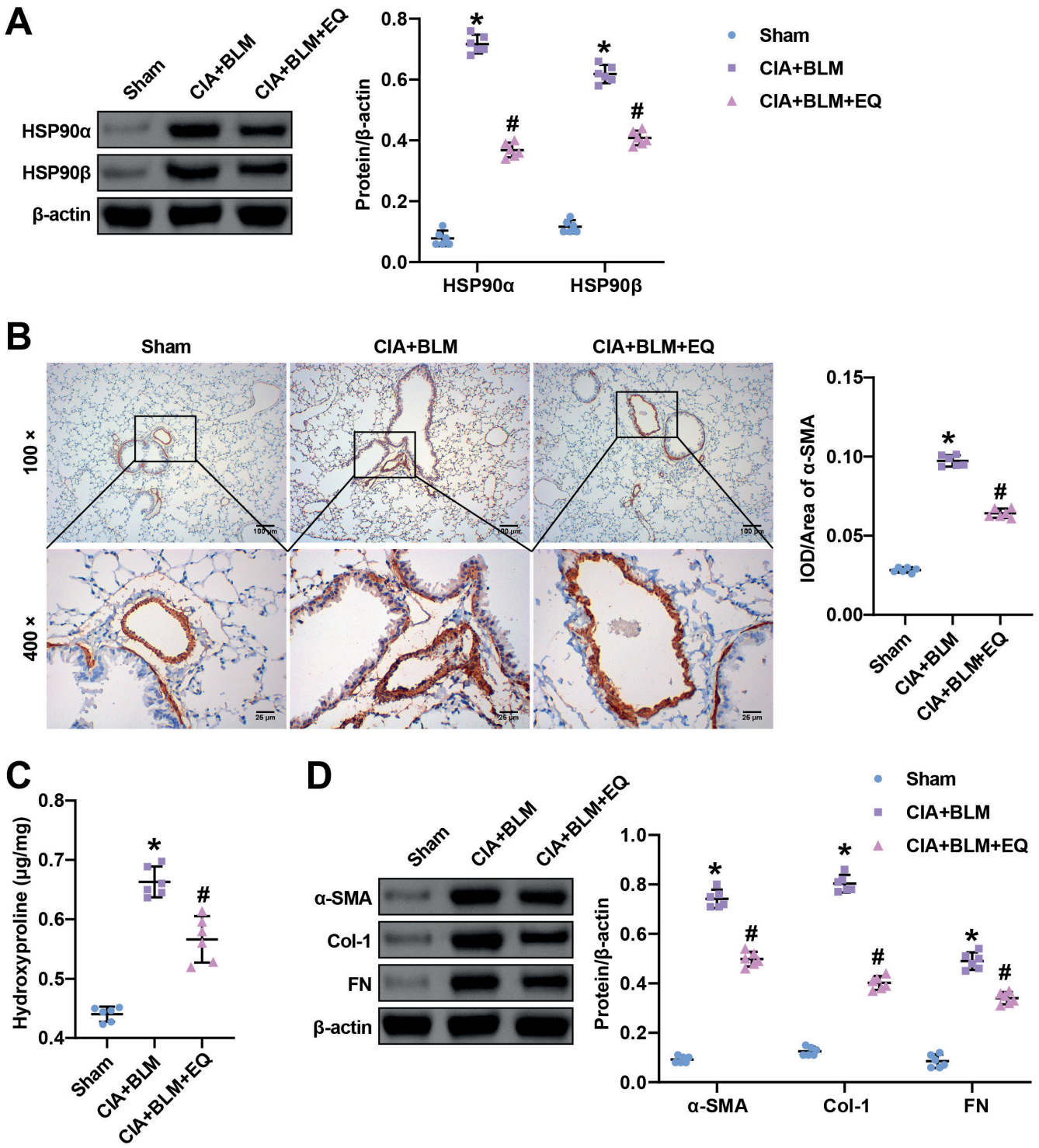

Fig. 3. Ethoxyquin (EQ) regulates HSP90 expression and collagen deposition in BLM-CIA mice. A. The abundance of HSP90α and HSP90β; B. The evaluation of alpha-smooth muscle actin (α-SMA) expression; scale bar = 100 μm (up) and 25 μm (down); C. Detection of hydroxyproline content in lung tissue; D. Analysis of the protein abundance of α-SMA, collagen I (Col-1) and fibronectin (FN)

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Data were expressed as the mean with a 95% confidence interval (95% CI); *p < 0.05 vs sham group; #p < 0.05 vs CIA+BLM group; CIA – collagen-induced arthritis; BLM – bleomycin.

exhibited a decreased survival rate; however, treatment with EQ restored the survival rate of the diseased mice (Fig. 2A). The CIA+BLM group displayed evident redness and swelling in the paws, while EQ treatment ameliorated arthritis and swelling in the affected mice (Fig. 2B). The body weight of the diseased mice decreased, while EQ partially restored their body weight on day 45 ($p < 0.001$, ANOVA, Fig. 2C). Moreover, the CIA+BLM group exhibited an increase in lung compliance reduction percentage compared to the sham group, indicating an exacerbated

reduction in lung compliance and restricted lung function in the CIA+BLM group. However, the CIA+BLM+EQ group showed a lower percentage than the CIA+BLM group ($p < 0.001$, ANOVA, Fig. 2D), suggesting that EQ increased lung compliance and was protective in the BLM-CIA mice. The CIA+BLM group exhibited a widened pulmonary septum, increased infiltration of macrophages and lymphocytes, elevated number of neutrophils, significant destruction of alveolar structure, and obvious inflammation. However, the severity of lung tissue lesions was notably reduced in the CIA+BLM+EQ group (Fig. 2E). In addition, H&E staining demonstrated marked joint synovial hyperplasia and infiltration of inflammatory cells in the CIA+BLM group, both of which were alleviated after EQ treatment (Fig. 2E). The CIA+BLM group mice showed significant collagen deposition in the lung interstitium, while EQ treatment alleviated this pathological phenomenon (Fig. 2F). Collectively, these results indicate that the BLM-CIA mice exhibited synovitis and pulmonary fibrosis, and treatment with EQ alleviated the physiological and pathological phenotypes associated with RA-ILD.

Effects of EQ on HSP90 expression and collagen deposition in BLM-CIA mice

Subsequently, compared with the sham group, we found that the expression of HSP90α and HSP90β was upregulated in the CIA+BLM group, while EQ decreased HSP90α and HSP90β levels ($p < 0.001$, ANOVA, Fig. 3A). The expression of α-SMA was elevated in BLM-CIA mice, while EQ inhibited its expression ($p < 0.001$, ANOVA, Fig. 3B). The hydroxyproline content in the lung tissues was increased in the CIA+BLM group, which was further suppressed by EQ ($p < 0.001$, ANOVA, Fig. 3C). The protein abundance of α-SMA, collagen I (Col-1) and fibronectin (FN) increased in the CIA+BLM group, but EQ downregulated the expression of these proteins ($p < 0.001$, ANOVA, Fig. 3D). Our data suggest that EQ blocked the expression of HSP90, α-SMA, Col-1, and FN in BLM-CIA mice.

Ethoxyquin affected the cellular immune status in the peripheral blood of BLM-CIA mice

Compared with the sham group, the CIA+BLM group exhibited a decrease in the proportion of M1 macrophages and an increase in M2 macrophages. Compared with the CIA+BLM group, EQ increased M1 macrophages and decreased M2 macrophages ($p < 0.001$, ANOVA, Fig. 4A). In the peripheral blood of the CIA+BLM group, iNOS and TNF-α levels were decreased, while the levels of VEGF-A and TGF-β1 increased. Treatment with EQ reversed these changes ($p < 0.001$, ANOVA, Fig. 4B). In addition, the CIA+BLM group exhibited a decrease in the proportion of Th1 and Treg cells and an increase in Th17 cells. However, EQ increased Th1 and Treg cells and decreased Th17 cells compared with the CIA+BLM group ($p < 0.001$, ANOVA, Fig. 4C). The level of IL-17A was increased in the CIA+BLM group, while the levels of IFN-γ and Foxp3 were decreased. The EQ treatment reversed these changes ($p < 0.001$, ANOVA, Fig. 4D). These results show that in the peripheral blood of BLM-CIA mice, EQ increased M1 macrophages, Th1 and Treg subsets and decreased M2 macrophages and Th17 cells.

Ethoxyquin regulated the cellular immune status in the lung of BLM-CIA mice

Compared with the sham group, the CIA+BLM group exhibited a decrease in the proportion of M1 macrophages and an increase in M2 macrophages in the BALF. Compared with the CIA+BLM group, EQ treatment increased M1 macrophages and decreased the M2 phenotype ($p < 0.001$, ANOVA, Fig. 5A). The CIA+BLM group showed reduced iNOS and TNF-α levels and elevated VEGF-A and TGF-β1 levels, while EQ reversed the levels of these factors ($p < 0.001$, ANOVA, Fig. 5B). In addition, the proportion of Th1 cells in the CIA+BLM group was lower, and the proportion of Th1 cells in the CIA+BLM+EQ group was higher than that of the CIA+BLM group ($p < 0.001$, ANOVA, Fig. 5C). The levels of *IFN-γ* and *Foxp3* were decreased, and *IL-17A* was increased in the CIA+BLM group, while EQ reversed these changes ($p < 0.001$, ANOVA, Fig. 5D). These results showed that EQ increased M1 macrophages and Th1 cells and decreased M2 macrophages in the lungs of BLM-CIA mice.

Effects of EQ on the TGF-β/Smad2/3 signaling pathway in BLM-CIA mice

We further investigated the effects of the TGF-β/Smad2/3 pathway on RA-ILD regulated by EQ. The CIA+BLM group exhibited increased *TGF-βR*, *Smad2* and *Smad3* mRNA levels in lung tissue, while EQ inhibited the increase of these factors ($p < 0.001$, ANOVA, Fig. 6A). The protein abundance of transforming growth factor beta type receptor I and II (TGF-βRI and TGF-βR II) was elevated in the CIA+BLM group, and EQ reversed these proteins' expression ($p < 0.001$, ANOVA). In addition, the ratios of p-Smad2/Smad2 and p-Smad3/Smad3 were increased in the CIA+BLM group, and EQ reversed these trends ($p < 0.001$, ANOVA, Fig. 6B). These data show that EQ inhibited the TGF-β/Smad2/3 pathway in BLM-CIA mice.

Ethoxyquin affected fibrosis-related protein expression through TGF-β/Smad2/3 pathway in vitro

Through in vitro experiments, the role of EQ was confirmed using TGF-β1-induced HLF1 cells. The toxic response of different concentrations of EQ (0, 1, 2, 4, 6, 8,

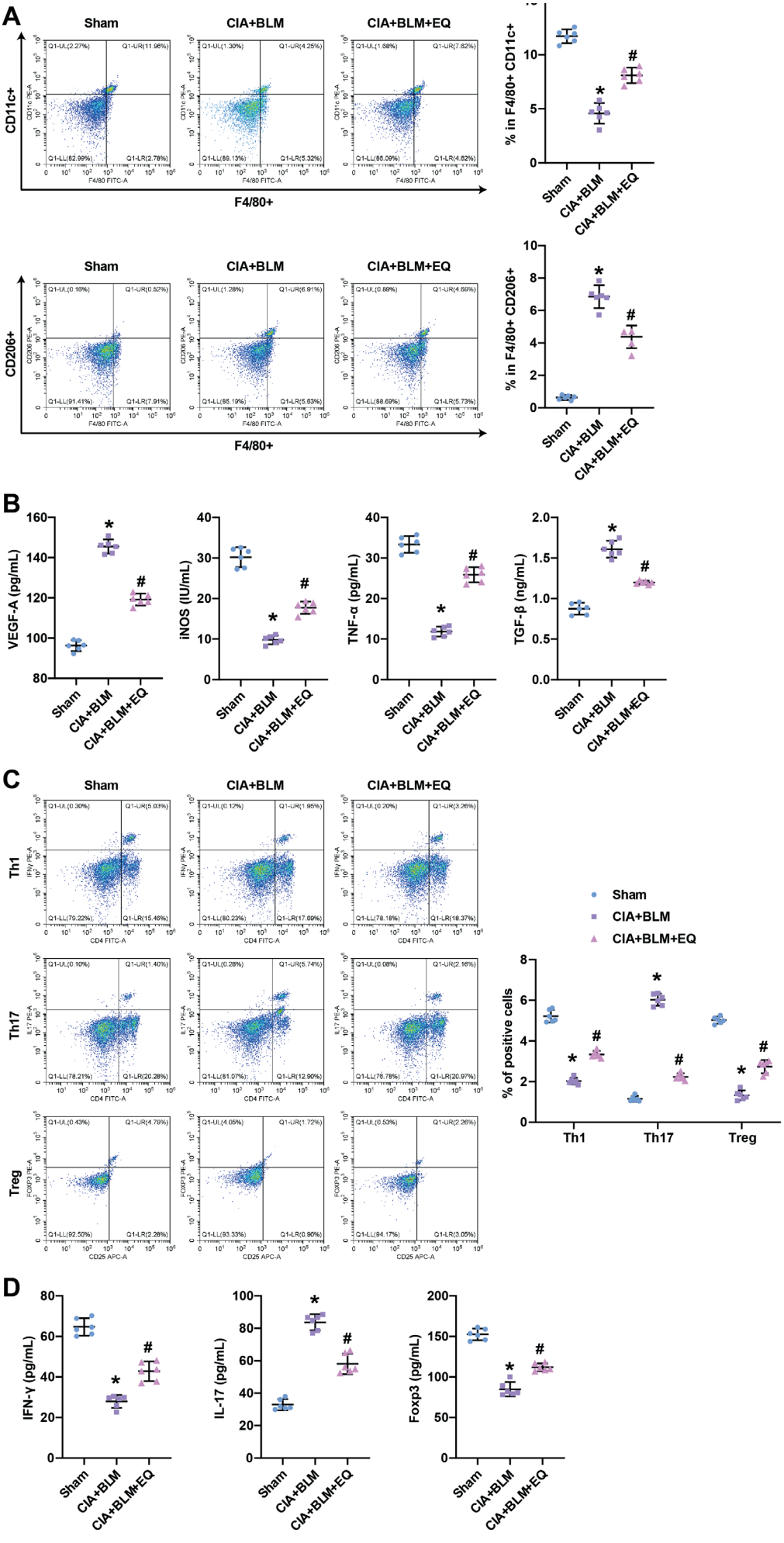

Fig. 4. Ethoxyquin (EQ) participates in rheumatoid arthritis-associated interstitial lung disease (RA-ILD) by regulating the proportion of immune cells in peripheral blood. A. The analysis of M1/M2 macrophages by F4/80$^+$ and CD11c$^+$/CD206$^+$ double staining; B. The detected inducible nitric oxide synthase (iNOS) tumor growth factor alpha (TNF-α), vascular endothelial growth factor (VEGF)-A, and transforming growth factor beta 1 (TGF-β1); C. Analysis of Th1, Th17 and Treg cell ratios; D. Detection of interferon gamma (IFN-γ), interleukin (IL)-17A and Foxp3

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Data were expressed as the mean with a 95% confidence interval (95% CI); *$p < 0.05$ vs sham group, #$p < 0.05$ vs CIA+BLM group; CIA – collagen-induced arthritis; BLM – bleomycin.

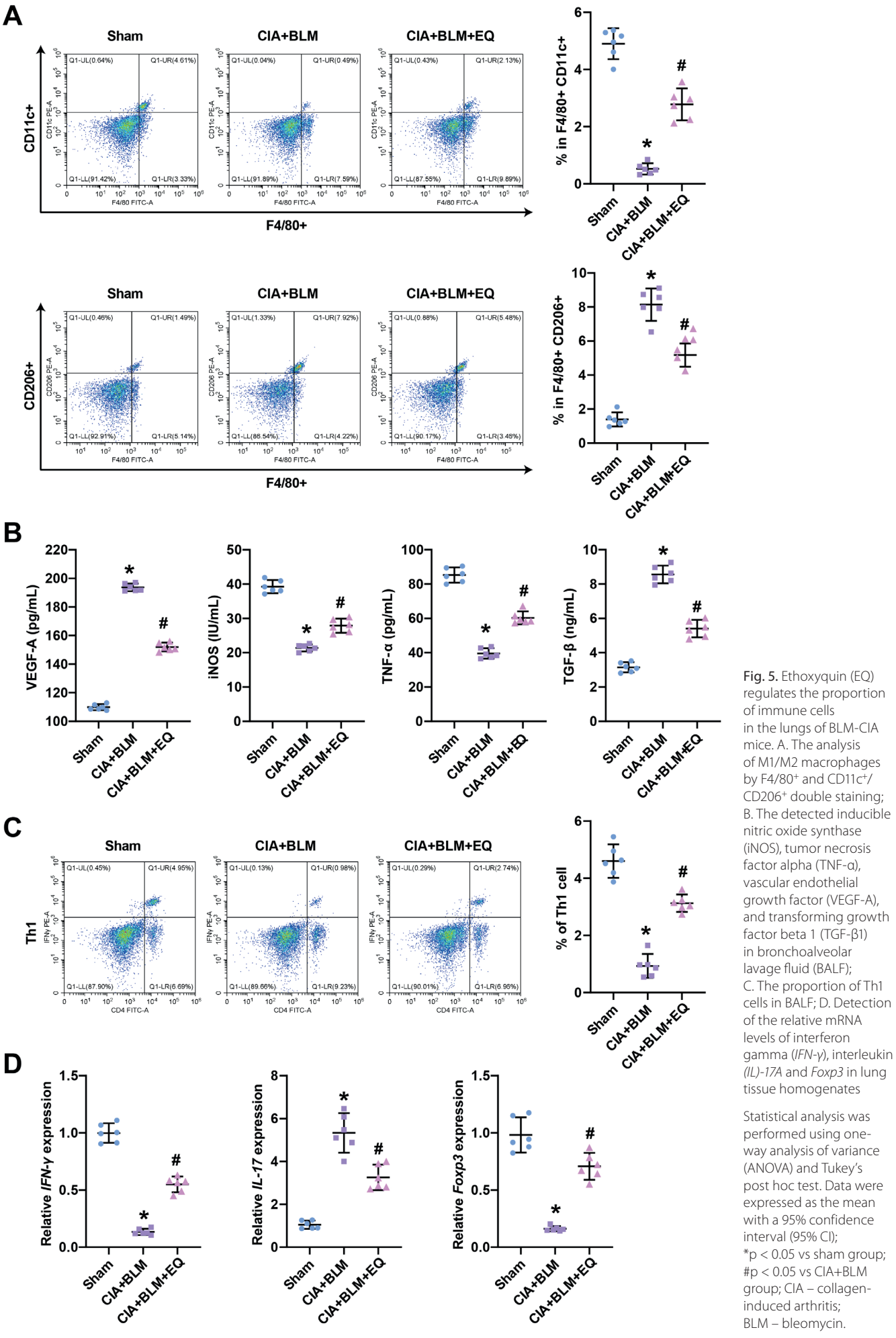

Fig. 5. Ethoxyquin (EQ) regulates the proportion of immune cells in the lungs of BLM-CIA mice. A. The analysis of M1/M2 macrophages by F4/80$^+$ and CD11c$^+$/CD206$^+$ double staining; B. The detected inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF-A), and transforming growth factor beta 1 (TGF-β1) in bronchoalveolar lavage fluid (BALF); C. The proportion of Th1 cells in BALF; D. Detection of the relative mRNA levels of interferon gamma (*IFN-γ*), interleukin *(IL)-17A* and *Foxp3* in lung tissue homogenates

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Data were expressed as the mean with a 95% confidence interval (95% CI); *p < 0.05 vs sham group; #p < 0.05 vs CIA+BLM group; CIA – collagen-induced arthritis; BLM – bleomycin.

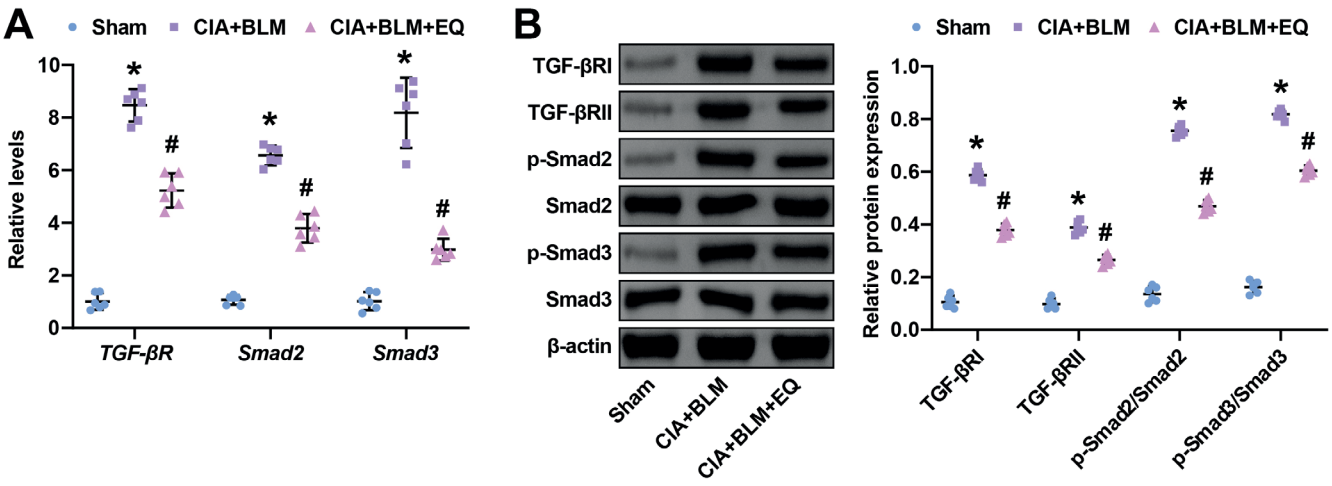

Fig. 6. Ethoxyquin (EQ) blocks TGF-β/Smad2/3 signaling to mediate the pathogenesis of rheumatoid arthritis-associated interstitial lung disease (RA-ILD). A. The relative levels of *TGF-βR* and *Smad2* and *Smad3*; B. The abundance of transforming growth factor beta type receptor I and II (TGF-βRI and TGF-βRII) p-Smad2, Smad2, p-Smad3, and Smad3 in lung tissue

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Data were expressed as the mean with a 95% confidence interval (95% CI); *p < 0.05 vs sham group; #p < 0.05 vs CIA+BLM group; CIA – collagen-induced arthritis; BLM – bleomycin.

and 10 μg/mL) to HLF1 cells was evaluated. Cell Counting Kit-8 assay revealed that there were no significant differences in the viability of HLF1 cells with an increase in EQ concentration compared with the control group, suggesting that EQ had no obvious toxic effect on HLF1 cells ($p = 0.088$, ANOVA, Fig. 7A). Therefore, 10 μg/mL EQ was chosen in subsequent experiments. Transforming growth factor beta 1 increased the expression of α-*SMA*, *Col-1* and *FN* in HLF1 cells, while EQ treatment reversed these changes ($p < 0.001$, ANOVA, Fig. 7B and 7C). Ethoxyquin suppressed TGF-β1-induced expression of α-SMA in HLF1 cells ($p < 0.001$, ANOVA, Fig. 7D). These results demonstrate that EQ reduced EMT-specific protein expression in TGF-β1-exposed HLF1 cells. In addition, TGF-β1 promoted the expression of HSP90α and HSP90β and increased the ratios of p-Smad2/Smad2 and p-Smad3/Smad3 in HLF1 cells, and EQ reversed the trends observed for these proteins ($p < 0.001$, ANOVA, Fig. 7E). These results suggest that EQ attenuated TGF-β1-induced expression of fibrosis-related factors in HLF1 cells.

Discussion

Interstitial lung disease is the most important comorbidity in RA, but there is still a lack of specific treatment strategies for RA-ILD.[38,39] Therefore, finding therapeutics that target pulmonary fibrosis and the adaptive immune response is critical. In this study, we investigated whether the HSP90 inhibitor EQ regulates BLM-induced pulmonary fibrosis in CIA mice. The results showed that EQ restricted the protein expression of HSP90 isoforms (HSP90α and HSP90β). Ethoxyquin ameliorated RA-associated pathological phenotypes, namely joint swelling and synovitis, in the BLM-CIA mice. Airway responsiveness can be used to reflect airway inflammation.[40,41] Pulmonary involvement in RA can cause airway impairment,[42] and pulmonary fibrosis also involves airway abnormalities and functional changes.[43] Fibrosis leads to a decrease in lung compliance, which in turn causes a decline in lung function.[44,45] Ethoxyquin reversed the reduction in lung compliance in BLM-CIA mice, as well as attenuated collagen deposition in the lungs of the BLM-CIA mice, as determined using H&E staining and Sirius staining. These results provide the first experimental evidence that HSP90 deficiency alleviated disease symptoms in BLM-CIA mice, thus providing novel insights and directions for the future treatment of RA-ILD.

The main pathology of pulmonary fibrosis involves the recruitment of inflammatory cells and the proliferation of lung fibroblasts, which leads to excessive deposition of ECM, mainly composed of collagen.[46] Col-1 is the major form of collagen in the ECM, and myofibroblasts expressing α-SMA are the major source of Col-1. In our study, we observed that EQ downregulated BLM-induced α-SMA expression and decreased the levels of collagen-related markers Col-1 and FN, as well as the collagen component hydroxyproline. These results confirm that EQ attenuated pulmonary fibrosis in BLM-CIA mice.

Transforming growth factor beta 1 occupies a central position in the pathogenesis of idiopathic pulmonary fibrosis (IPF). It promotes the transformation of fibroblasts into myofibroblasts and the EMT and accelerates collagen formation.[34] At present, evidence that the classic TGF-β1/Smad2/3 pathway is involved in idiopathic pulmonary fibrosis has gradually increased.[47,48] In our report, EQ inhibited the expression of the TGF-β receptors TGF-βRI and TGF-βRII, and hindered the phosphorylation of Smad2 and Smad3. Previous reports have supported that HSP90 can stabilize TGF-β receptors and Smads and that inhibition

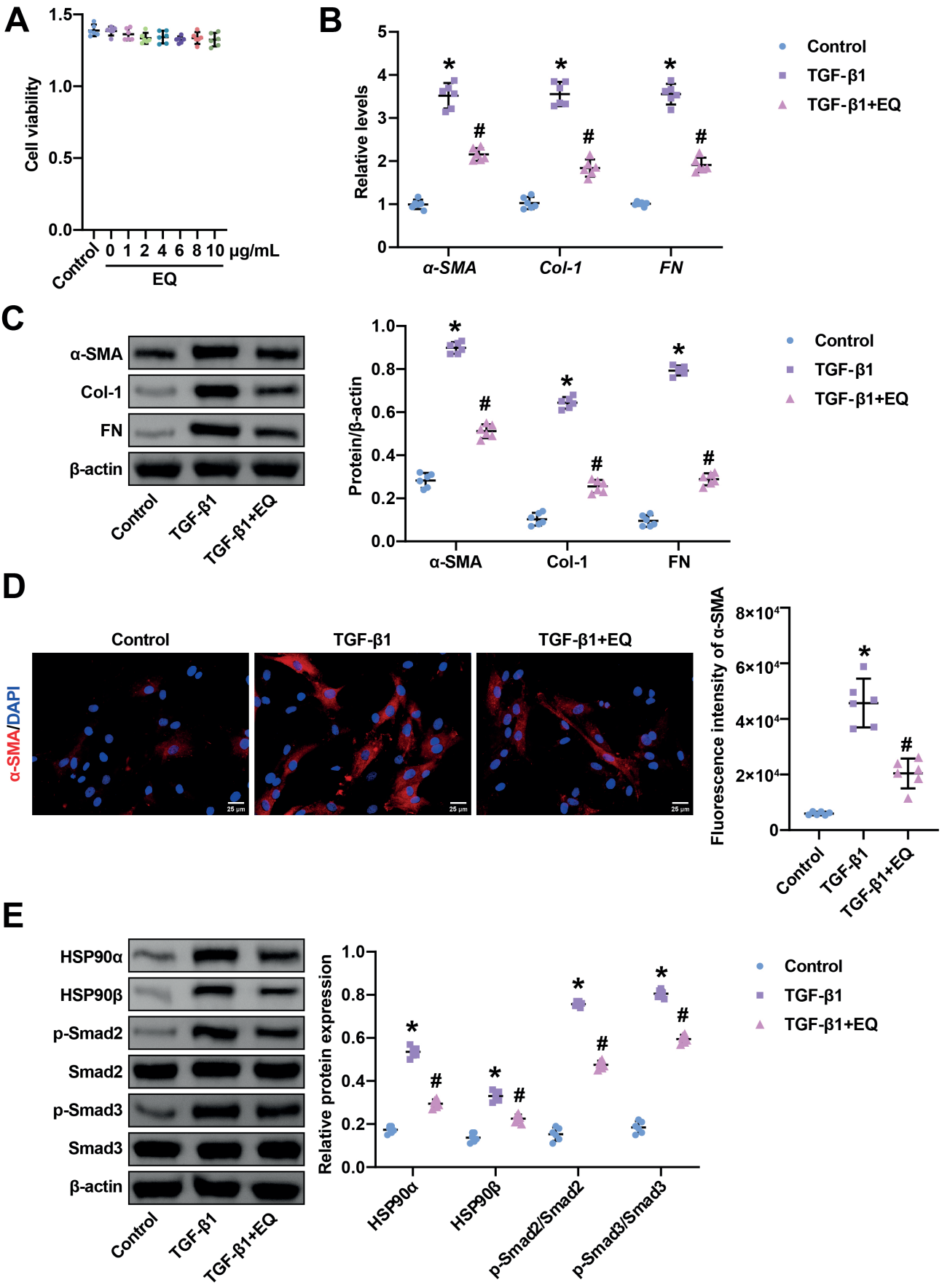

Fig. 7. Ethoxyquin (EQ) regulates the progression of rheumatoid arthritis-associated interstitial lung disease (RA-ILD) through the transforming growth factor beta (TGF-β)/Smad2/3 pathway in vitro. A. The analysis of HLF1 cells in response to EQ cytotoxicity; B. Relative mRNA levels of alpha-smooth muscle actin (*α-SMA*), collagen I (*Col-1*) and fibronectin (*FN*) in cells; C. Relative protein levels of α-SMA, Col-1 and FN in cells; D. Assessment of α-SMA expression is shown; scale bar = 25 μm; E. The abundance of HSP90α, HSP90β, p-Smad2, Smad2, p-Smad3, and Smad3 in cells

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Data were expressed as the mean with a 95% confidence interval (95% CI); *$p < 0.05$ vs control group; $^{\#}$ $p < 0.05$ vs TGF-β1 group.

of HSP90 attenuated TGF-β-driven myofibroblast transformation and EMT deposition.[49,50] We found that EQ suppressed TGF-β1-induced expression of α-SMA, Col-1 and FN in HLF1 cells. Taken together, our data reveal that EQ alleviated pulmonary fibrosis in RA-ILD by impeding the TGF-β1/Smad2/3 pathway.

The number and phenotype of macrophages are critical to the fibrotic process, and induction of M2 macrophage polarization aggravates the development of pulmonary fibrosis.[51] Studies have shown that MBD2 stimulates PI3K/Akt signaling to enhance the macrophage M2 phenotype, and MBD2 knockdown protects BLM-induced lung fibrosis by depleting M2 macrophages.[52] The M2 macrophage-derived microRNAs (miRNAs) are thought to promote lung fibrosis.[53] Furthermore, M2 macrophages are the main source of TGF-β1, which induces fibroblast differentiation and proliferation.[54] Based on the above data, we hypothesized that EQ might impair macrophage M2 polarization to protect CIA mice from BLM-induced pulmonary fibrosis. As expected, we observed that EQ increased the proportion of M1 macrophages and decreased M2 in peripheral blood and BALF, promoting the switch of macrophages from an M2 to an M1 phenotype. Examination of the levels of markers associated with M1 and M2 macrophages supported this finding. In BLM-CIA mice, EQ upregulated iNOS and TNF-α and downregulated VEGF-A and TGF-β1.

In this study, we also found an imbalance in the T cell subsets in BLM-CIA mice. The BLM increased Th1 cells (IFN-γ) and Treg cells (Foxp3) and decreased Th17 cells (IL-17A) in peripheral blood and BALF. Ethoxyquin reversed the effect of BLM on Th1/Th17/Treg cells in BLM-CIA mice. T cells are key players in pulmonary fibrosis.[55] An increase in the ratio of Th1/Th2 cells is widely thought to exert an anti-fibrotic effect.[56,57] Recent evidence supports the pro-fibrotic role of Th17 cells, and inhibition of Th17 production can prevent BLM-induced pulmonary fibrosis.[58,59] Tregs seem to have a dual role in pulmonary fibrosis, which may be related to different models and different stages of pulmonary fibrosis.[60] In addition, TGF-β1 is very important for T cell response in pulmonary fibrosis,[61,62] but whether EQ mediates T cell differentiation and functional maintenance in RA-ILD through the TGF-β1/Smad2/3 pathway needs further evidentiary support.

Limitations

There are some limitations to this study. A constrained timeframe and financial constraints prevented the application of TGF-β1 in an animal model to explore the potential involvement of the TGF-β1/Smad2/3 pathway in the function of EQ. Different animal models could produce varying outcomes, and further analysis is required to understand the inhibitory effects of EQ on HSP90 fully. The inability to examine the efficacy and safety of EQ in therapeutic animal models is also a limitation of this study. It should be noted that the results from the BLM-CIA mouse model and the TGF-β1-induced HLF1 cell model may not necessarily reflect the same effects of EQ in human RA-ILD, and further validation in clinical samples is required. The role of the airway responsiveness test in assessing the development of RA-ILD is limited. Therefore, additional evidence is still needed to fully evaluate the effect of EQ on lung function in BLM-CIA mice. The association between RA-ILD and RA-airway diseases is worth exploring. The optimal administration route and dosage of EQ for BLM-induced CIA mice still needs to be determined. Moreover, additional research is needed to elucidate the significance of macrophage polarization and T cell responses in the development of RA-ILD, as well as to gather more evidence supporting the regulatory role of EQ on macrophage polarization and T cell responses. Sample sizes for the in vitro and in vivo experiments were also limited. We plan to utilize larger sample sizes in future studies.

Conclusions

Our work identified EQ's previously unrecognized critical role in RA-ILD. We confirmed that EQ inhibited the TGF-β1/Smad2/3 pathway to attenuate synovitis, joint destruction and pulmonary fibrosis in BLM-CIA mice. Furthermore, we also illustrated that EQ promoted M2-to-M1 programming of macrophages and affected the differentiation of Th1/Th17/Treg cells. These results support the possible application of EQ as a therapy addressing the pathophysiology of RA-ILD.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5072/zenodo.34794. The package includes the following files:

Supplementary Table 1. Normality and uniformity test results of data and main test results in Fig. 2.

Supplementary Table 2. Normality and uniformity test results of data and main test results in Fig. 3.

Supplementary Table 3. Normality and uniformity test results of data and main test results in Fig. 4.

Supplementary Table 4. Normality and uniformity test results of data and main test results in Fig. 5.

Supplementary Table 5. Normality and uniformity test results of data and main test results in Fig. 6.

Supplementary Table 6. Normality and uniformity test results of data and main test results in Fig. 7.

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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Small RNA sequencing highlights a potential regulatory network mediated by Gecko miRNA affecting the prognosis of hepatocellular carcinoma

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Conflict of interest
None declared

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Abstract

Background. Gecko has been widely documented in Chinese scientific literature as an anti-tumor agent for various illnesses for thousands of years, and more recently, it has been examined for its anti-tumor effects on several cancers. The effect of Gecko microRNAs (miRNAs) on hepatocellular carcinoma (HCC) has not yet been reported.

Objectives. This study was designed to identify miRNAs in Gecko through small RNA sequencing and utilize bioinformatics techniques to construct a potential regulatory network and explore the possible mechanisms of exogenous miRNAs involved in HCC.

Materials and methods. RNA was extracted from Gecko tablets, and we screened the Gecko miRNA expression dataset after high-throughput sequencing. Bioinformatics analysis was used to identify novel Gecko and HCC survival-related miRNA-mRNA cross-species regulation networks.

Results. miR-100-5p, miR-99a-5p and miR-101-3p were identified as critical for the role of Geckos in HCC. Nine downstream mRNAs (*EZH2*, *KPNA2*, *LMNB1*, *LRRC1*, *MRGBP*, *SMARCD1*, *STMN1*, *SUB1*, and *UBE2A*) were identified as target genes for critical miRNAs. A miRNA-mRNA regulatory network was constructed, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed these key mRNAs might be associated with both the suppression and progression of HCC. The novel network significantly correlated with the abundance of multiple immune cells, as determined with immune infiltration analysis.

Conclusions. These findings suggest that Gecko may inhibit progression and exert a therapeutic effect on HCC by targeting critical miRNA-mRNA networks for cross-species regulation. It also provides a reference for future research and development of traditional Chinese medicine (TCM).

Key words: hepatocellular carcinoma, miRNA, immunity, traditional Chinese medicine, Gecko

Acknowledgements
We would like to express our gratitude to The First Hospital of Hunan University of Chinese Medicine for providing the Gecko samples and conducting the experimental analysis. Additionally, we would like to thank all study participants from the School of Integrated Chinese and Western Medicine of Hunan University of Chinese Medicine for their assistance with data organization and equipment support.

Background

MicroRNAs (miRNAs) are small, endogenous noncoding RNAs approx. 22 bases in length that primarily exert negative regulation on target protein-coding genes. They exhibit specific complementary binding to their targets, enabling a single miRNA to target numerous genes. The mechanism of this multi-target regulation coincides with the therapeutic theory of traditional Chinese medicine (TCM).[1] As tumor suppressors, miRNAs are associated with various biological processes, including cell proliferation, differentiation and apoptosis. Meanwhile, research on using miRNAs for tumor therapeutics is proliferating and has received extensive attention and investigation.[2] Recent studies have revealed that miRNAs derived from herbal medicines can enter mammalian cells upon ingestion, where they stabilize and modulate gene expression, thereby influencing the functions of tissues and cells.[3–5]

In recent years, most of the anti-tumor studies of exogenous miRNAs have originated from medicinal plant miRNAs or compounds, while relatively few studies have been conducted on the effects of animal-based miRNAs on tumors.[6] Gecko is commonly used in TCM, and numerous clinical studies have shown that it exhibits good efficacy against cancers of the digestive tract, especially esophageal, liver and gastric cancers, inhibiting tumor development and improving patient immunity.[7,8] The aqueous Gecko extract can inhibit the growth of liver cancer cells, and the Gecko protein can induce apoptosis of cancer cells without adversely affecting normal cells.[9,10] Current studies have focused only on Gecko protein crude extracts and clinical efficacy, but there has been no extraction and identification of Gecko miRNAs and exploration of their molecular anti-tumor mechanisms. Herein, we acquired the miRNA sequences of Gecko through small RNA sequencing, and we used bioinformatics data integration analysis to construct a potential regulatory network of Gecko miRNAs affecting the prognosis of hepatocellular carcinoma (HCC), which was validated using multi-pathway enrichment analysis and immune infiltration correlation.

Objectives

The aim of the study was to screen the prognostic core miRNAs of Gecko and to establish a model of Gecko miRNA regulatory network by combining the survival analysis, differential expression and immune infiltration analysis, in order to better explore the mechanism of Gecko miRNAs in HCC.

Materials and methods

Gecko miRNA sequencing

Chinese medicinal geckos were purchased from the First Hospital of the Hunan University of Chinese Medicine (Changsha, China), dried after removing the viscera and processed by powdering. The total RNA of Gecko powder was extracted with the TRIzol method to estimate RNA concentration and integrity, and sRNA libraries were constructed using the Truseq Small RNA Library Prep Kit tool. The library was tested for quality and then sequenced using the Illumina HiSeq 2500 high-throughput sequencing platform (Beijing Novogene Technology Co., Beijing, China). The miRNAs with a read count value higher than 105 and a Transcripts per million (TPM) value higher than 7,000 were included in the miRNA sequencing data of Gecko in descending order of expression. The selected miRNA base sequences were compared with human miRNAs to obtain co-expressed miRNAs for subsequent studies.

Data collection

To screen out differential miRNAs in HCC, we downloaded human miRNA expression profiles GSE147889 (including 2565 miRNAs from 97 cancer samples and 97 adjacent standard samples) from the Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo),[11] which is based on the GPL21263 platform (3D-Gene Human miRNA V21_1.0.0).[12] Furthermore, miRNA expression profiles of HCC patients (including 374 cancer and 50 normal samples) and clinical information of tumor samples (including 374 samples) were also obtained from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov) database to improve the reliability of the results.[13]

Identification of key miRNAs

Gecko and human identically expressed miRNAs were intersected with prognostically relevant differential miRNAs in HCC patients to obtain Gecko and human co-expressed key miRNAs for follow-up studies. In addition, we further validated the expression patterns of key miRNAs in multiple cancers using the CancerMIRNone (CancerMIRNone; http://bioinfo.jialab-ucr.org/CancerMIRNome/) database.[14] This database is a web-based tool that encapsulates a range of cutting-edge bioinformatics tools and machine learning algorithms that allow analysis of miRNAs of interest in multiple cancer types to identify divergent miRNAs and develop diagnostics, prognostic signatures, comparisons, and visualizations.

Enrichment analysis

The obtained miRNAs were enriched using miRTarBase (https://mirtarbase.cuhk.edu.cn),[15] miRDB (http://www.mirdb.org) and TargetScan (http://www.targetscan.org)[16] databases for downstream target gene prediction, while the predicted downstream target genes from the 2 databases were compared with the dataset and the intersection of target genes was obtained. The regulatory interactions between miRNAs and mRNAs helped to form a basic regulatory network of miRNA-targeting mRNAs. The miRNA-mRNA targeting network was viewed using Cytoscape (v. 3.8.2) software (https://cytoscape.org).[17] To better understand the roles of key Gecko miRNAs, we analyzed these miRNAs for Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment using the R package (R Foundation for Statistical Computing, Vienna, Austria).

Validation of gene expression and survival analyses

Utilizing gene expression and clinical data from the TCGA database, we performed univariate Cox regression analysis using the "survival" package to screen for target genes significantly associated with prognosis. Subsequently, we assessed the downstream mRNA expression levels targeted by miRNAs in HCC to identify prognostically relevant upregulated target genes for further analysis. The expression differences of these genes were illustrated with boxplot, and the clinical prognostic significance between high and low expression groups was depicted with Kaplan–Meier (KM) survival curves, with the hazard ratio outlined in a forest plot. All figures were generated using RStudio software (v.2023.12.1+402; https://posit.co/products/open-source/rstudio).

Survival-related miRNA network identification and annotation

Based on the aforementioned predictions and assessments, we constructed and visualized a novel miRNA-mRNA regulatory network associated with Gecko and HCC patient prognosis using Cytoscape. By further analyzing the role of Gecko miRNAs in this regulatory network, KEGG and GO enrichment analysis of downstream mRNAs were explored through the SangerBox database (http://sangerbox.com/Tool). The DisGeNET database (http://www.disgenet.org/) was utilized to investigate the molecular underpinnings of human diseases and their complications, analyze the features of disease-related genes, and evaluate the associations between genes and diseases.[18]

Immune infiltration analyses

In this study, we utilized CIBERSORT (https://cibersortx.stanford.edu/) to investigate the association between Gecko miRNA regulatory networks and immune cell infiltration in HCC. CIBERSORT is a deconvolution algorithm to predict the proportion of 22 tumor-infiltrating immune cells in each sample based on gene expression data.[19] The immune cell infiltration matrix was divided into 2 groups according to the median expression of key genes, and thus, the association between key genes and tumor-infiltrating immune cells was analyzed using Spearman's correlation analysis.

Statistical analyses

R software (v. 4.3.0; http://www.r-project.org/) was used to convert miRNA probe IDs into mature miRNA names, based on the platform annotation file. Adjusted p-values for differential expression of miRNAs and genes were computed as false discovery rate (FDR) using the "limma" package.[20] Wilcoxon test was employed to compare differences between 2 groups containing non-parametric data. The KM method and a log-rank test were used to measure overall survival (OS) employing the "survival" package of R. Univariate Cox regression analysis was applied to test the risk factors. Martingale residuals were used for the linearity assumption testing. The proportionality of the hazard function was checked based on the Schoenfeld residuals using the R function "cox.zph".[21] The results of all hypotheses are presented in Supplementary Table 1 and Supplementary Fig. 1. The correlation between target gene expression and immune infiltration was analyzed using Spearman's method. The ranked data from the Spearman's correlation analysis are detailed in Supplementary Table 4, and the results of the Spearman correlation analysis are provided in Supplementary Table 5. Only enrichment terms with $p < 0.05$ were deemed statistically significant and reported in our analysis.

Results

Sequencing and screening of Gecko miRNAs

By sequencing Gecko miRNAs and comparing them with the same miRNA bases in human, a total of 19 co-expressed miRNAs were obtained after taking into account the miRNAs with read count values greater than or equal to 105 and TPM values greater than 7000 in the study, including miR-1a-3p, miR-143-3p, miR-148a-3p, miR-21-5p, miR-26-5p, miR-203-3p, let-7f-5p, miR-100-5p, miR-206-3p, miR-184-3p, miR-10b-5p, miR-99a-5p, miR-140-3p, let-7e-5p, miR-122-5p, miR-9-5p, let-7i-5p,

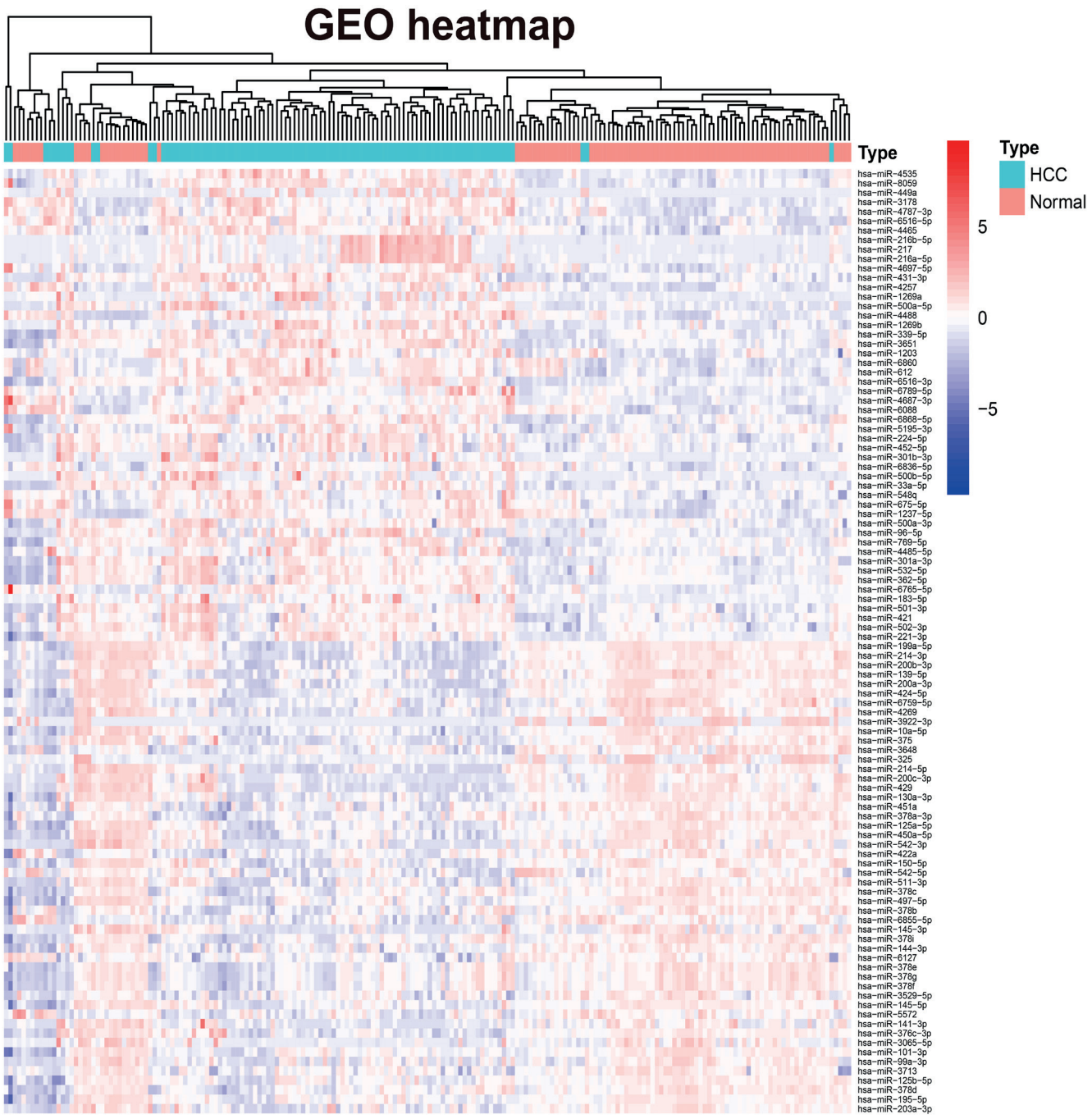

Fig. 1. Heatmap of differentially expressed miRNAs in GSE147889 dataset of Gene Expression Omnibus (GEO). Upregulation is indicated by red, while downregulation is represented by blue. The columns and rows respectively correspond to samples and miRNAs

miR-101-3p, and miR-200a-3p. The whole read counts, TPM values and sequence numbers are shown in Table 1.

Certification of DE-miRNAs in HCC

Differentially expressed miRNAs in the GEO and TCGA datasets were identified using empirical Bayes moderation in the "limma" package with adjusted $p < 0.05$ and $|\log2\ FC| > 0.5$, as depicted in the heat maps (Fig. 1,2) and volcano plots (Fig. 3A). In the TCGA dataset, 394 differential miRNAs were selected, while the GEO database identified 189 differential miRNAs. Using the Venn diagram intersection (Fig. 3B), 92 miRNAs were differentially expressed in both datasets and were selected for subsequent analysis.

Key miRNA identification

To assess the prognostic value of differential miRNAs on the OS of HCC, clinical information of corresponding patients, including the survival status and survival time

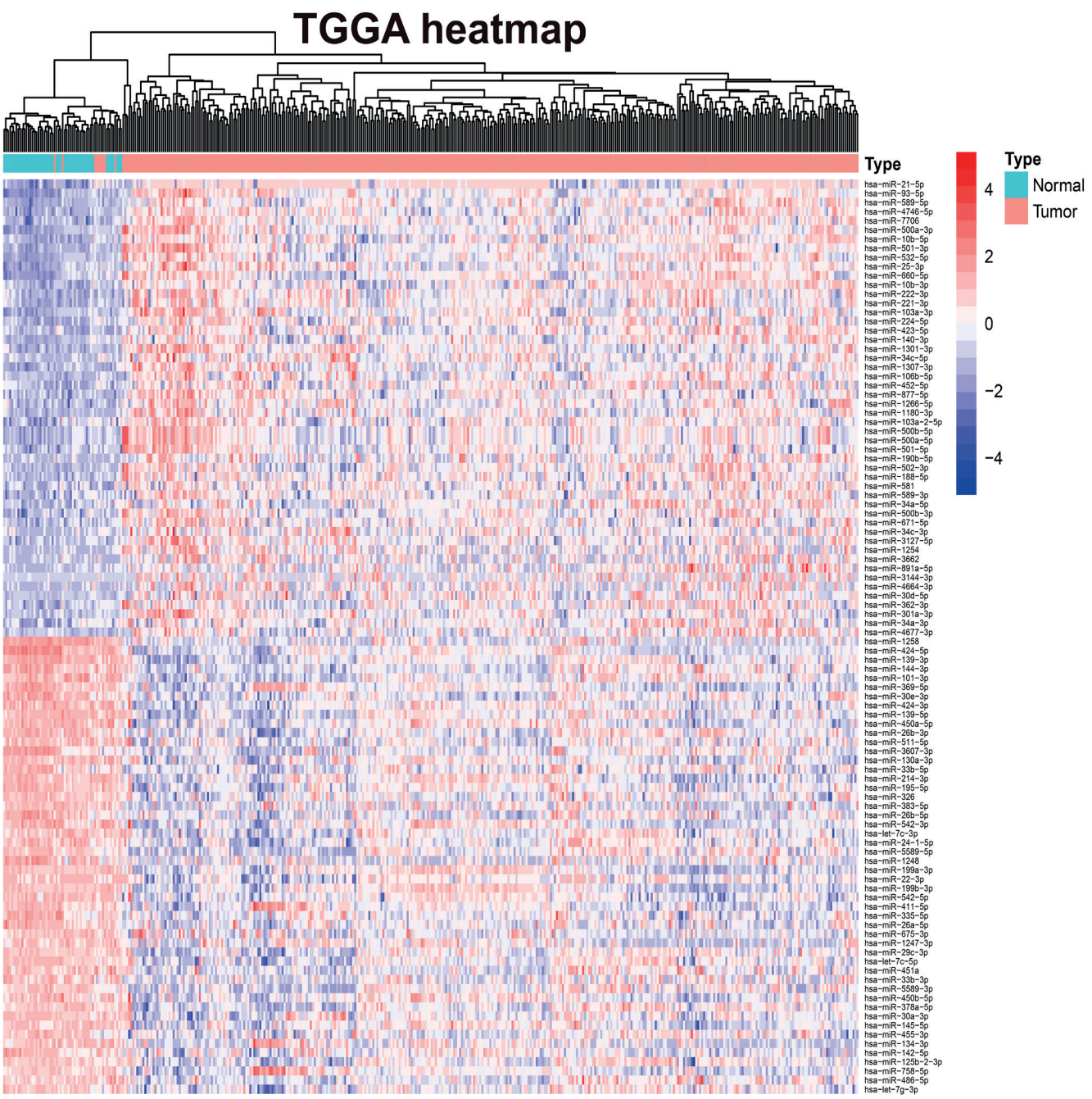

Fig. 2. Heatmap of differentially expressed miRNAs in The Cancer Genome Atlas (TCGA) database. Upregulation is indicated by red, while downregulation is represented by blue. The columns and rows respectively correspond to samples and miRNAs

of 373 HCC patients, was gathered from the TCGA database. Survival-related univariate Cox analysis and KM survival analysis were performed, and 24 miRNAs out of 92 differentially expressed miRNAs were significantly associated with the prognosis of HCC patients (Fig. 4A). Taking the intersection of the above 24 miRNAs with the 19 co-expressed miRNAs of Gecko showed that 4 differential miRNAs were most likely to be significantly associated with the prognosis of HCC patients (miR-9-5p: $p = 0.008$, miR-99a-5p: $p = 0.001$, miR-100-5p: $p < 0.001$, and miR-101-3p: $p = 0.032$). Meanwhile, we further compared the expression levels of these survival-related miRNAs between cancer and normal samples in the TCGA dataset (Fig. 4B). Among them, miR-100-5p, miR-99a-5p and miR-101-3p exhibited low expression and were associated with poor prognosis in human HCC ($p < 0.001$) (Fig. 4C). To enhance the reliability of the screening results, we performed a pan-cancer analysis in the CancerMIRNome database to validate these 3 differential miRNAs (Fig. 4D). miR-100-5p, miR-99a-5p and miR-101-3p differed in multiple cancer types, including liver cancer. Therefore, these 3 miRNAs were selected for subsequent analysis.

Table 1. Gecko miRNA sequencing values and screening table (n = 19)

Gecko mature miRNA	ReadCount	TPM	Mature sequences	Human Identical Sequences miRNA
aca-miR-1a-3p	5254	351602.7571	UGGAAUGUAAAGAAGUAUGUAU	miR-1a-3p
aca-miR-143-3p	2357	157732.7177	UGAGAUGAAGCACUGUAGCUC	miR-143-3p
aca-miR-148a-3p	546	36538.84762	UCAGUGCACUACAGAACUUUGU	miR-148a-3p
aca-miR-21-5p	492	32925.11544	UAGCUUAUCAGACUGAUGUUGA	miR-21-5p
aca-miR-26-5p	440	29445.22519	UUCAAGUAAUCCAGGAUAGG	miR-26-5p
aca-miR-203-3p	369	24693.83658	GUGAAAUGUUUAGGACCACUUG	miR-203-3p
aca-let-7f-5p	346	23154.65435	UGAGGUAGUAGAUUGUAUAGUU	let-7f-5p
aca-miR-100	324	21682.39309	AACCCGUAGAUCCGAACUUGUG	miR-100-5p
aca-miR-206-3p	223	14923.37549	UGGAAUGUAAGGAAGUGUGUG	miR-206-3p
aca-miR-184-3p	204	13651.87713	UGGACGGAGAACUGAUAAGGGU	miR-184-3p
aca-miR-10b-5p	203	13584.95617	UACCCUGUAGAACCGAAUUUGU	miR-10b-5p
aca-miR-99a-5p	178	11911.93201	AACCCGUAGAUCCGAACUUGCGG	miR-99a-5p
aca-miR-140-3p	177	11845.01104	UACCACAGGGUAGAACCACGG	miR-140-3p
aca-let-7e-5p	127	8498.962725	UGAGGUAGUAGAUUGAAUAGUU	let-7e-5p
aca-miR-122-5p	117	7829.753062	UGGAGUGUGACAAUGGUGUUUG	miR-122-5p
aca-miR-9-5p	116	7762.832095	UCUUUGGUUAUCUAGCUGUA	miR-9-5p
aca-let-7i-5p	113	7562.069196	UGAGGUAGUAGUUUGUGCUGUU	let-7i-5p
aca-miR-101-3p	107	7160.543398	UACAGUACUGUGAUAACUGA	miR-101-3p
aca-miR-200a-3p	105	7026.701466	UAACACUGUCUGGUAACGAUGU	miR-200a-3p

Both Gecko miRNAs and human miRNAs were derived from the mature sequences that ranked in the top 19 of sequencing results compared with the comprehensive database. TPM – transcripts per million.

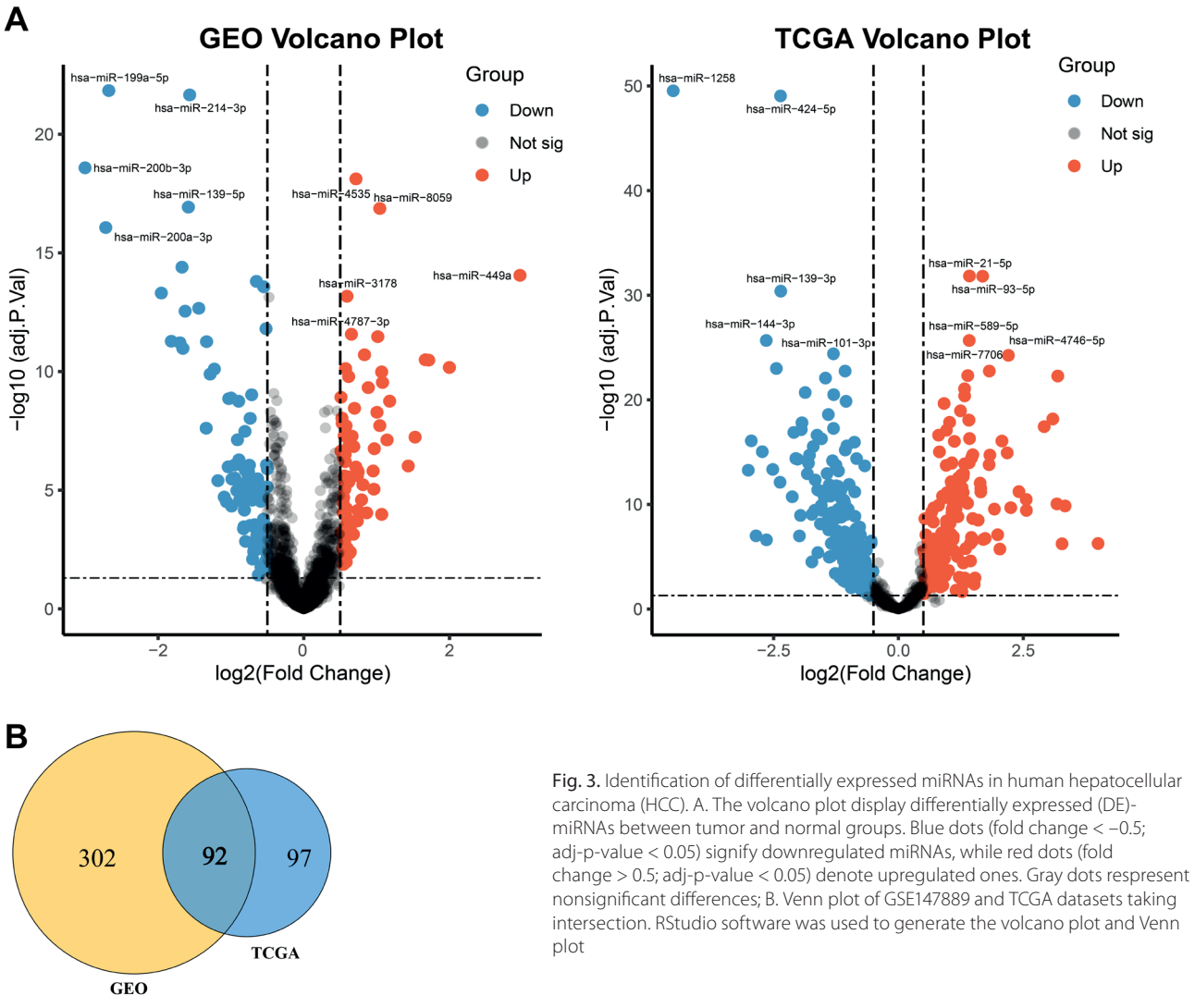

Fig. 3. Identification of differentially expressed miRNAs in human hepatocellular carcinoma (HCC). A. The volcano plot display differentially expressed (DE)-miRNAs between tumor and normal groups. Blue dots (fold change < −0.5; adj-p-value < 0.05) signify downregulated miRNAs, while red dots (fold change > 0.5; adj-p-value < 0.05) denote upregulated ones. Gray dots respresent nonsignificant differences; B. Venn plot of GSE147889 and TCGA datasets taking intersection. RStudio software was used to generate the volcano plot and Venn plot

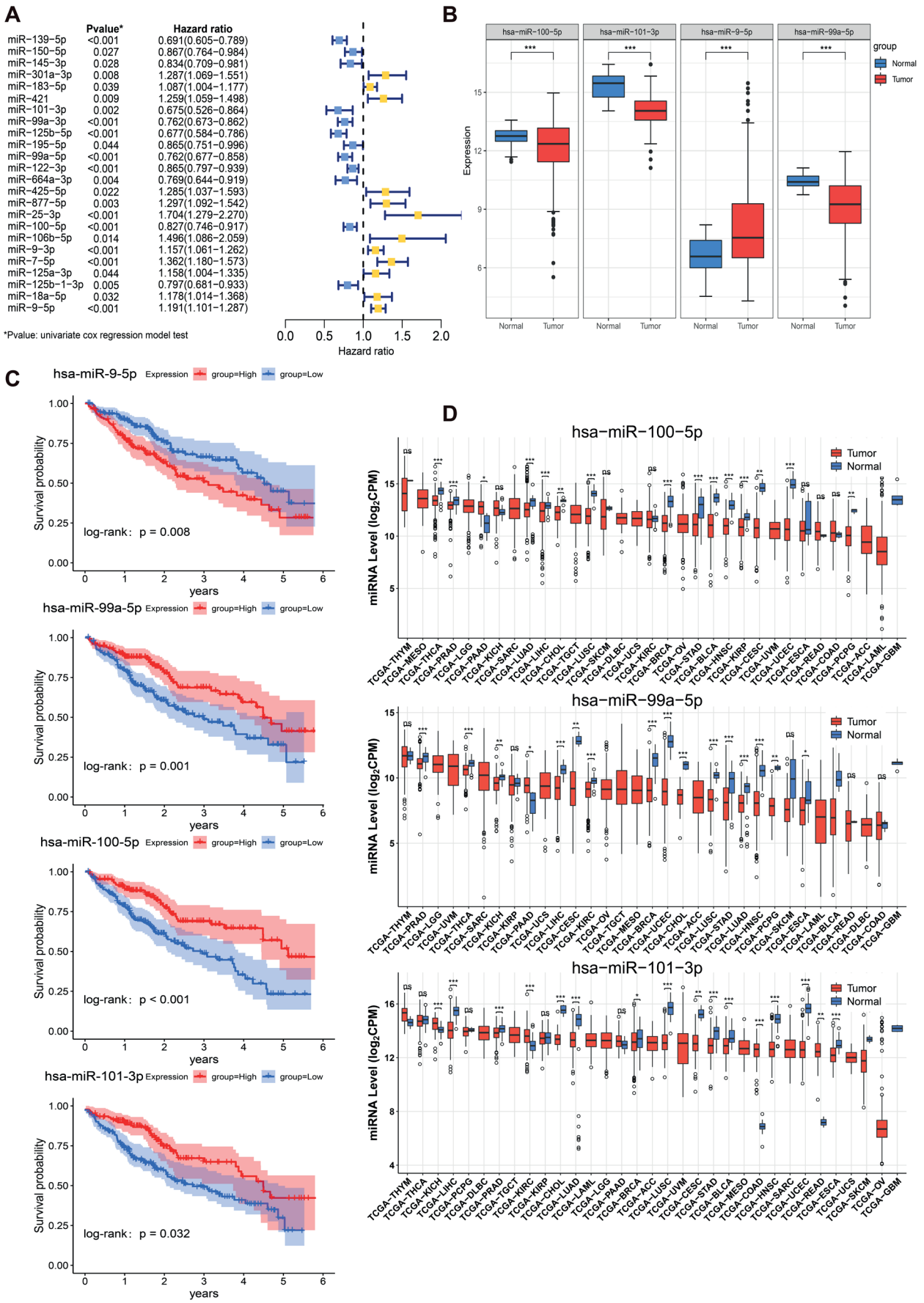

Fig. 4. Identification of crucial Gecko miRNAs in hepatocellular carcinoma (HCC). A. Forest plot demonstrated the correlations between the prognostic miRNAs and survival. The p-value was calculated using univariate Cox regression analysis; B. Expression of Gecko key miRNAs. The boxplot whiskers connect the minimum and maximum values of the overall data (excluding outliers), with a middle horizontal line inside representing the median. The upper and lower horizontal lines indicate the maximum and minimum values, while the dots denote outliers; C. Kaplan–Meier curves of key miRNAs. The log-rank test was used for statistical analysis; D. Expression boxplots in multiple cancer types of has-miR-100-5p, has-miR-99a- 5p and has-miR-101-3p. Wilcoxon rank-sum test was used to analyze the data

ns – no significance; ***p < 0.001; **0.001 < p < 0.01; *0.01 < p < 0.05.

Construction of miRNA regulatory network

To identify the downstream mRNAs that these 3 key miRNAs might target, we applied miRTarBase, targetScan and miRDB databases to improve the confidence of the predictive results. The 3 databases intersected a total of 198 mRNAs, of which 35 downstream mRNAs were targeted by miR-99a-5p, while there were 195 downstream mRNAs likely to be targeted by miR-101-3p and 68 downstream mRNAs likely to be targeted by miR-100-5p. Based on the above predictions, the miRNA regulatory network consisting of 3 miRNAs and 198 target mRNAs were established and visualized using Cytoscape (Fig. 5A). The intersection targets of the databases are presented using a Venn diagram (Fig. 5B).

Further enrichment analysis

To better understand these candidate target genes, GO function, and KEGG pathway enrichment analysis were performed using R software. A total of 33 KEGG pathways and 234 GO terms were enriched, showing each group's top 10 GO-enriched terms and the top 10 KEGG-enriched pathways in terms of p-value. In the biological process (BP), the miRNA regulatory network was mainly enriched for regulating transmembrane receptor protein serine/threonine kinase signaling pathways, cellular response to transforming growth factor β stimulation, glycerolipid biosynthesis process, and regulation of the Wnt signaling pathway. In the cellular component (CC), the network was mainly enriched in ribonucleoprotein granules, fiber centers and cytoplasmic stress granules. It demonstrated significant enrichment in molecular functions (MF), including phosphatase activity, transcriptional co-regulatory activity, activin binding, and ubiquitin-protein transferase regulatory activity. These findings suggested that the miRNA network play a crucial role in cell apoptosic, migration and proliferation (Fig. 5C). Furthermore, KEGG enrichment analysis revealed significant enrichment of miRNA regulatory networks in various cancer-related pathways, including the MAPK and PI3K-Akt signaling pathways, miRNA in cancer, HCC, Th17 cell differentiation, and transcriptional dysregulation in cancer pathways (Fig. 5D).

Identification of key mRNAs

Based on miRNA regulation theory, miRNAs may be adversely correlated with downstream mRNAs. Consequently, we collected the expression profiles of 198 target mRNAs in the regulatory network from The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) database and assessed the expression levels of these mRNAs using the "limma" package. Additionally, the prognostic value of these mRNAs on OS was evaluated using the "survival" package. Among them, 15 target genes were found to be significantly upregulated in LIHC (Supplementary Table 2). Additionally, 38 target genes were found to be significantly associated with HCC prognosis using univariate Cox survival model analysis (Supplementary Table 3). We finally took the intersection to obtain 9 upregulated genes (*EZH2*, *KPNA2*, *LMNB1*, *LRRC1*, *MRGBP*, *SMARCD1*, *STMN1*, *SUB1*, and *UBE2A*). The KM survival curves and forest plots indicated that 9 genes were significantly correlated with the prognosis of patients with HCC (Fig. 6A,B). The boxplot showed differential expressions (Fig. 6C).

Survival-related miRNA regulatory network and enrichment analysis

Based on the above validation, a survival-related miRNA regulatory network of Gecko was established, including 3 miRNAs and 9 target genes (Fig. 7A). This regulatory network showed significant prognostic value, consistent with the miRNA hypothesis. Additionally, we investigated biological functions of the target genes using the Sangerbox database. Kyoto Encyclopedia of Genes and Genomes enrichment analysis revealed significant enhancement in cancer-related pathways, including miRNA in cancer signaling, MAPK pathway signaling and apoptosis signaling pathways. Gene Ontology enrichment in BP was supplemented in the regulation of the thrombin-activated receptor signaling pathway, multi-organism nuclear import and hepatocyte growth factor receptor signaling pathway. The CC pathway was enriched in a nuclear lumen, chromatin, nuclear part, and nucleoplasm, and the MF pathway was enriched in transcription coregulator activity, histone methyltransferase activity and primary miRNA binding (Fig. 7B). In the DisGeNET database, the miRNA regulatory network was considerably enriched for several cancer diseases, including follicular lymphoma, solid tumors, small cell carcinoma, and gallbladder cancer. The Gecko miRNA regulatory network significantly affects both tumor pathways and diseases (Fig. 7C).

Correlation of Gecko miRNA regulatory network with immune infiltration level in HCC

CIBERSORT analysis quantified immune cell proportions in tumor samples. Tumor specimens were categorized based on median gene expression, and the impact of differential gene expression on immune cell abundance was analyzed. The results indicated a significant influence on immune cell infiltration, including memory B cells, $CD4^+$ memory-activated T cells, T follicular helper cells, M0 macrophages, and others on high and low expression of 4 core target genes (*KPNA2*, *STMN1*, *EZH2*, and *LMNB1*) when compared to other genes (Fig. 8,9). Furthermore, we evaluated the correlation between the Gecko miRNA regulatory network and immune cell infiltration using Spearman's correlation analysis in the HCC microenvironment. As expected, we observed a negative monotonic

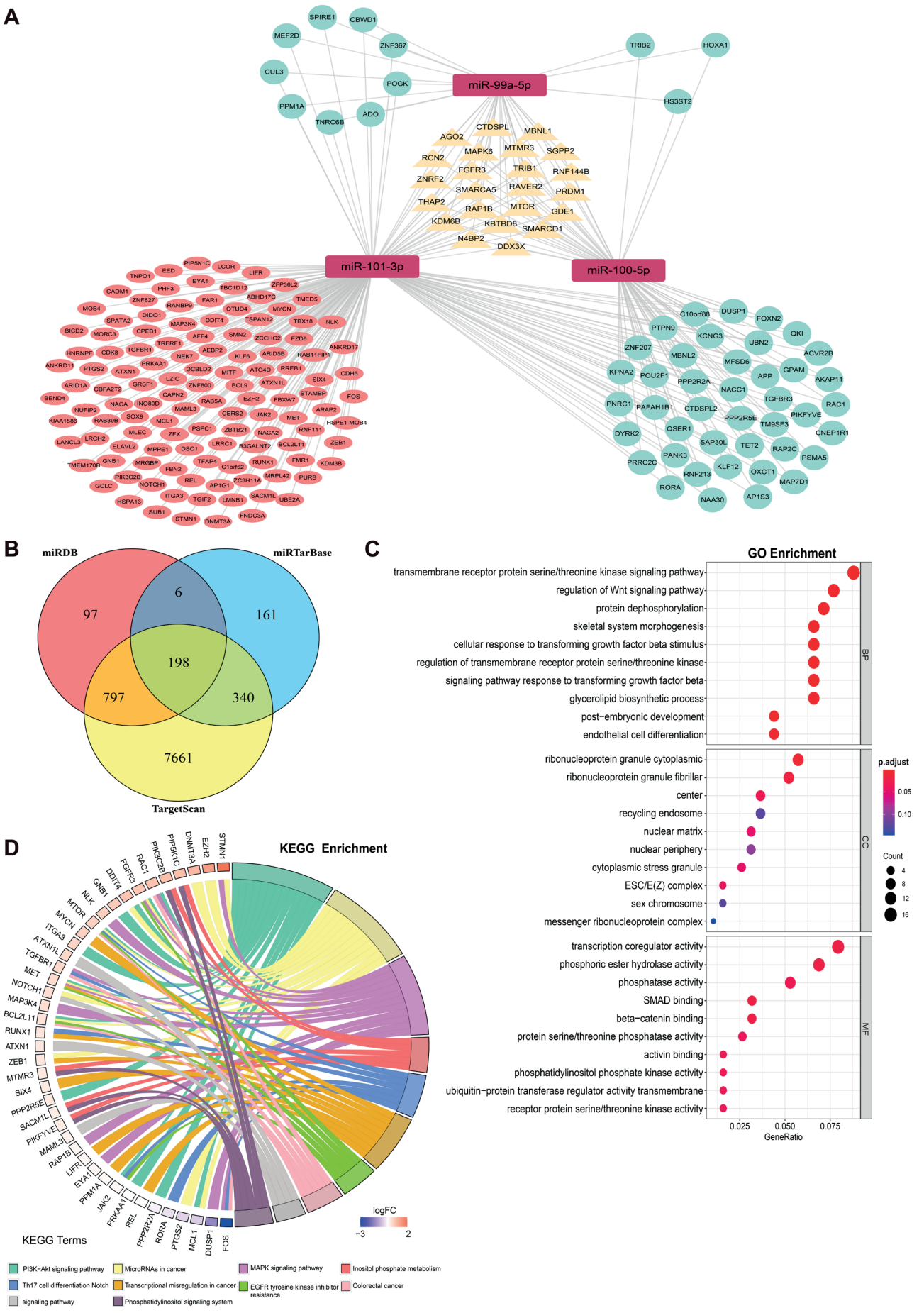

Fig. 5. Gecko miRNA target gene network construction and enrichment analysis. A. Network of Gecko key miRNA target genes constructed using Cytoscape software. The dark red rectangles in the network represent miRNAs, the yellow triangles represent genes targeted by 3 miRNAs together, the cyan circles represent genes targeted by 2 of the miRNAs, and the pink ovals represent genes targeted by 1 miRNA; B. Intersection of downstream mRNAs targeted by miR-99a-5p, miR-101-3p and miR-100-5p in miRDB, miRTarBase and Targetscan databases; C. Gene Ontology (GO) analysis of the Gecko miRNA network analysis: D. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the Gecko miRNA network

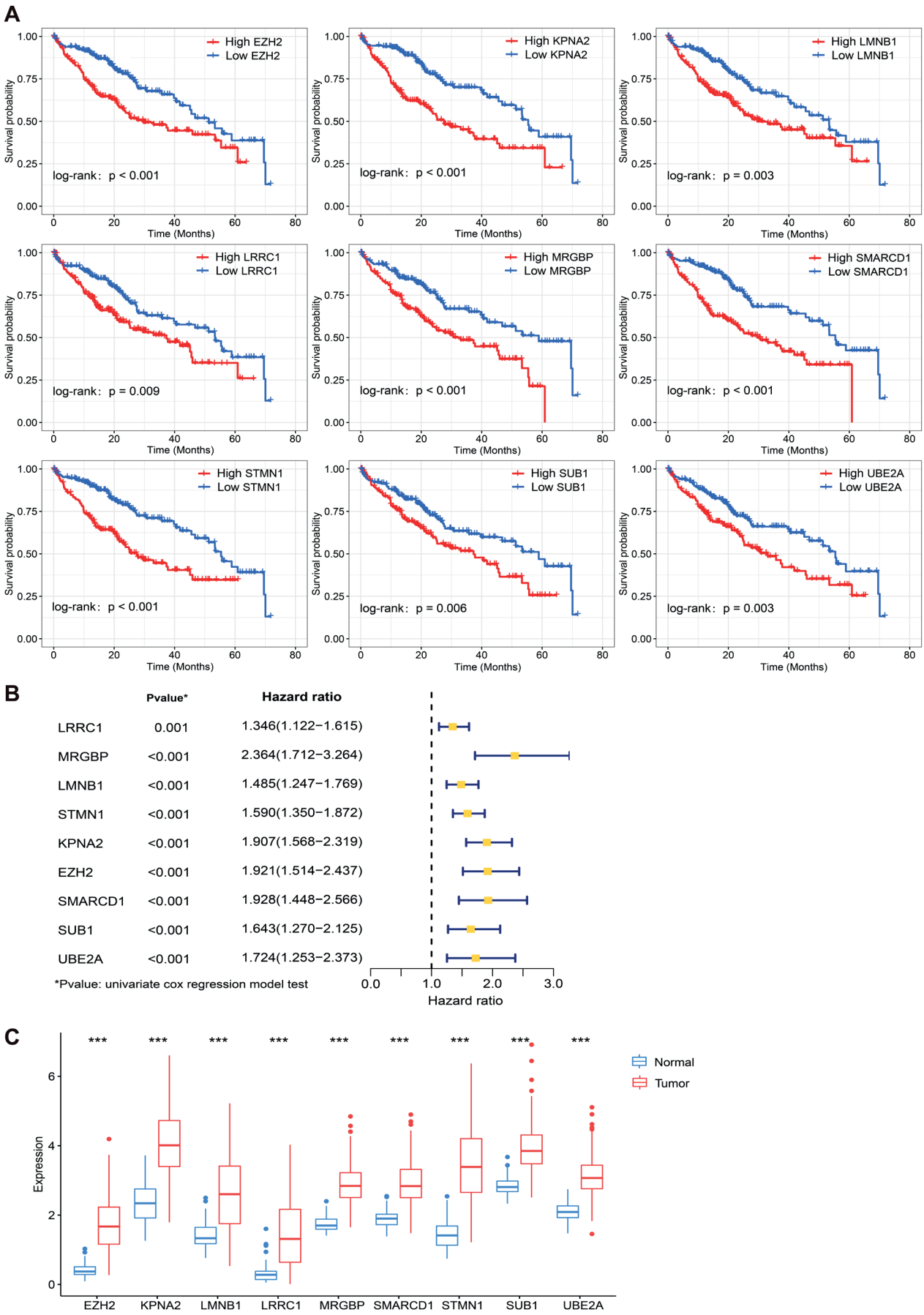

Fig. 6. Identification of Gecko-targeted key mRNAs in hepatocellular carcinoma (HCC). A. Downstream mRNAs' survival curves. The log-rank test was used for statistical analysis; B. Forest plot demonstrated the correlations between the 9 upregulated key mRNAs and survival. The p-value was calculated using univariate Cox regression analysis; C. The boxplot of downstream mRNA differential expression levels. Wilcoxon rank-sum test was used to analyze the data. The whiskers represent the range from the minimum and maximum values of the expression data, connected to the box. The middle horizontal line denotes the median, while the dots indicate outliers

ns – no significance; ***p < 0.001; **0.001 < p < 0.01; *0.01 < p < 0.05.

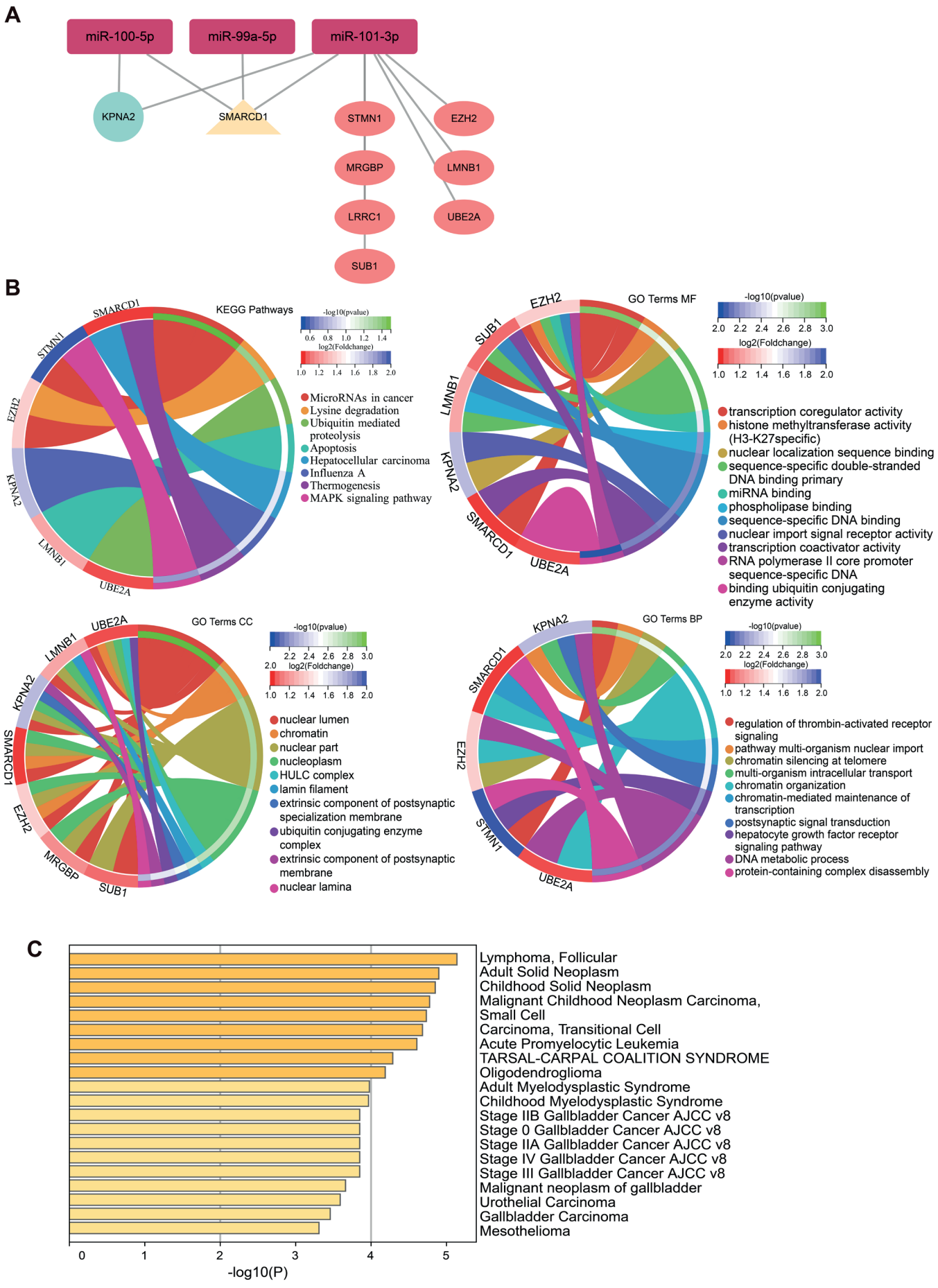

Fig. 7. Construction of Gecko survival-related miRNA-mRNA regulatory network in human hepatocellular carcinoma (HCC). A. Targeted regulatory network is associated with the prognosis of HCC patients; B. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis of mRNAs in the regulatory network; C. Disease enrichment analysis of mRNAs in the regulatory network

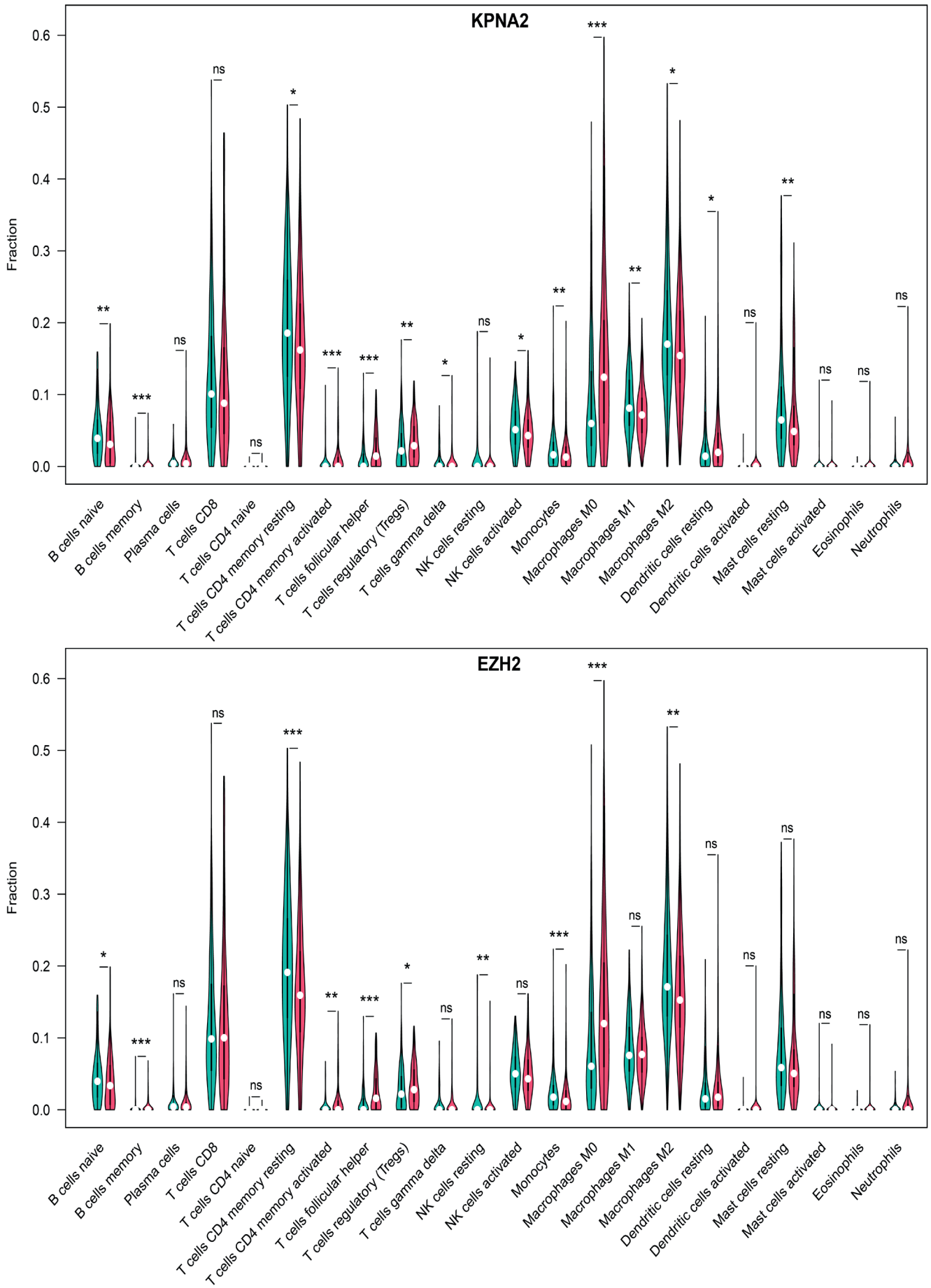

Fig. 8. Association of Gecko miRNA regulatory network expression with the level of immune infiltration. A. Violin plot of the correlation between core mRNA expression downstream KPNA2 and EZH2 of the Gecko regulatory network and the level of immune infiltration in hepatocellular carcinoma (HCC); p-values for expression differences were calculated using the Wilcoxon rank-sum test. Red indicates high expression, while green denotes low expression

ns – no significance; ***p < 0.001; **0.001 < p < 0.01; *0.01 < p < 0.05.

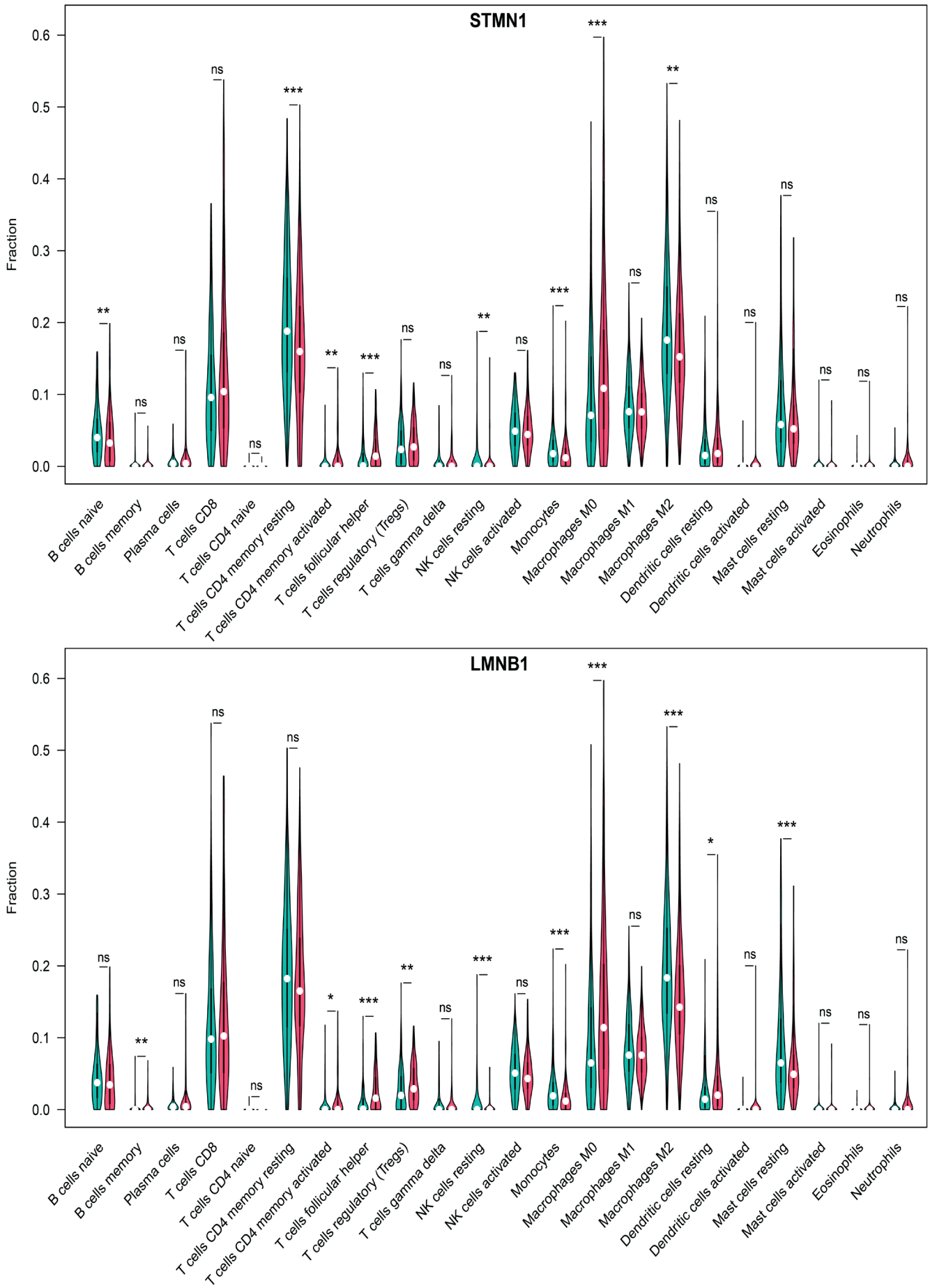

Fig. 9. Violin plot of the correlation between core mRNA STMN1 and LMNB1 of the Gecko regulatory network and the level of immune infiltration in hepatocellalar carcinoma (HCC); p-values for expression differences were calculated using the Wilcoxon rank-sum test. Red indicates high expression, while green denotes low expression

ns – no significance; ***$p < 0.001$; **$0.001 < p < 0.01$; *$0.01 < p < 0.05$.

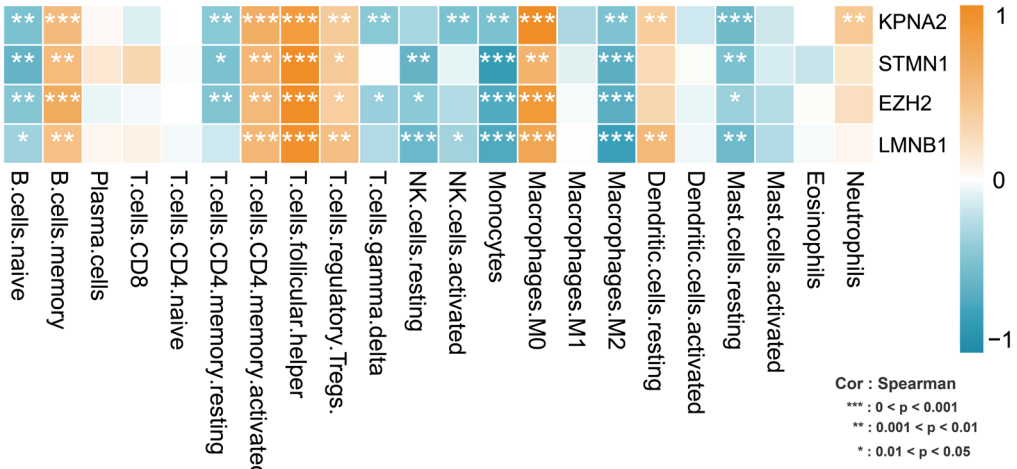

Fig. 10. Correlation heatmap analysis between core miRNA expression and 22 immune cells in hepatocellular carcinoma (HCC). The correlation analysis was conducted using Spearman's method. Positive correlations were depicted in orange, while negative in blue

ns – no significance; ***p < 0.001; **0.001 < p < 0.01; *0.01 < p < 0.05.

component of correlation with naïve B cells, monocytes, M2 macrophages, and resting mast cells, and a positive monotonic component with memory B cells, CD4 memory activated T cells, T follicular helper cells, T regulatory cells, and M0 macrophages (Fig. 10). This suggested a close link between the Gecko miRNA regulatory network and immune infiltration in HCC, potentially influencing immune cell function to inhibit HCC progression and development.

Discussion

Recent studies have demonstrated the potential of miRNA to therapeutically target cancer-related genes. Better targeting is provided by miRNA-based medicines, which can also be combined with other treatment methods, including chemotherapy, radiation therapy and immunotherapy, to increase their efficacy.[22,23] However, addressing several downstream targets and identifying harmful side effects are still difficult tasks. The construction and delivery of endogenous miRNA mimics are required to increase clinical usability,[24] while exogenous miRNAs derived from dietary sources exhibit potential as gene expression regulators with fewer negative consequences.[25] The role of exogenous miRNAs in tumor regulation has, however, received relatively little research, and there is still potential for further investigation. Moreover, various etiologies may also affect the role of systemic therapies and response markers such as miRNAs, thereby limiting their effect in a clinical setting.[26]

As a traditional animal-based Chinese medicine, Gecko has clear effects in inhibiting tumor proliferation and metastases and promoting tumor apoptosis, especially in digestive system tumors such as esophageal, liver and gastric cancers.[27] While current research on Gecko is mainly focused on herbal prescriptions and the utility of proteins, there is a lack of studies focusing on the extraction of small molecules such as miRNA. Furthermore, there are few studies on the mechanism of Gecko as an ingestible exogenous miRNA heterologous regulator of anti-tumor effects in humans. Therefore, exploring the regulatory network of Gecko-related miRNAs will aid our comprehensive understanding of the molecular mechanisms of gene interactions and regulation in human HCC, response to complex HCC and treatment through the multi-targeting effect of exogenous miRNAs in cells. It will also screen effective novel anti-tumor miRNA components, find alternative ways to synthesize the active ingredients of animal drugs, and provide new ideas for the inhibition mechanism of HCC. Finally, discovering circulating prognostic markers and predictive miRNAs in conjunction with efficacious drugs such as sorafenib is a future direction in the exploration of TCM.[28,29]

In this investigation, we discovered that Gecko miRNA components such as miR-100-5p, miR-99a-5p and miR-101-3p are associated with HCC prognosis. These miRNAs may serve as biomarkers for the anti-HCC actions of Gecko. By targeting Polo-like kinase 1 (PLK1), miR-100 overexpression slows the growth of HCC and causes apoptosis.[30] Moreover, by preventing lamellipodia and matrix metalloproteinase 2 (MMP2) activation, it also prevents HCC metastasis.[31] Additionally, miR-100 regulates mTOR and insulin-like growth factor-1 receptor (IGF-1R) to promote autophagy and prevent the growth of HCC.[32] MiR-99a, the 6th most abundant microRNA in normal human liver, is markedly reduced in HCC. Cholesterol-bound miR-99a mimics efficiently suppressed tumor growth and reduced α-fetoprotein levels in HCC-bearing mice upon intra-tumor injection.[33] Moreover, miR-99a overexpression inhibited Argonaute 2 (AGO2) upregulation in HCC, suggesting that targeting AGO2 could significantly inhibit HCC growth in vivo.[34] Furthermore, miR-101 functions as a liver tumor suppressor by regulating abnormal Nemo-like kinase (NLK) activity.[35] Additionally, miR-101 overexpression inhibits proliferation, invasion, colony formation, and HCC progression in humans by directly targeting the *EZH2* oncogene.[36] The identified Gecko miRNA regulatory network is reliable, and potentially facilitates the regulation of HCC proliferation, metastasis and invasion, with therapeutic implications. Further exploration is needed as the focus on these 3 networks remains limited.

Although 9 critical prognostic downstream mRNAs regulated by miR-100-5p, miR-99a-5p and miR-101-3p were identified in HCC, only *KPNA2*, *STMN1*, *EZH2*, and *LMNB1* were further related to progression as well as immunity in HCC. For instance, *KPNA2* knockdown has been shown to reduce HCC cell migration and proliferation, while its overexpression increased malignancy of hepatoma carcinoma cell.[37] The expression of *STMN1* is significantly correlated with *E2F1/TFPD1* and *KPNA2* expression, as well as poor prognosis in HCC patients.[38] *EZH2*, an epigenetic modifier, reduces the expression of the immunological checkpoint inhibitor PD-L1.[39] Furthermore, *LMNB1* promotes HCC progression by regulating the PI3K and MAPK signaling pathways.[40] We constructed a Gecko miRNA network that is enriched in important tumorigenic pathways, such as PI3K-Akt[41] and MAPK signaling pathways.[42,43] Additionally, it is associated with HCC, gallbladder cancer and other closely related oncological diseases. In addition, we observed a significant association between the above core targets of the Gecko miRNA regulatory network and immune cell infiltration, including B cells, T cells, macrophages, NK cells, and monocytes. The heterogeneous nature of immune cells in the tumor microenvironment compared to normal tissues highlights their importance in improving patient survival and the efficacy of immunotherapy for HCC.[44] These reports improve the credibility of our study and further indicate the value of studying the effects of novel Gecko miRNA regulatory networks on HCC and immunotherapy.

Limitations

There is currently a lack of in vivo experimental evidence supporting the role of Gecko miRNAs. Furthermore, it is necessary to conduct further in-depth studies to determine whether Gecko have novel non-human miRNAs that also have anti-tumor effects.

Conclusions

We have constructed a miRNA-mRNA regulatory network of Gecko in HCC. This network exhibits significant correlations with patient prognosis and immune regulation in the tumor microenvironment. Our findings have opened up new insights into the use of herbal medicine to improve targeted therapy and immunotherapy for human HCC. In addition, this study provides a new direction for understanding the subsequent mechanism of tumor suppression by small molecules of TCM.

Supplementary data

The Supplemenary materials are available at https://doi.org/10.5281/zenodo.10624641. The package includes the following files:

Read_me_data.txt. A brief description of the database's content sources.

Supplementary Fig. 1. Linearity assumption testing for univariate Cox regression models.

Supplementary Table 1. Proportional hazards hypothesis testing for univariate Cox analysis.

Supplementary Table 2. 15 target genes were found to be significantly upregulated in the regulatory network.

Supplementary Table 3. Downstream 38 target genes identified by univariate Cox regression analysis model were associated with prognosis.

Supplementary Table 4. The ranked data after transformation of sample expression.

Supplementary Table 5. The results of Spearman's rank correlation coefficient.

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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Inhibiting circ_0000673 blocks the progression of colorectal cancer through downregulating CPSF6 via targeting miR-548b-3p

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Abstract

Background. Colorectal cancer (CRC) is one of the most common cancers, and its progression is regulated by several factors, including circular RNA (circRNA).

Objectives. The objective of this study was to determine the role, or roles, of circ_0000673 in CRC.

Materials and methods. We used quantitative real-time polymerase chain reaction (qPCR) to detect the expression of circ_0000673, miR-548b-3p and cleavage and polyadenylation specific factor 6 (CPSF6) in DLD-1 and RKO cells. Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EdU) assays were used to determine circ_0000673 roles in proliferation. Wound healing and transwell assays were used to detect cell migration and invasion abilities. Expression of CPSF6 protein and stem cell-associated proteins were examined using western blot. The putative relationship between miR-548b-3p and circ_0000673 or CPSF6 was verified with dual-luciferase reporter assay. The role of circ_0000673 in CRC was also investigated in a tumor xenograft assay in nude mice.

Results. Circ_0000673 expression was increased in CRC tissues and cancer cells. Silencing circ_0000673 reduced tumor cell proliferation, migration and invasion, while also decreasing cell stemness. MiR-548b-3p was found to be a target of circ_0000673, while CPSF6 was a downstream target of miR-548b-3p. The tumor-regulatory effects of si-circ_0000673, anti-miR-548b-3p and oe-CPSF6 were partially reversed by anti-miR-548b-3p, si-CPSF6 and si-circ_0000673, respectively, in rescue assays. Downregulation of circ_0000673 reduced solid tumor growth in vivo.

Conclusions. Circ_0000673 inhibition reduced CPSF6 expression by targeting miR-548b-3p, thereby blocking proliferation, migration and invasion of CRC tumor cells.

Key words: colorectal cancer, CPSF6, circ_0000673, miR-548b-3p

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Background

Progression of colorectal cancer (CRC) begins with abnormal transformation of epithelial cells in the colon or rectum and has the 3rd highest incidence and 2nd highest mortality rate of all malignancies.[1,2] In developing countries, the incidence of CRC is quickly growing and is now more than 4 times that of industrialized countries.[3] Moreover, the prognosis for individuals with advanced CRC remains poor, with recurrence and distant metastases being the primary causes of death following surgery.[4,5] The mechanism of CRC carcinogenesis is complex and entails numerous steps in the disease evolution. Numerous genes and signaling pathways have been implicated in tumor cell invasion and metastasis, yet the underlying mechanisms of CRC are not thoroughly understood. Thus, exploring the molecular pathways integral to the pathogenesis of CRC and identifying novel biomarkers are pivotal for enhancing early diagnosis and prognosis for patients.

Circular RNA (circRNA), a type of non-coding RNA, possesses a covalently closed-loop structure at the 3' and 5' untranslated regions (UTRs), resulting from the reverse splicing of precursor messenger RNA (pre-mRNA).[6–8] Circular RNA is more stable than linear RNA in cells due to its unique structure, which prevents cleavage from exonucleases. Circular RNA is involved in various aspects of cancer biology and can play a crucial role in regulating gene expression by acting as a sponge for microRNAs (miRNAs).[9–11] It is also linked to malignant tumor characteristics, including cell proliferation, the cell cycle and invasion. For instance, silencing of circ_RNF121 can reduce cell proliferation, metastasis and glycolysis through the miR-1224-5p/*FOXM1* axis, which plays a significant role in CRC progression.[12] Circ-LECRC functions as a competitive endogenous RNA (ceRNA) to regulate *KLF4* expression and acts as a "brake signal" to reduce the over-activation of oncogenic *YAP* signaling, thereby inhibiting CRC tumor growth.[13] However, whether circ_0000673 plays a role or participates in particular mechanisms in CRC requires further research.

Objectives

By conducting an extensive review of existing literature and conducting meticulous bioinformatics analysis, our goal was to explore the potential significance of circ_0000673 as a pivotal circRNA in the regulation of CRC progression, specifically through the miR-548b-3p/*CPSF6* pathway. Unraveling these intricate molecular mechanisms in the context of CRC tumorigenesis not only enhances our comprehension but also sets the stage for potential clinical applications in this domain.

Materials and methods

Tissues collection and study approval

A cohort of 34 CRC patients was enlisted from the Second Affiliated Hospital of Harbin Medical University, China, for this study. The resected tissues were immediately preserved in liquid nitrogen for RNA analysis. Pathological sections confirmed that all tumor tissues belonged to CRC, and the patients' clinicopathological information was fully documented. Informed consent forms were signed by all patients or their family members, indicating their awareness and approval of sample usage. The collection of clinical specimens was conducted in accordance with the ethical standards set forth by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (approval No. KY2021-230).

Cell culture

The CRC cell lines DLD-1 and RKO, alongside the normal colorectal epithelial cell lines NCM460, were procured from the Institute of Type Culture, Chinese Academy of Sciences, Shanghai, China. After retrieving the frozen storage tube from liquid nitrogen, it was immediately thawed in a water bath at 37°C to ensure rapid lysis. The contents were then transferred to an ultra-clean operating table for further processing. Initially, the cell suspension was pipetted from the freezing tube into a 15 mL centrifuge tube, and 1 mL of complete culture medium was added and mixed. The mixture was then centrifuged at 1,000 rpm for 5 min, and the supernatant was carefully decanted. Subsequently, 5 mL of complete medium was added to the centrifuge tube, and the cells were gently mixed and transferred to cell culture dishes or flasks. The cells were finally incubated in an incubator at 37°C with 5% CO_2. All cells were maintained for less than 6 months.

Quantitative real-time polymerase chain reaction

Cells were lysed and centrifuged, and the precipitated material was transferred to a centrifuge tube and centrifuged at 1,000 rpm for another 5 min, after which the supernatant was discarded, and TRIzol (Invitrogen, Waltham, USA; 15596026) reagent was added to the pellet. The solution was then gently mixed several times to rupture the cells and shear the DNA. The Reverse Transcription Kit (Roche, Penzberg, Germany; 11483188001) was then used to generate complementary DNA (cDNA). The PCR system with FastStart Universal SYBR Green aster (Roche; FSSGMMRO) and a C1000 thermal cycler were used to perform quantitative real-time polymerase chain reaction (qPCR). Internal controls were *GAPDH* and *U6*, and the data were evaluated using the $2^{-\Delta\Delta Ct}$ technique.[14] All primer sequences are listed in Supplementary Table 1.

Cell transfection

To perform cell transfection, 125 uL of serum-free Dulbecco's modified Eagle's medium (DMEM) was added to each Eppendorf (EP) tube and labeled accordingly. Next, 5 uL of Lipofectamine 3000 (Invitrogen; V518472) was added along with 5 uL of diluted siRNA to each of the 2 EP tubes. The contents of the EP tubes were mixed thoroughly by gentle pipetting to homogenize and then allowed to stand at room temperature for 15 min. When the cells reached 40–60% confluency, the supernatant was discarded, and the cells were washed twice with sterile phosphate-buffered saline (PBS). Next, the serum-free DMEM medium was added to the cells, and then the mixture from the EP tubes was put into the corresponding well of the 6-well plate. The contents were mixed well, and the plate was placed in the incubator for further cultivation. After 6–8 h, the medium was replaced with complete medium. To detect the RNA content, transient transfection was performed for 48–72 h, and for protein content and functional experiments, it was performed for 72 h.

Ribonuclease R (RNase R) and actinomycin D treatment

The loop structure of circ_0000673 was confirmed via RNase R treatment. A total of 5 μg of RNA, extracted from DLD-1 and RKO cells, was treated with 2 U/μg of RNase R (Geneseed, Guangzhou, China; R0301) at 37°C for 30 min. Subsequently, the enzyme was inactivated at 70°C for 5 min. DEPC-treated water (MilliporeSigma, St. Louis, USA) served as a control. Actinomycin D (2 μg/mL) or dimethyl sulfoxide (DMSO; MilliporeSigma; D2650-100ML) were introduced to media containing DLD-1 or RKO cells for 0, 4, 8, and 12 h to analyze actinomycin D disposal. Then, we used qPCR to detect the expression of circ_0000673 and corresponding linear RNA.

Cell Counting Kit-8 assay

Colorectal cancer cells post-transfection were grown in 96-well plates with a density of 1×10^5 cells per well. At each designated time point (0, 24, 48, and 72 h), 10 μL of Cell Counting Kit-8 (CCK-8) reagent (Beyotime Biotechnology, Shanghai, China; C0037) was incorporated per well and then incubated for an additional 2 h at 37°C within a humidified incubator. Finally, the absorbance at 450 nm was read using a microplate reader (imark; Bio-Rad, Hercules, USA).

5-ethynyl-2'-deoxyuridine assay

The target cell line in logarithmic growth was selected, and a cell suspension was prepared. The cells were inoculated into a 96-well plate, and 100 μL of diluted 5-ethynyl-2'-deoxyuridine (EdU) in complete medium (50 μM) (Beyotime Biotechnology; C0071S) was added and incubated for 2 h. In the dark, cells were subjected to flow incubation, fixed with 4% paraformaldehyde, and successively treated with permeabilizing solution, Click-Interaction mixture and fluorescent nuclear stains. After each treatment, cells were washed with an immunostaining blocking buffer. Finally, images of the cells were captured using a fluorescent microscope (Leica DMI4000 B; Leica Camera AG, Wetzlar, Germany) and the cells were counted with different fluorescent colors.

Transwell assay

The cells to be treated were initially resuspended in serum-free medium. Subsequently, cell counting was performed, and the cells were appropriately diluted to obtain a concentration of 1×10^5/mL. Then, 200 uL of the cell suspension was aspirated and added dropwise to the upper chamber of the transwell. Following this, complete medium (0.7 mL) was added to the lower chamber. For invasion experiments, a diluted layer of Matrigel (BD Biosciences, San Jose, USA; 353095) was applied to the surface of the chambers. After a 24-h incubation period, the underlying cells were fixed for 30 min with 4% paraformaldehyde and stained for 1 h with 0.5% crystal violet. Finally, images were acquired using an inverted optical microscope (Leica DMI4000 B) and the quantity of migrating or invading labeled cells was counted using ImageJ software (National Institutes of Health (NIH), Bethesda, USA).

Wound healing assay

DLD-1 and RKO cells were seeded at a density of 6×10^5 per well in 6-well plates and allowed to grow for 48 h. Once 90% confluence was achieved, a linear scratch was made in each well using a 200 μL pipette tip. Then, cells were washed in PBS and incubated in complete medium. After 24 h of incubation, the width of the wound was analyzed.

Spheroid formation assay

Spheroid formation assay was performed in serum-free medium supplemented with ×1 B27, 20 ng/mL each of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF, Invitrogen; 17504-044, 266775, 266475), along with 100 U/mL penicillin and 0.1 mg/mL streptomycin (Beyotime Biotechnology; c0222) and required special ultra-low attachment plates (Corning Company, Corning, USA; 7007). The quantity and size of spheroids were measured using an inverted optical microscope (Leica DMI4000 B) after 10 days. The efficiency of spheroid formation was calculated using the formula as the number of colonies divided by the number of seeded cells.

Dual-luciferase reporter assay

The binding sequence of circ_0000673 or *CPSF6* to miR-548b-3p was predicted, and genomic DNA from CRC cells was extracted and amplified. The double-enzymatic luciferase reporter gene empty plasmid used was pGL3 (Promega, Madison, USA). The luciferase reporter vector generated (pmirGLO-circ_0000673-WT, pmirGLO-circ_0000673-MUT, pmirGLO-*CPSF6*-WT, pmirGLO-*CPSF6*-MUT) and miR-NC or miR-548b-3p mimics were co-transfected into DLD-1 and RKO cells. Luciferase activity was detected using the dual-luciferase reporter detection system kit (Promega; E1910) after transfection for 48 h.

Western blot assay

After lysing cells or tissues with RIPA Lysis Buffer (Beyotime Biotechnology; P0013B) and 1% phenylmethylsulfonyl fluoride (PMSF) (Seven, Beijing, China; SW106) to collect proteins, the protein concentration was determined using the BCA Protein Concentration Assay Kit (Beyotime Biotechnology; P0009), and the proteins subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on polyacrylamide gels were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, USA) using electrophoretic techniques. The PVDF membranes were sealed with a sealing solution at room temperature for a period adjusted for the different antibodies, followed by overnight incubation at 4°C in a cold room immersed in primary antibody. The next day the membranes were washed 3 times with phosphate-buffered saline with Tween (PBST) and then incubated with the secondary antibody for 1.5 h at room temperature and washed again in PBST. The expression level of the target protein was assessed using a BeyoECL kit (Beyotime Biotechnology; P0018S), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as an internal reference. All antibodies were sourced from Immunoway (Texas-Plano, USA; NESTIN: YN2050, SOX2: YM0594) or Abcam (Cambridge, USA; CPSF: ab175237, OCT4: ab200834). The antibody was diluted in accordance with the manufacturer's instructions.

Xenograft assay

We purchased nude BALB/c mice from Vital River (Beijing, China) and housed them in a barrier system at 45–50% humidity and 25–27°C. A CRC xenograft model was established by subcutaneously injecting 5×10^6/150 µL of transfected CRC tumor cells into the anterolateral groin of the left hind limb and 5×10^6/150 µL of sh-NC transfected tumor cells into the anterolateral groin of the right hind limb of each mouse. Tumor volume was measured once a week. After 4 weeks, the mice were euthanized by cervical dislocation, and the tumors were dissected and weighed, and the expression of genes under study was also assessed. The Ethics Committee of The Second Affiliated Hospital of Harbin Medical University approved the study (approval No. SYDW2021-052).

Statistical analyses

The data were analyzed using IBM SPSS v. 27.0 (IBM Corp., Armonk, USA). Data visualization was performed using GraphPad Prism v. 9 (GraphPad Software, San Diego, USA). Data were expressed as mean ± standard deviation (±SD) and underwent analysis based on the grouping or pairing conditions, employing distinct Mann–Whitney test, Kruskal–Wallis test and post hoc multiple comparisons. The clinicopathologic data in Table 1 were examined using Fisher's exact test. Due to the limited sample size, non-parametric tests were used, leading to marginal

Table 1. Correlation between circ_0000673 expression and clinicopathological characteristics of colorectal cancer (CRC) patients

Clinicopathological characteristics		Total cases (n = 34)	Circ_0000673 expression		p-value
			low (n = 17)	high (n = 17)	
Age [years]	<60	12	5	7	0.360
	≥60	22	12	10	
Gender	male	20	11	9	0.364
	female	14	6	8	
Tumor size [cm]	<5	23	13	10	0.232
	≥5	11	4	7	
Differentiation grade	well/moderate	17	10	7	0.274
	poor/undifferentiated	17	7	10	
TNM stage	I–II	14	10	4	0.039*
	III–IV	20	7	13	
Lymph node invasion	positive	19	6	13	0.018*
	negative	15	11	4	

*p < 0.05. TNM – tumor-node-metastasis staging system.

significance in the reported results, and the statistical methods and detailed parameters utilized for all results were documented in Supplementary Table 2.

Results

Circ_0000673 was upregulated in CRC tissues and cells

We first identified the basic characteristics of circ_0000673, which is 251 nucleotides in length produced by exons 4 and 5 of *RSL1D1* mRNA (Fig. 1A). To explore the relevance of circ_0000673 to CRC progression, we examined its expression in CRC cells. Quantitative real-time polymerase chain reaction assays pointed to higher circ_0000673 enrichment being detected in CRC cell lines (DLD-1, RKO and HT-29), compared to NCM460 cells (Fig. 1B). After RNase R treatment, we found that circ_0000673 was resistant to RNase R digestion when compared to *RSL1D1* (Fig. 1C,D). After total RNA synthesis was inhibited by actinomycin D, the expression of circ_0000673 and *RSL1D1* mRNA were detected at different time points, and again, the stability of circRNA was found to be much stronger than that of linear mRNA (Fig. 1E,F).

The relationship between circ_0000673 and clinicopathologic data of CRC patients

Considering the elevated expression of circ_0000673 in CRC tissues compared to normal tissues (Fig. 2A), we investigated the relationship between circ_0000673 expression and the pathological advancement of CRC. Initially, we categorized the patients based on the presence of lymph node metastasis or TNM (tumor-node-metastasis) stage

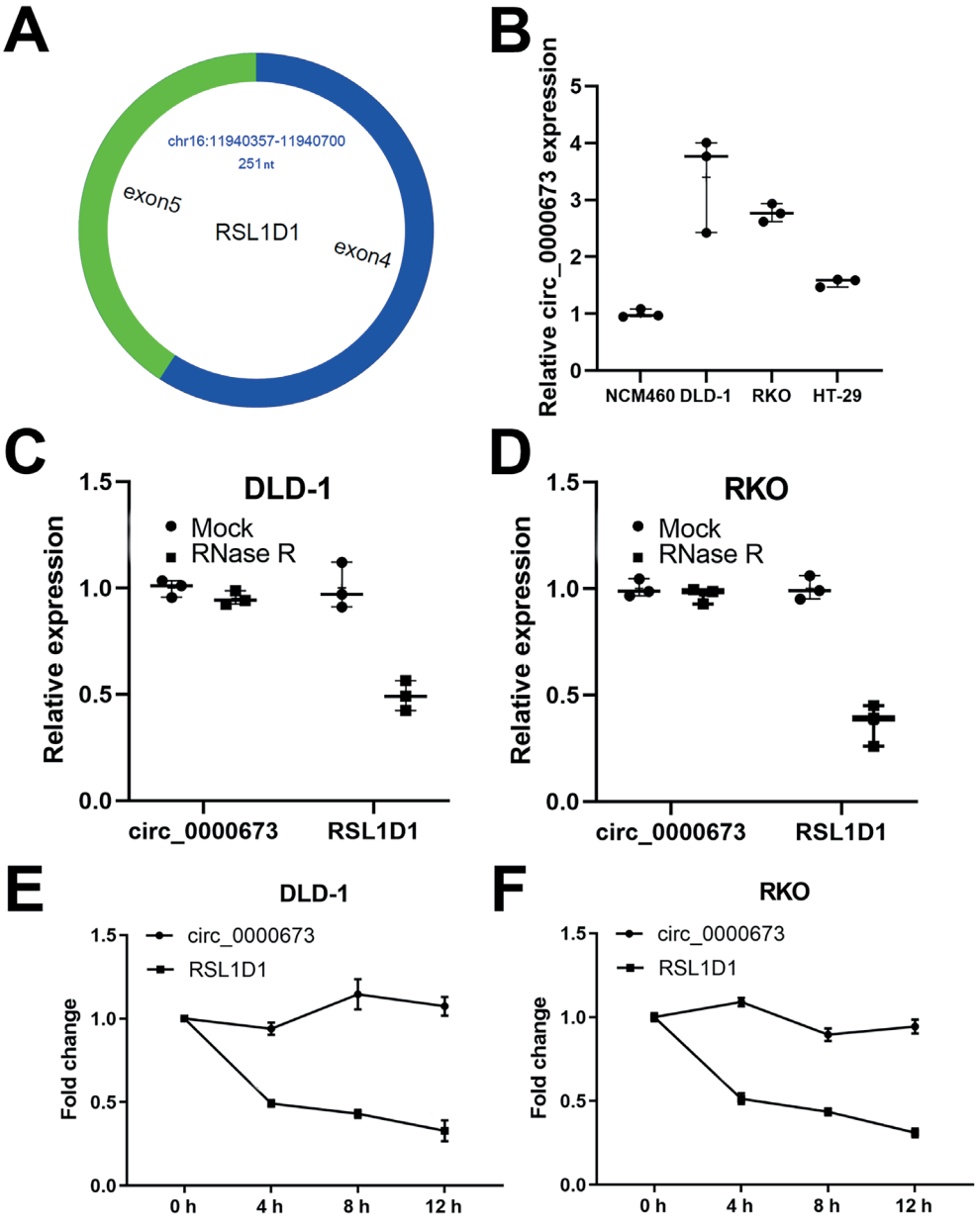

Fig. 1. Circ_0000673 was upregulated in CRC cells. A. Diagram showing the structure of circ_0000673 derived from *RSL1D1* mRNA; B. qPCR assay was used for the expression of circ_0000673 in *NCM460*, *DLD-1*, *RKO* and *HT-29* cells; C,D. The relative circ_0000673 and *RSL1D1* expression were detected using the qPCR in CRC cells treated with RNase R; E,F. Actinomycin D assay in DLD-1 and RKO cells. Kruskal–Wallis test was employed for the comparison of circ_0000673 among different cell types, followed by the Bonferroni post hoc test. The comparison between circ_0000673 and *RSL1D1* was conducted using the Mann–Whitney test

CRC – colorectal cancer; qPCR – quantitative real-time polymerase chain reaction; RNase – ribonuclease.

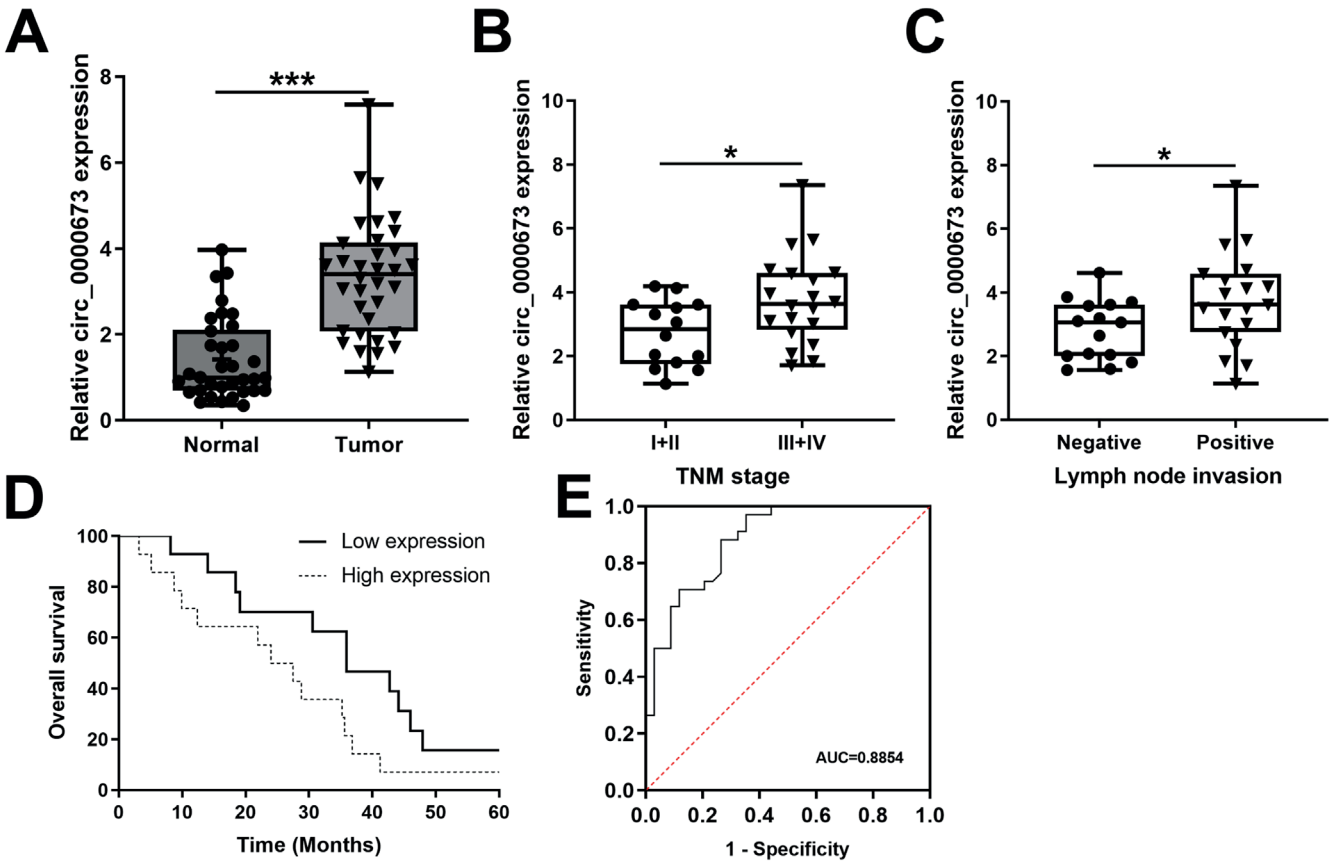

Fig. 2. Circ_0000673 expression in CRC tissues and its correlation with clinical features and prognosis. A. qPCR assay for the expression of circ_0000673 in tumor and adjacent normal tissues; B,C. Association of circ_0000673 expression in patients with TNM stage or presence of lymph node invasion; D. Overall survival (OS) comparison between high and low circ_0000673 expression groups; E. The ROC curve of circ_0000673 detection as a prognostic biomarker. The comparison of circ_0000673 among different tissues and stages was conducted using a paired t-test

*p < 0.05, ***p < 0.001; CRC – colorectal cancer; qPCR – quantitative real-time polymerase chain reaction; TNM – tumor-node-metastasis staging system; ROC – receiver operating characteristic.

and subsequently performed quantitative comparisons of circ_0000673 expression levels within each group using qPCR (Fig. 2B,C). However, to explore whether circ_0000673 could play a role in the clinical setting, we also analyzed its correlation with pathological data after dividing all cases into circ_0000673 high- and low-expression groups (Table 1). Survival correlation, as analyzed using a Kaplan–Meier estimation, revealed that patients exhibiting high circ_0000673 expression experienced poorer overall survival (OS) compared to their counterparts with low circ_0000673 expression (log-rank p = 0.043; Fig. 2D). To evaluate the prognostic efficacy of circ_0000673, we plotted receiver operating characteristic (ROC) curves, and the results confirmed that circ_0000673 had clinical prognostic significance (p < 0.001; Fig. 2E).

Circ_0000673 downregulation restricted the proliferation, invasion and migration of cancer cells

Once the transfection efficacy of si-circ_0000673 was assessed (Fig. 3A), CCK-8 and EdU assays revealed a significant reduction in the vitality of DLD-1 and RKO cells following si-circ_0000673 transfection compared to the si-NC group (Fig. 3B–D). Building upon the findings presented above, the downregulation of circ_0000673 exhibits the capacity to curb the proliferation of CRC cells. We extended our investigation to assess the influence of circ_0000673 on the invasive and migratory capabilities of CRC cells, employing both wound healing and transwell assays. The results indicated that in cells transfected with si-circ_0000673, the migration distance of tumor cells and the number of migrating cells were notably lower compared to the control group (Fig. 4A,B). Moreover, the transwell assay showed a reduction in the number of invasive cells in the experimental group following the removal of the stromal gel layer (Fig. 4C). The number of invasive cells in the transwell layer was also reduced (Fig. 4D). A subsequent attenuation of tumor cell stemness after knockdown of circ_0000673 can be seen in the spheroid formation assay, and some stemness markers like *NESTIN*, *OCT4* and *SOX2* were also downregulated at the protein expression level (Fig. 5A,B).

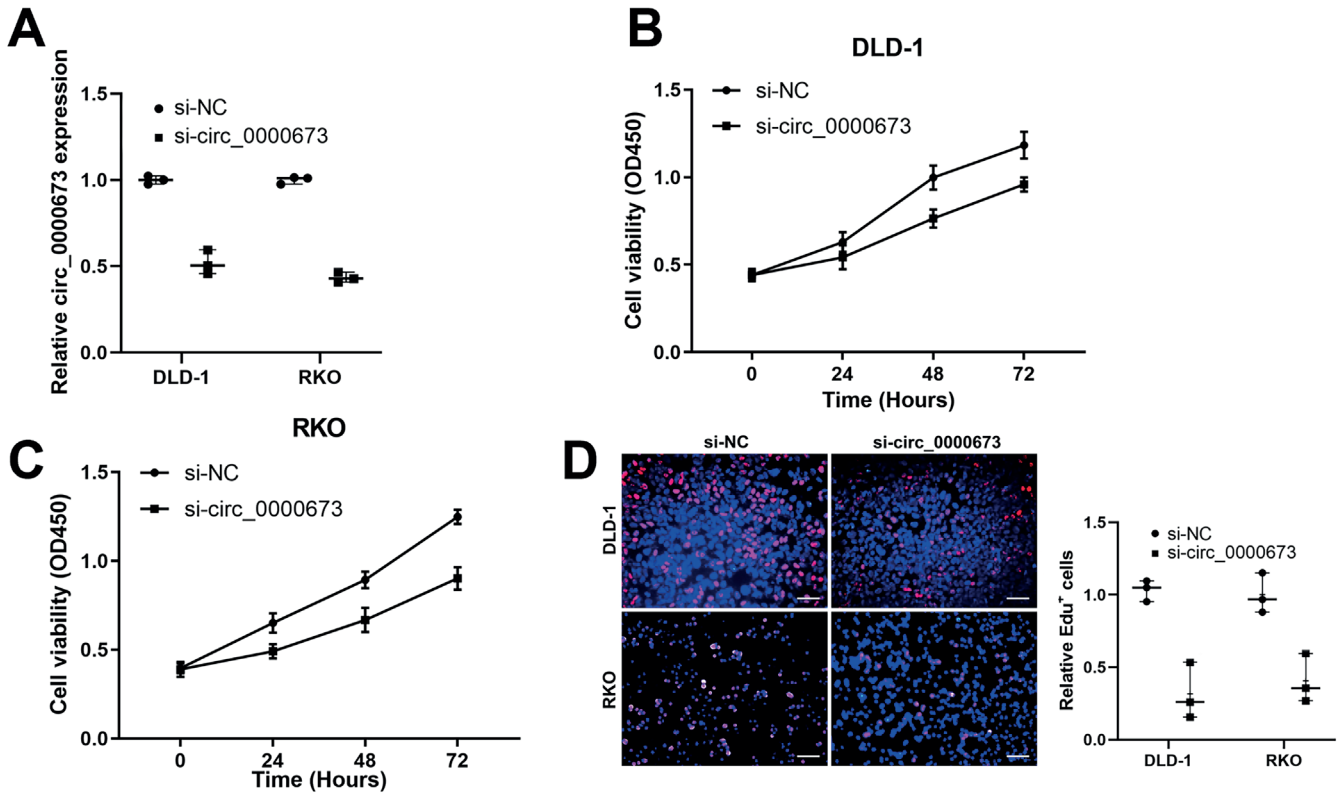

Fig. 3. Downregulation of circ_0000673-inhibited CRC cell proliferation. A. qPCR determined circ_0000673 expression in transfected cells; B,C. CCK-8 assay was performed to examine the cell viability of transfected CRC; D. EdU assay was used to determine cell proliferation. All statistical analyses employed in this figure were performed using the Mann–Whitney test

CRC – colorectal cancer; qPCR – quantitative real-time polymerase chain reaction; CCK-8 – Cell Counting Kit-8; EdU – 5-ethynyl-2′-deoxyuridine.

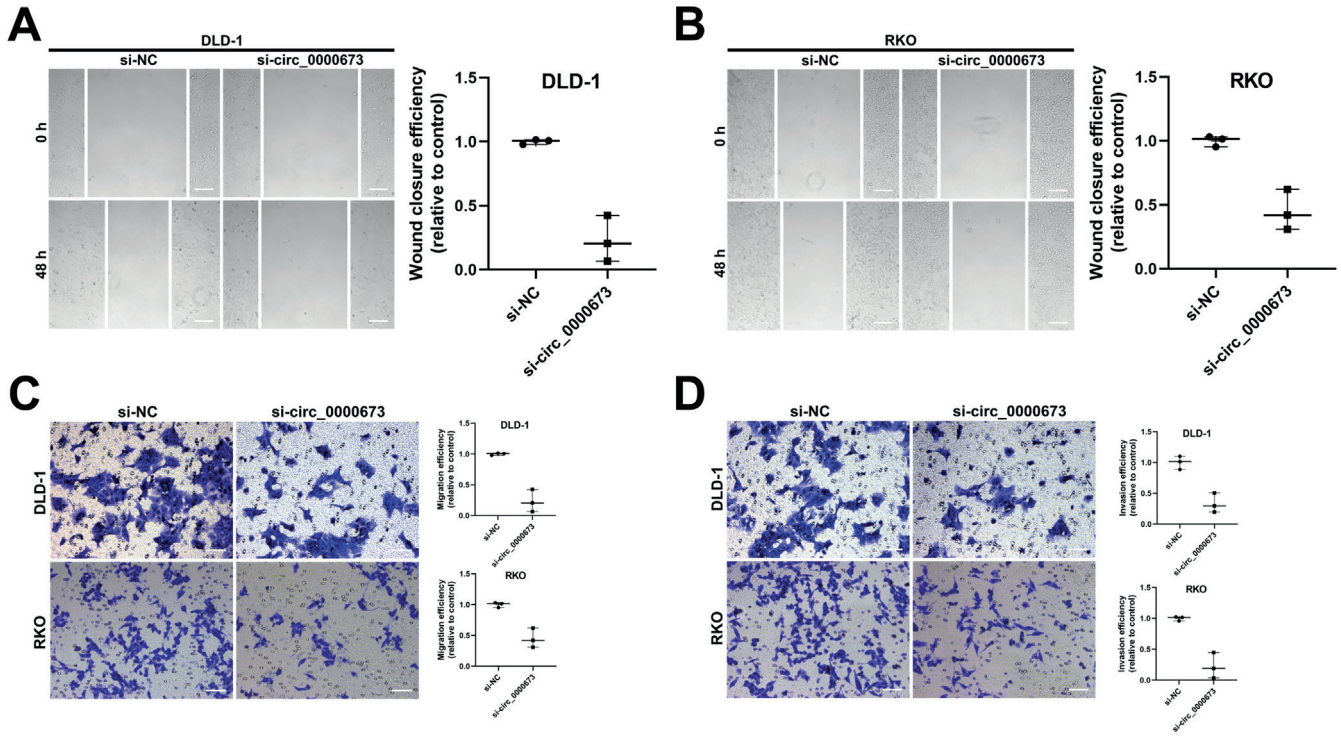

Fig. 4. Knockdown of circ_0000673 expression suppressed migration and invasion in CRC cells. A,B. Wound healing assay was used to detect the migration ability of CRC cells after circ_0000673 knockdown; C,D. Transwell assay gauged cell migration and invasion. All statistical analyses employed in this figure were performed using the Mann–Whitney test

CRC – colorectal cancer.

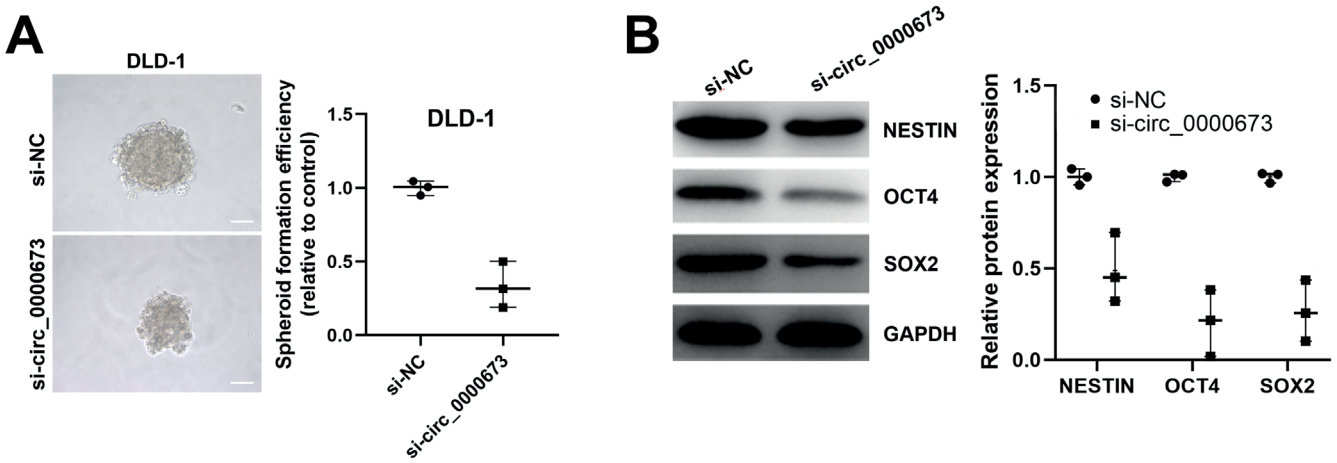

Fig. 5. Inhibited circ_000673 could affect tumor cell stemness. A. Spheroid formation was used to evaluate the spheroid-forming abilities of transfected DLD-1 cells; B. Western blot assay was conducted to analyze the protein expression of NESTIN, OCT4 and SOX2. All statistical analyses employed in this figure were performed using the Mann–Whitney test

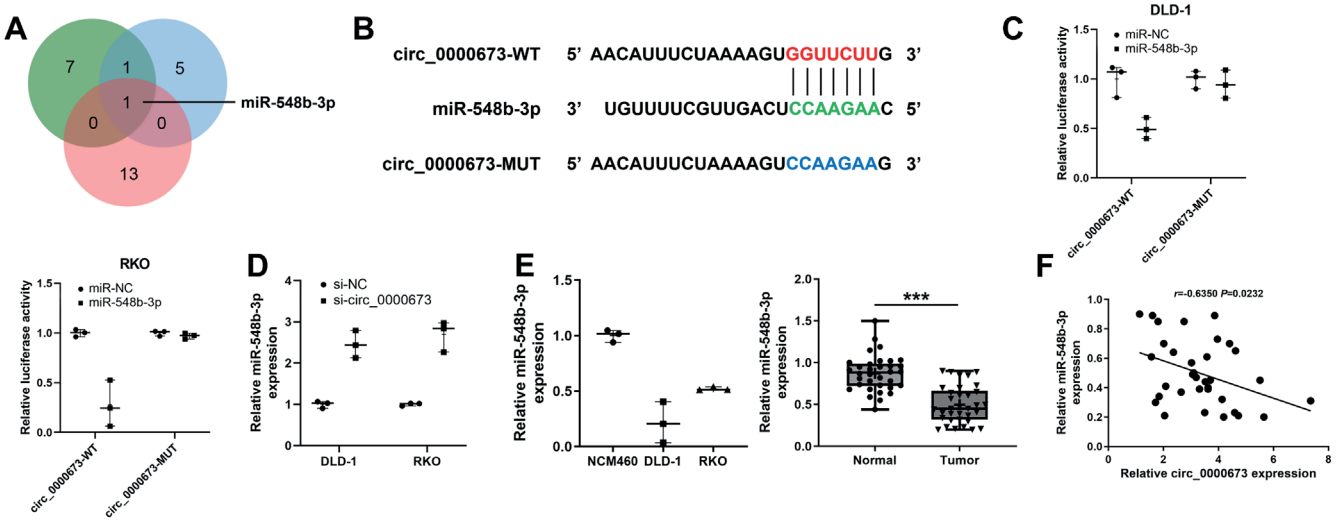

Fig. 6. MiR-548b-3p was a direct target of circ_0000673. A Venn diagram was used to screen miRNAs targeted circ_0000673 from circinteractome, Starbase and circBANK; B. The binding sets between miR-548b-3p and circ_0000673 were presented; C. Dual-luciferase reporter assay confirmed the miR-548b-3p and circ_0000673 interaction; D. MiR-548b-3p expression in cells transfected with a negative control vector or circ_0000673 inhibitor was detected using qPCR; E. The expression of miR-548b-3p in NCM460, DLD-1 and RKO cells was detected with qPCR. The miR-548b-3p expression in CRC tumor tissues and normal tissues was detected using qPCR; F. The correlation between miR-548b-3p and circ_0000673 expression was analyzed using the Pearson's correlation coefficient. The statistical analysis for the dual-luciferase reporter gene experiment and the differential expression of miR-548b-3p in cells were both assessed using the Mann–Whitney test. The expression differences of miR-548b-3p among different tissues were analyzed using a paired t-test

***p < 0.001; qPCR – quantitative real-time polymerase chain reaction; CRC – colorectal cancer.

Targeted regulation of miR-548b-3p expression by circ_0000673 in colorectal cancer cells

Circinteractome, starBase and circBANK predicted that miR-548b-3p may act downstream of circ_0000673 in regulating CRC (Fig. 6A,B). To validate this hypothesis, we conducted a dual-luciferase reporter assay. The experimental results confirmed our hypothesis, showing that the luciferase activity was significantly reduced in DLD-1 and RKO cells co-transfected with miR-548b-3p mimics and the recombinant plasmid circ_0000673-WT (Fig. 6C). Using qPCR assay, miR-548b-3p expression level was significantly increased after transfection with si-circ_0000673 compared to that with small interfering RNA negative control (si-NC) group (Fig. 6D), and miR-548b-3p expression was found to be downregulated in CRC cells and tissues (Fig. 6E). It is worth noting that the expression of circ_0000673 and miR-548b-3p in CRC tissues presented a negative relationship (Fig. 6F).

CPSF6 severed as a downstream effector of miR-548b-3p in CRC

Bioinformatics analysis disclosed that miR-548b-3p could target *CPSF6* (Fig. 7A). The results obtained from the experiment indicate that luciferase activity was considerably

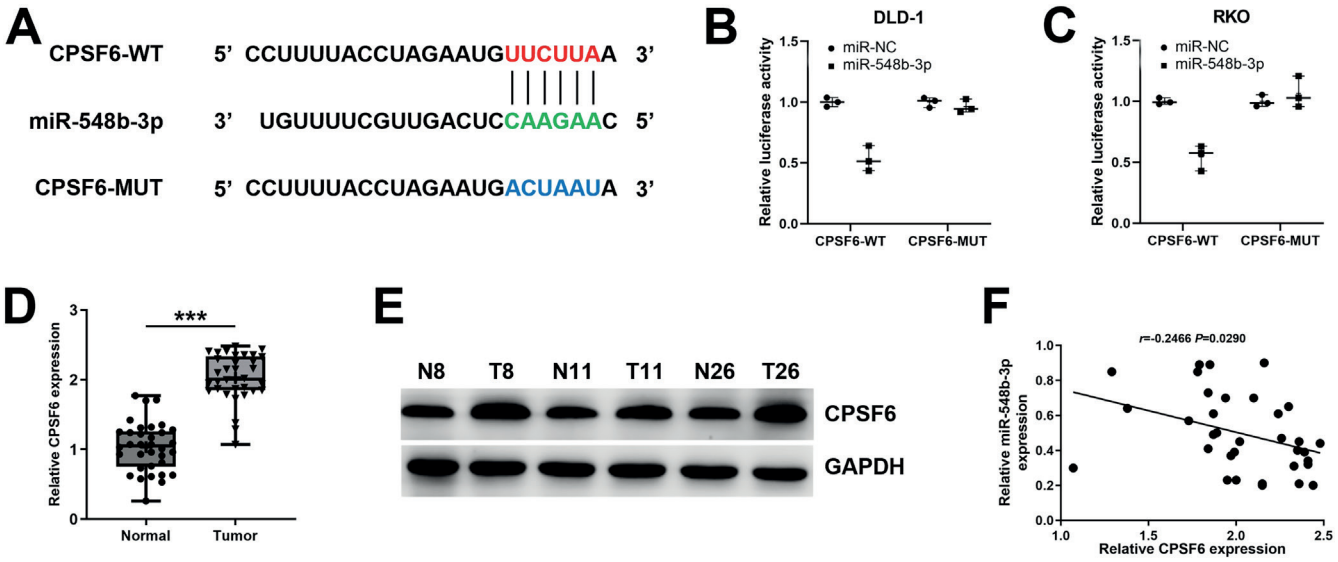

Fig. 7. *CPSF6* was a target gene of miR-548b-3p in CRC tissues. A. The binding sets between *CPSF6* and miR-548b-3p were analyzed using Starbase; B,C. The relationship between *CPSF6* and miR-548b-3p was validated with dual-luciferase reporter assay; D,E. *CPSF6* mRNA and protein expression in normal tissues and CRC tissues were detected using qPCR and western blot, respectively; F. The correlation between *CPSF6* mRNA and miR-548b-3p expression was analyzed in CRC tissues. The analysis of the dual-luciferase reporter gene experiment, the regulation of *CPSF6* expression by miR-548b-3p, and the assessment of *CPSF6* tissue protein expression levels were all conducted using the Mann–Whitney test. The mRNA level differences of *CPSF6* between normal and tumor tissues were evaluated using a paired t-test

***p < 0.001; CRC – colorectal cancer.

weaker in the *CPSF6*-WT cells transfected with miR-548b-3p compared with the control group, while the luciferase activity was not significantly changed in the *CPSF6*-MUT group cells transfected with miR-548b-3p mimics (Fig. 7B,C). In CRC tissues, we observed that both *CPSF6* mRNA and protein were increased and *CPSF6* expression was negatively correlated with miR-548b-3p expression (Fig. 7D–F). Similarly, the *CPSF6* nucleic acid and protein expression levels were elevated in CRC cells (DLD-1 and RKO) (Fig. 8A,B). The addition of anti-miR-548b-3p significantly increased *CPSF6* abundance, but miR-548b-3p overexpression decreased *CPSF6* expression (Fig. 8C).

Circ_0000673 regulated CRC malignant biological behaviors via miR-548b-3p/*CPSF6*

We performed rescue experiments to further characterize the regulatory relationship between circ_0000673 and *CPSF6*. It was found that circ_0000673 knockdown suppressed *CPSF6* expression at both the nucleic acid and protein levels, and then rescued *CPSF6* repression by silencing miR-548b-3p in part (Fig. 8D). The findings suggest that circ_0000673 could function as a ceRNA, potentially binding to miR-548b-3p to elevate *CPSF6* in CRC cells. Subsequent rescue experiments regarding cellular phenotype were also conducted. Cell Counting Kit-8 and EdU rescue assays provided further confirmation that the reduced cell proliferation due to circ_0000673 knockdown could be restored by suppressing miR-548b-3p (Fig. 9A–C). Additionally, transwell assays showed that the circ_0000673 knockdown-induced inhibition of migration and invasion capabilities could be partially reversed by inhibiting miR-548b-3p. Moreover, the downregulation of cell proliferation, migration and invasion by miR-548b-3p was partially mitigated upon transfection with si-*CPSF6* (Fig. 10A–C). Finally, overexpression of *CPSF6* significantly amplified the proliferation, migration and invasion of tumor cells, while the knockdown of circ_0000673 partly countered these effects (Fig. 11A–C). In conclusion, circ_0000673/miR-548b-3p/*CPSF6* axis is involved in the tumorigenic process of CRC.

Circ_0000673 silencing restrains xenograft tumor growth in vivo

A CRC xenograft model was established to confirm the effects of circ_0000673 on CRC tumorigenicity. RKO cells at the logarithmic growth stage were inoculated into nude mice after transfection with sh-circ_0000673 or short hairpin RNA negative control (sh-NC) and monitored for 4 weeks. Both tumor volume and weight of the sh-circ_0000673 transfected group were decreased (Fig. 12A,B), indicating that circ_0000673 silencing was able to inhibit the growth of xenograft tumors in vivo. Quantitative real-time polymerase chain reaction and western blot on tumor tissues showed circ_0000673 expression was severely decreased in sh-circ_0000673 group tumors (Fig. 12C). Conversely, the expression of miR-548b-3p was upregulated due to the silenced circ_0000673 (Fig. 12D). In addition, mRNA and protein expression of *CPSF6* were significantly decreased in sh-circ_0000673 tumor tissues compared to sh-NC group (Fig. 12E,F).

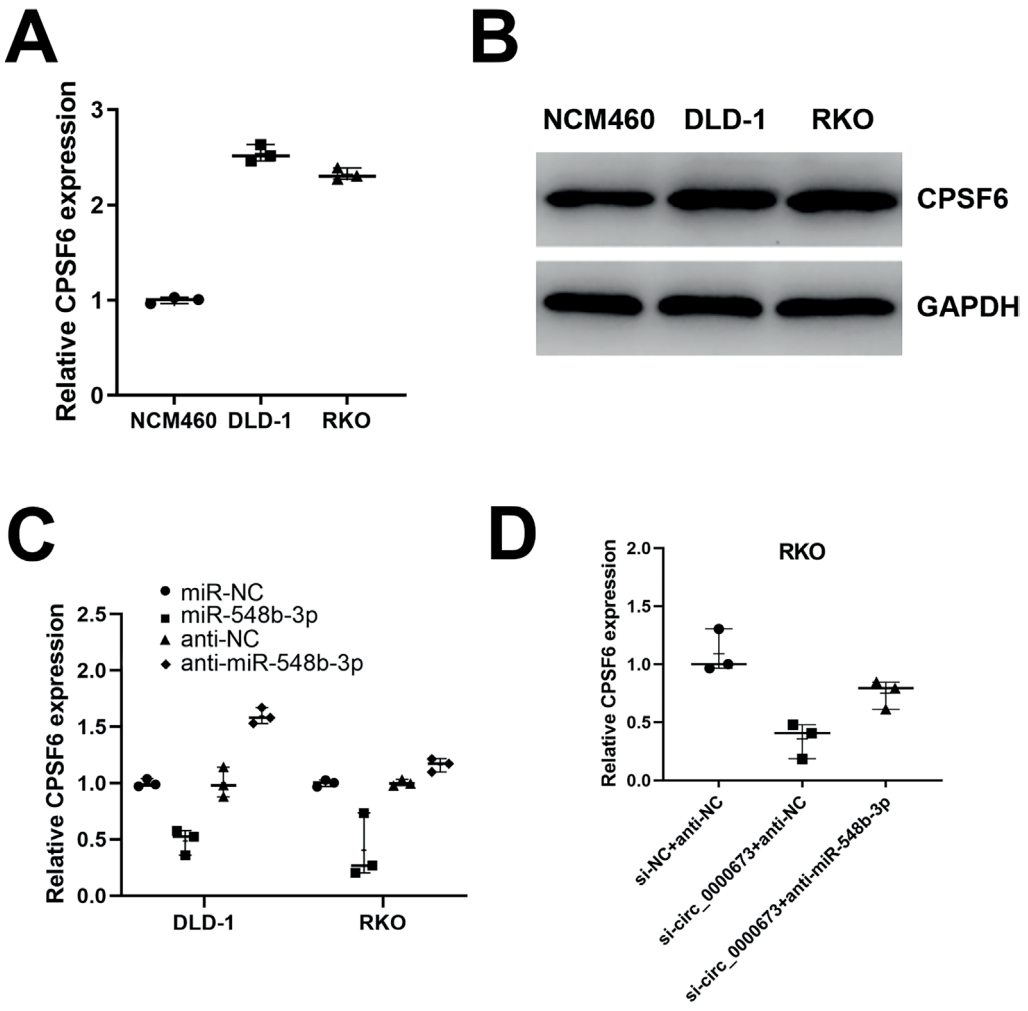

Fig. 8. Circ_0000673 regulated the expression of *CPSF6* by targeting miR-548b-3p in CRC cell lines. A,B. The mRNA and protein levels of *CPSF6* in NCM460, DLD-1 and RKO cells were measured using qPCR and western blot, respectively; C. The expression of *CPSF6* in DLD-1 and RKO cells after transfected with miR-548b-3p mimics or anti-miR-548b-3p was measured with qPCR; D. After co-transfection of si-circ_0000673 and miR-548b-3p inhibitor, the *CPSF6* expression was detected using qPCR. All statistical methods employed in this figure were performed using the Kruskal–Wallis test, followed by the Dunn's test with Bonferroni correction post hoc test

CRC – colorectal cancer; qPCR – quantitative real-time polymerase chain reaction.

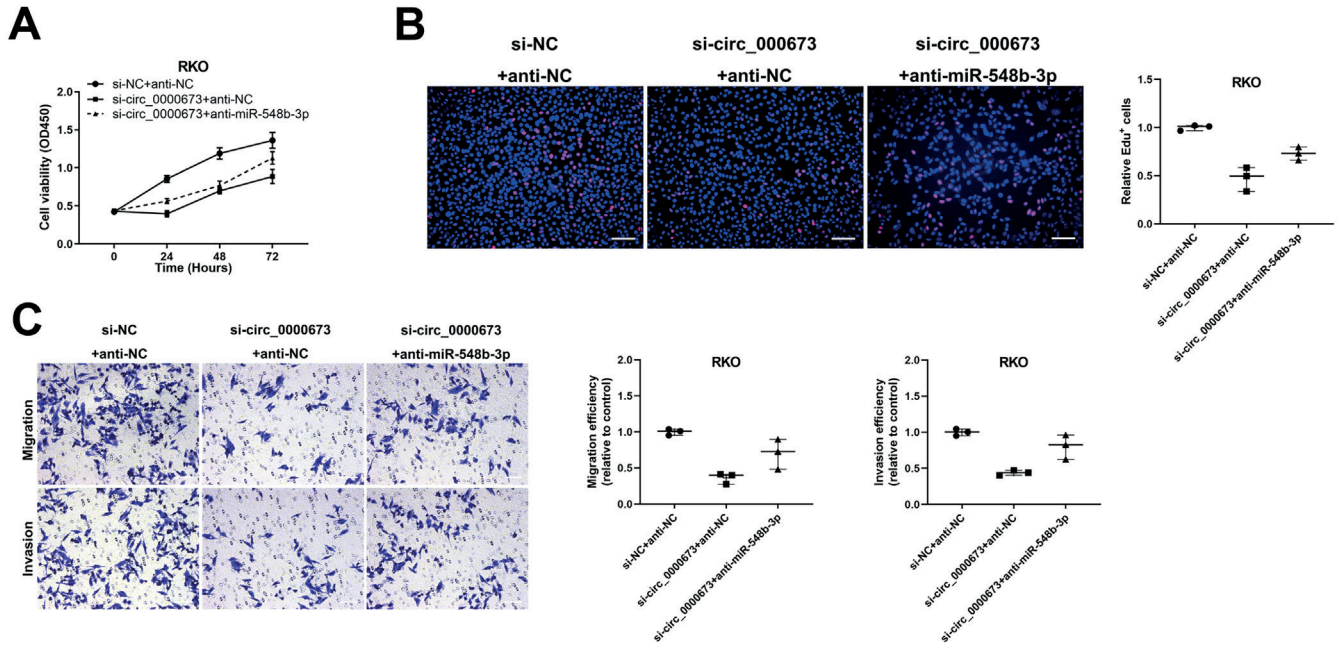

Fig. 9. CCK-8, EdU and transwell assays confirmed that the effects of proliferation, migration and invasion induced by circ_0000673 inhibitor were partly reversed by anti-miR-548b-3p. All statistical methods employed in this figure used the Kruskal–Wallis test as a preliminary analysis, followed by the Bonferroni post hoc test

CCK-8 – Cell Counting Kit-8; EdU – 5-ethynyl-2'-deoxyuridine.

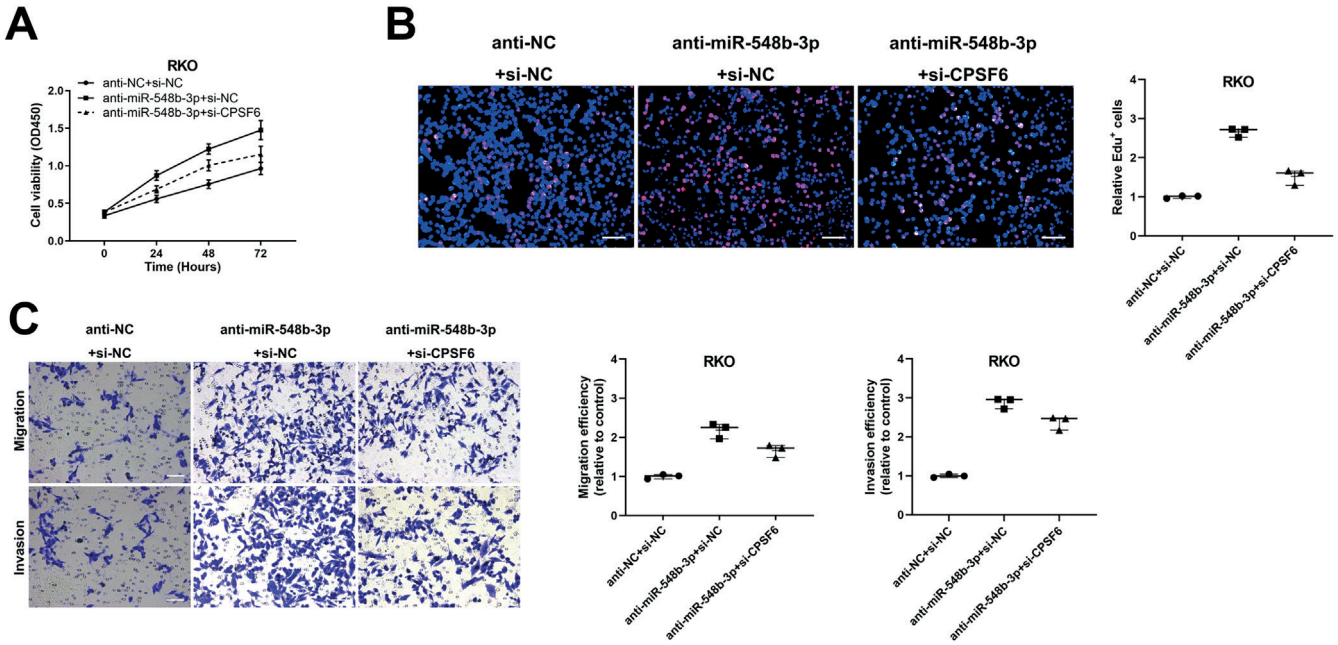

Fig. 10. Silencing *CPSF6* could partially reverse the proliferation, migration and invasion induced by anit-miR-548b-3p in CCK-8, EdU and transwell assays. All statistical methods employed in this figure used the Kruskal–Wallis test as a preliminary analysis, followed by the Bonferroni post hoc test

CCK-8 – Cell Counting Kit-8; EdU – 5-ethynyl-2′-deoxyuridine.

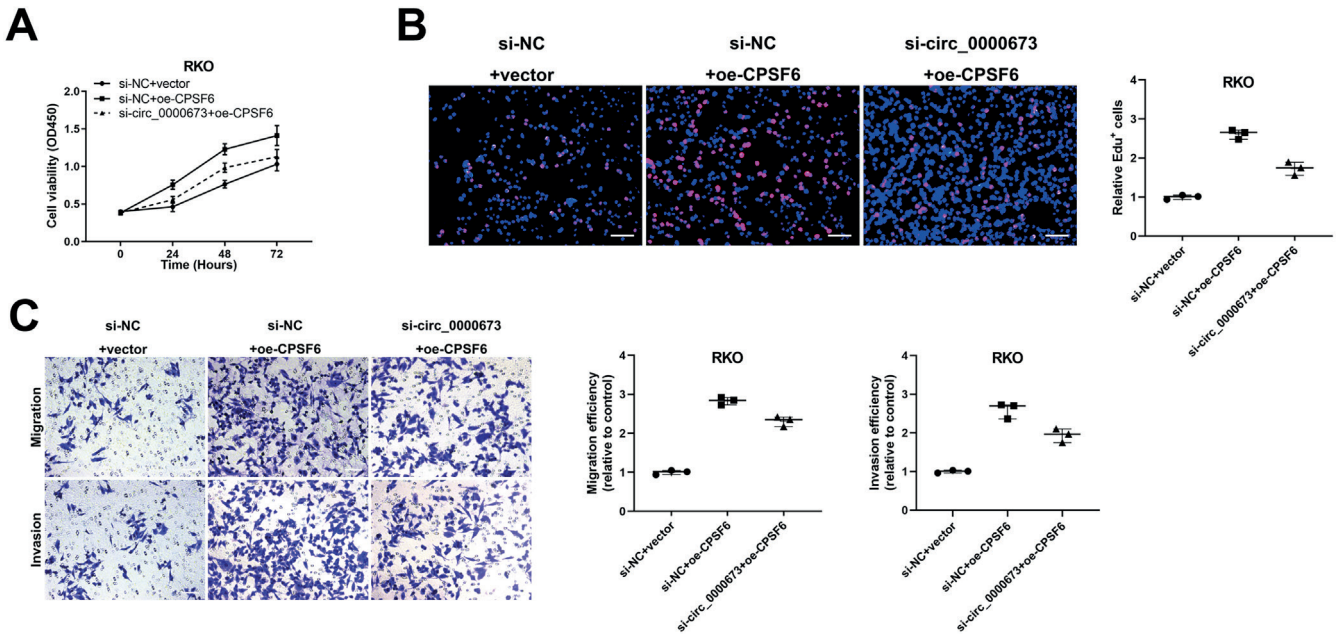

Fig. 11. Restoration of *CPSF6* expression rescued the promotion of proliferation, migration and invasion generated through circ_0000673 inhibition in CCK-8, EdU and transwell, respectively. All statistical methods employed in this figure used the Kruskal–Wallis test as a preliminary analysis, followed by the Bonferroni post hoc test

CCK-8 – Cell Counting Kit-8; EdU – 5-ethynyl-2′-deoxyuridine.

Discussion

The incidence of CRC has remained consistently high over time.[15] Emerging evidence highlights the significant roles that circRNAs play in various human cancers. For instance, circ_0120175 has been shown to promote the proliferation, migration and invasion of lung squamous cell carcinoma (LSCC) cells through the miR-330-3p/SLC7A11 axis, while concurrently inhibiting apoptosis.[16] Similarly, circ-ZNF609 has been demonstrated to selectively bind to miR-432-5p, leading to increased *LRRC1* expression and facilitating cholangiocarcinoma (CCA).[17,18] In this study, we have confirmed the elevated expression of circ_0000673 in CRC tissues and cells. Moreover,

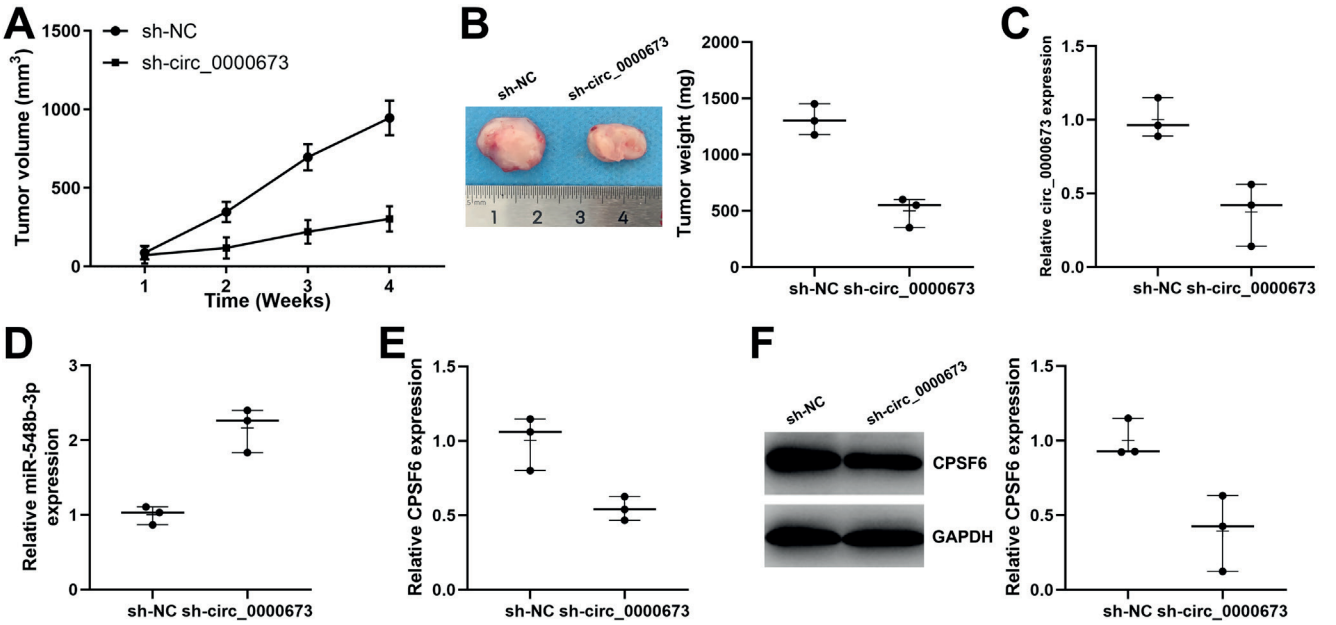

Fig. 12. Downregulated circ_0000673 inhibited tumor growth in vivo. A. Tumor volume was measured every week after injection; B. Tumor weight in nude mice at 4 weeks post-injection; C–E. The expression of circ_0000673, miR-548b-3p and *CPSF6* were detected using qPCR in tumor tissue; F. The CPSF6 protein expression in the collected tissues was determined with western blot assay. All statistical analyses employed in this figure were performed using the Mann–Whitney test

qPCR – quantitative real-time polymerase chain reaction.

we observed that high circ_0000673 expression was associated with shorter OS among CRC patients, and ROC curve analysis suggested that circ_0000673 might hold potential as a diagnostic biomarker. Notably, the inhibition of circ_0000673 effectively curtailed the proliferation, migration and invasion of CRC cells while diminishing their stemness. Finally, circ_0000673 knockdown resulted in the suppression of tumor growth in in vivo experiments. Collectively, these findings underscore the regulatory role of circ_0000673 as a pro-cancer factor in CRC.

In recent years, accumulating evidence has underscored the pivotal role of circRNAs in gene expression regulation, often achieved through their capacity to sponge miRNAs.[19] In our study, we unveiled that circ_0000673 functions as a miR-548b-3p sponge in CRC cells. Previous research has highlighted the tumor-suppressive effects of elevated miR-548b-3p in breast cancer, achieved by inhibiting *MDM2* expression.[20] In the context of lung cancer, Wang et al. demonstrated that miR-548b-3p acts as an oncogenic suppressor by regulating the PI3K/AKT signaling pathway,[21] and miR-548b-3p suppresses the malignancy of tumor cells by inhibiting the expression of *CIP2A* and *SP1*.[22] Our study has provided conclusive evidence that miR-548b-3p is downregulated in CRC tissues and cells. Notably, anti-miR-548b-3p partially counteracted the effects of circ_0000673 silencing on the malignant behaviors of CRC cells. These data collectively suggest that miR-548b-3p plays a tumor-suppressive role in CRC, whereas circ_0000673 functions as an accelerator of CRC progression by sponging miR-548b-3p.

Our study uncovers the role of *CPSF6* as a target gene of miR-548b-3p in CRC cells. In gastric cancer, *CPSF6* has been shown to negatively regulate *VHL* expression through alternative polyadenylation (APA), and the *VHL* short 3'UTR heterodimer induced apoptosis and hindered the growth of gastric cancer cells.[23] Similarly, *CPSF6* has been implicated in the progression of hepatocellular carcinoma (HCC) by upregulating *NQO1* expression through APA.[24] In a study by Liu et al., *CPSF6* was found to inhibit *BTG2* expression, promote glycolysis and suppress apoptosis in HCC cells.[25] In our investigation, we observed an upregulation of *CPSF6* in CRC tissues and cells, and the increased *CPSF6* levels counteracted the inhibitory effects of miR-548b-3p overexpression and si-circ_0000673 on the malignant behavior of CRC cells. These findings collectively suggest that *CPSF6* plays an oncogenic role in CRC. Therefore, we conclude that circ_0000673 regulates the progression of CRC by sponging miR-548b-3p to modulate the expression of *CPSF6*.

Limitations

Our study revealed the function and mechanism of circ_0000673 in CRC development. However, we did not investigate further the upstream regulatory mechanisms of circ_0000673, such as whether there is a strongly associated transcription factor causing abnormal expression of circ_0000673 in CRC. Furthermore, studies related to the clinical application of circ_0000673 have not been performed, including those examining its relationship with tumor drug resistance capacity.

Conclusions

To sum up, circ_0000673 assumes a tumor-promoting role within the context of CRC, actively driving its progression by orchestrating the miR-548b-3p/*CPSF6* axis. These findings propose that circ_0000673 holds potential as a promising target for the diagnosis and treatment of CRC in clinical practice.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10867861. The package includes the following files:

Supplementary Table 1. Primers used in this study.

Supplementary Table 2. Statistical methods and test results used.

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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Which risk factors are involved in a distal biceps tendon injury? A systematic review

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Abstract

Distal biceps tendon rupture is a rare injury predominately occurring in middle-aged men. This study aimed to collect relevant risk factors associated with distal biceps tendon rupture from the published literature. This systematic review aimed to collect and tabulate the risk factors for distal biceps tendon rupture. Studies published in English were searched concerning risk factors for distal biceps tendon ruptures until July 2022; cohort studies, case series and randomized controlled trials were subjected to analysis. Case studies, cadaveric studies and reviews in any form were excluded. The studies were quantitatively and qualitatively reviewed. One hundred twenty-one articles presenting risk factors for distal biceps tendon ruptures were identified, recruiting a total of 7,484–7,576 patients. The average age of the individuals was 46.8 years, with 96.7% being males and 94.7% having affinity for sports activities. In 56.7% of the cases, the dominant arm was involved, and in 54.6%, the right arm was affected. The use of tobacco was found in 20.8% of cases and of anabolic steroids in 2.5% of cases. On average, 55.8% of distal biceps tendon rupture patients had a physical occupation and the most common mechanism of the injury was related to heavy weight lifting observed in 53.2% of subjects. The most common and outstanding reported risk factors for distal biceps tendon ruptures were age, sex and sports activity, i.e., middle-aged males being still physically active and practicing sports. Steroid usage does not seem to increase significantly the risk of the distal biceps tendon rupture.

Key words: risk factor, tendon, rupture, distal biceps

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Introduction

The biceps brachii muscle originates as a 2-headed muscle, with the long head originating from the supraglenoid tubercle and the short head from the coracoid process. The muscle inserts on the radial tuberosity as 1 tendon. The fibers of the long head insert more proximally than the fibers of the short head, leading to greater function in supination of the long head and greater function in flexion of the short head.[1] A rupture of the distal biceps tendon usually leads to immediate weakness in supination and flexion. Additionally, a popping sound can be heard, followed by ecchymosis and edema. In most cases, retraction of the muscle is visible as a reverse Popeye sign.

Distal biceps tendon ruptures are uncommon injuries, with an incidence of 1.2 in the past and up to 6 per 100,000 nowadays.[2,3] This is likely explained by improved diagnostic procedures and consistent documentation of records nationwide.

Different methods have been implemented to diagnose and treat distal biceps tendon ruptures. Usually, clinical evaluation is sufficient, such as the hook test or the biceps squeeze test, yet in cases of doubt, ultrasonography (USG) or magnetic resonance imaging (MRI) can support the diagnosis.[4,5] Acute ruptures are characterized by a better prognosis, as chronic cases tend to have increased complication rates. The anatomical reinsertion should be targeted to recover most of the strength in flexion and supination. In older patients, conservative treatment is a viable alternative. The single- and the double-incision approaches have been described in the literature as equally viable methods with slight differences in complication rates.[6–9] The most common and major complications are lateral antebrachial cutaneous nerve (LABCN) injury, posterior interosseous nerve (PIN) injury, pain, and heterotrophic ossification.[10] A graft can be used for tendon reconstruction in cases of chronic rupture or insufficient tendon quality.[6,9] Overall, literature has shown that surgery on the ruptured distal biceps tendon can lead to a very satisfactory outcome with a slight loss in supination and flexion strength.[11–13]

Yet, data collection about risk factors for distal biceps tendon injuries in the current literature is limited to a few reviews without quantification of the cases.

Objectives

In the published literature, risk factors for distal biceps tendon injuries have been presented, but not quantified as a systematic review. This systematic review aimed to determine the risk factors associated with distal biceps tendon ruptures, as well as to collect the data and articles currently available in the literature.

Material and methods

This systematic review was designed according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement from 2020. A registration and review protocol was not prepared.

Literature search

The databases of Scopus, Embase and MEDLINE were searched on July 15, 2022, for risk factors associated with distal biceps tendon ruptures. A starting date for the search was not defined. The terms applied for bibliographic search in these databases were "distal", "biceps", "rupture", and "tear", with the following Boolean line: (distal) AND (biceps) AND ((rupture) OR (tear)). Filters were set according to the inclusion and exclusion criteria mentioned below. The search was performed by the principal investigator and reviewed by 2 co-investigators independently. The abstracts were then screened to identify articles involving the distal biceps tendon. The full-text articles were then reviewed by the authors independently. The data was collected independently and then compared between the investigators. Differences in data collection were discussed, and the articles were rescreened. Inclusion and exclusion criteria were set before data collection. The inclusion criteria included articles published in English, retrospective/prospective cohort studies, case series, and randomized controlled trials. Exclusion criteria included reviews in any form, cadaveric studies, case reports, and patients with tendinopathy or tendinitis without rupture.

The data were extracted and tabulated into age, sex, dominant arm, lateralization of injury, tobacco smoking, alcohol consumption, steroid usage, occupation, body mass index (BMI), drug usage (fluoroquinolones and statins), radial tuberosity, sports, comorbidities, and mechanism of injury.

Search results

The initial search of all before mentioned databases combined yielded 1,237 results, of which 596 were from Scopus, 132 from MEDLINE and 509 from Embase. After screening for duplicates, 649 articles were excluded. After screening the abstracts according to the exclusion criteria, 114 additional articles were excluded. Four hundred seventy-four articles were left for full-text article review, and of these, 119 articles met the inclusion standards, and 2 additional articles were identified by reviewing the references of these articles, resulting in a total of 121 for the systematic review (Fig. 1,2).

Analysis of articles

The articles that did not report an absolute number of participants in the subgroups but instead reported a percentage of participants were calculated to the absolute number by rounding the number to the next full digit.

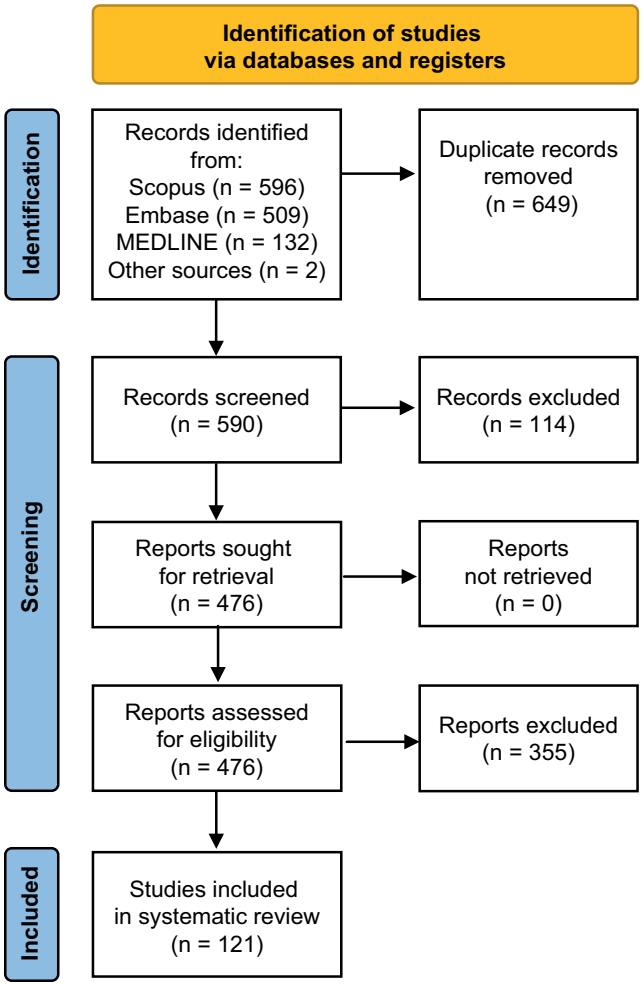

Fig. 1. Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 flowchart for a systematic review of risk factors for the distal biceps tendon rupture

Age

In 1 study, the age was not given as an average but instead in brackets. We assumed the middle value for each bracket and calculated the average value according to the provided data.

Occupation

This subgroup was divided into physical, mixed and non-physical groups. According to this classification, we assigned the occupation to their intensity in physical work. When an article categorized their cohort as physical and non-physical, the mixed category was counted as 0. Additionally, when an article partially did not report the occupation of participants, the amount of unknown occupations was deducted from the total number.

BMI

Some articles only reported height and weight either of each patient or as an average, so that was used to calculate the BMI.

Mechanism of injury

Martial arts, boxing and wrestling were considered to be fighting. Sports that did not belong to a fighting discipline, like football, baseball and rugby, were classified as sports. Weightlifting and bodybuilding were considered as lifting a heavy object as the mechanism. Additionally, when 2 mechanisms were mentioned for 1 number, this number was divided by 2, and each mechanism got half of the original number accredited.

Results

We analyzed the characteristics of 7,484–7,576 patients. The range is due to 1 article reporting a different/inconsistent number of patient information for different baseline characteristics.

Age

Of the 121 articles, 118 (97.5%) mentioned the age of the patients. In cases of bilateral ruptures, the age of the initial

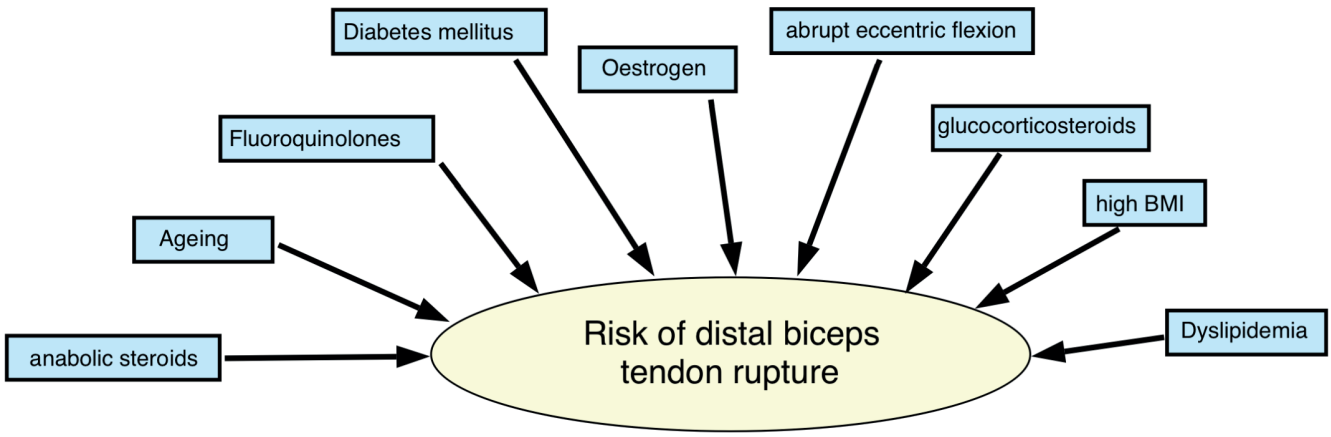

Fig. 2. Risk factors for distal biceps tendon ruptures

rupture was used in the average. The age of 7,408 patients at the time of the distal biceps tendon rupture was reported with an average age of 46.8 years. Furthermore, 7 articles reported the ages of female participants. The average age for 54 female patients was reported to be 60.1 years.

Sex

In total, 117 (96.7%) articles reported sex of the individuals. Of 7,355 participants, 7,112 were male (96.7%) and 243 (3.3%) were female.

Dominance and laterality

Information regarding injury to the dominant upper extremity was present in 88 articles (72.7%), while the laterality of the injury was addressed in 42 articles (34.7%). Of 3,798 patients analyzed in the 88 articles reporting dominance, 2,153 patients (56.7%) injured their dominant upper extremity and 1,645 patients (43.3%) injured their non-dominant upper extremity. From the point of laterality, 877 patients (54.6%) out of 1,606 injured their right upper extremity, while 729 patients (45.4%) injured their left side.

Tobacco smoking, steroids and alcohol

Overall, 33 (27.3%) articles included data on 3,573 patients, of which 743 (20.8%) were categorized as smokers. Regarding steroid use, 18 (14.9%) articles reported on 1,104 patients, in which 31 (2.8%) study participants were administered steroids, 28 (2.5%) patients admitted abusing anabolic steroids and 3 patients were used therapeutic glucocorticosteroids (lateral epicondylitis, sciatica and herniated disc). Only 3 (2.5%) articles reported alcohol consumption. Of 37 patients in the 1st study, 3 (8.1%) drank alcohol occasionally, 24 (64.9%) drank alcohol regularly and 10 (27%) abused alcohol. The 2nd study included 8 participants, of which no addiction to alcohol could be associated. The 3rd study reported 45 patients, of which 15 (33.3%) declared drinking alcohol regularly.

Occupation

In total, 29 (24%) articles reported either occupation of the patients or physical activity in their occupation. Of the 981 patients, 547 (55.8%) declared having a physical occupation, 122 (12.4%) a mixed occupation and 312 (31.8%) a non-physical occupation.

BMI

Body mass index was the subject in 10 (8.3%) articles during the time of rupture of the distal biceps tendon. The average BMI of 372 patients was 29.6, which is considered on the border between overweight to obese. Two articles categorized the BMI of their patients. Thus, 1 article concluded that 58 (15.5%) patients were obese in their cohort of 373 patients, and another article concluded that 6 (8.7%) had normal weight, 17 (24.6%) were overweight and 46 (66.7%) were obese in a cohort of 69 patients.

Statins and fluoroquinolones

Two articles included information about statin usage, and 2 articles included fluoroquinolones. In the aspect of statins, 1 study gave a quantitative measurement with 32 out of 104 (30.8%) patients reported using statins. The other study with 10 participants mentioned the usage of statins, yet the quantitative number is unclear. For fluoroquinolones, 36 patients were evaluated, of which 1 (2.8%) was administered levofloxacin at the time of injury.

Radial tuberosity

Hilgersom et al. concluded that radial tuberosity volume and height are significant risk factors for distal biceps tendon ruptures. In his cohort of ruptured tendons, 9 participants had a mean radial tuberosity size of 705 mm^3 compared to 541 mm^3 in the control group of 18 study participants. The mean radial tuberosity height in the rupture group was 4.6 mm, while in the control group it was 3.7 mm.[14] On the other hand, Kodde et al. concluded that radial tuberosity size did not correlate with distal biceps tendon ruptures in a cohort size of 22 patients in the rupture group and 22 in the control group.[15]

Sports

Of the 11 (9.1%) articles that reported on activity in sports, 177 (94.7%) of 187 were active sportsmen. Six of the 11 articles included data about the type of sports with 76 participants. The most common sports among those were weight lifting (n = 16), fitness (n = 15), boxing (n = 10), cycling (n = 7), martial arts (n = 5), and rugby (n = 5). Other types of sports reported were running (n = 3), soccer (n = 2), swimming (n = 2), triathlon (n = 2), tennis (n = 1), skiing (n = 1), rowing (n = 1), kabaddi (n = 1), bodybuilding (n = 1), wrestling (n = 1), climbing (n = 1), baseball (n = 1), volleyball (n = 1), golf (n = 1), and bow hunting (n = 1).

Comorbidities

In total, 10 (8.3%) articles included information about comorbidities. Two of those only mentioned the coexistence of systemic comorbidities but did not specify the comorbidities themselves. Of 139 patients, 35 (25.2%) had comorbidities. Four articles provided data about diabetes as a comorbidity; upon evaluating 364 patients, 17 (4.7%) were diagnosed with diabetes. Another study by Kelly et al. identified a 2.7% rate of diabetes in their cohort for patients below the age of 65 and a 19.8% rate for patients above the age of 65.[2] Hypercholesterolemia was a subject

Table 1. Summary of risk factors with references

Risk factors	Result	Reference
Age	46.8 years	3, 9, 11, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128
Age of women	60.1 years	3, 15, 23, 32, 65, 95, 103
Sex	96.7% men, 3.3% women	2, 3, 9, 11, 14, 15, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130
Dominance	56.7% dominant arm, 43.3% non-dominant arm	3, 9, 16, 17, 18, 19, 23, 26, 27, 28, 29, 30, 31, 32, 33, 34, 36, 37, 40, 41, 42, 43, 44, 46, 47, 48, 49, 52, 53, 54, 56, 59, 61, 62, 63, 64, 65, 66, 67, 68, 71, 72, 74, 75, 76, 77, 78, 81, 82, 83, 84, 85, 86, 87, 89, 90, 91, 92, 94, 95, 96, 97, 98, 101, 102, 103, 104, 106, 107, 108, 110, 111, 112, 113, 114, 116, 117, 118, 119, 121, 122, 123, 124, 125, 127, 128, 129, 130
Laterality	54.6% right arm, 45.4% left arm	3, 11, 14, 15, 17, 20, 21, 23, 24, 30, 31, 34, 43, 45, 49, 52, 53, 54, 55, 57, 61, 69, 70, 72, 74, 78, 82, 84, 86, 89, 92, 93, 101, 107, 108, 114, 119, 120, 124, 125, 127
Smoking	20.8%	2, 3, 9, 11, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 32, 34, 35, 36, 39, 41, 42, 43, 77, 102, 118, 128, 129, 130
Steroid	2.8%	3, 9, 15, 28, 32, 33, 34, 35, 37, 38, 39, 42, 61, 71, 102, 117, 129, 130
Occupation	55.8% physical, 12.4% mixed, 31.8% non-physical	2, 3, 21, 23, 31, 32, 33, 34, 35, 36, 43, 48, 65, 66, 67, 70, 71, 72, 75, 86, 95, 99, 100, 113, 116, 119, 120, 126, 130
BMI	29.6 kg/m^2	2, 14, 16, 23, 25, 34, 35, 36, 50, 68, 106, 119
Statin	30.8%	30, 35
Fluoroquinolone	2.8%	15, 35
Sports	94.7%	16, 21, 31, 33, 35, 37, 38, 39, 102, 113, 129
Alcohol	–	20, 32, 119
Radial tuberosity	–	14, 128
Comorbidities	–	2, 3, 17, 18, 21, 23, 24, 36, 37, 43
Mechanism of injury	–	3, 9, 11, 15, 17, 21, 28, 31, 34, 36, 42, 44, 47, 48, 59, 63, 67, 70, 86, 91, 94, 95, 96, 99, 101, 102, 106, 111, 113, 115, 116, 117, 119, 120, 123, 124, 125, 127

BMI – body mass index.

of 2 articles with 246 patients, of which 5 were diagnosed with hypercholesterolemia. Further comorbidities were addressed in 2 studies involving 241 patients. Hypertension (12), asthma (9) and diabetes (5) were the most common ones. Ischemic heart disease (3), tendinitis (3), hypercholesterolemia (2), inflammatory joint disease (1), and renal dysfunction (1) were also reported.

Mechanism of injury

Of the 121 articles, 39 (33.1%) articles reported about the mechanisms of injury, of which 5 qualitatively described the mechanism of injury. All 5 articles described an eccentric, excessive and sudden load in an acute setting as the most common. In the other 34 articles, the mechanism of injury for 43 patients out of 1,645 was unknown. The most common way of rupturing the distal biceps described was lifting a heavy object with 852 (53.2%) ruptures, followed by sporting activities with 196 (12.2%) ruptures. Also, quite common mechanisms of rupture were falling with 93 (5.8%) ruptures and forceful elbow extension-catching a falling object with 81 ruptures (5.1%). Less common were fighting and assault with 46 ruptures (2.9%), insidious ruptures with 34 ruptures (2.1%), pulling movements with 29 ruptures (1.8%), blunt impact with 10 ruptures (0.6%), and other causes in 243 ruptures (15.2%, Table 1).[3–130]

Discussion

This systematic review summarizes the knowledge about current risk factors for distal biceps tendon ruptures. The average male age associated with the incidence of rupture was 46.8 years, while in female patients, the average age of rupture was more advanced at 60.1 years. We suspect that decreased loads posed on the arm due to everyday activities, along with increased tendon degeneration with aging, precipitates conditions in which a minimal load on the arm is sufficient to cause the distal biceps tendon to rupture. Likewise, the sex distribution indicates that men as more associated with ruptures. Dunphy et al. and Ford et al. reported a similar incidence of rupture

in male and female participants with 98.5% and 97.6%, respectively.[9,23]

Additionally, we wanted to point out the risk factor of arm dominance in the literature. In most of the literature, the dominant side is affected by the rupture,[131–135] yet in this systematic review, the dominant arm was involved in only 56.7% of the reported cases.

In this systematic review, it was observed that the majority of participants (94.7%) engaged in sports activities, either as professionals or as a leisure pursuit. The meaningfulness of BMI becomes less accurate when muscle mass is highly increased, such as in sportsmen. In these cases, BMI value would indicate higher than normal body mass, yet in some healthy states, the increase of BMI is due to muscle and not fatty tissue. Therefore, BMI alone is an inaccurate risk factor as the body composition of muscle and other structures is disregarded. Due to the high rate of study participants involved in sports activities, we believe that muscle mass is the major contributor to the increased BMI. Additionally, most of the patients reported at least a partially physical occupation (68.2%), leading to the possibility that muscle mass might be increased in those subjects.

The literature ascribes distal biceps tendon ruptures to middle-aged men, usually with an active lifestyle.[2,136–138] This systematic review confirms the general knowledge, and reports the rupture incidence in men at 96.7%, at the mean age of 46.8 years, with 94.7% of individuals being involved in an active lifestyle and 68.2% having a physical or mixed physical occupation.

Regarding tobacco smoking, Kelly et al. reported findings that are not in line with our study. They used a national database and reported that 4.3% of the injured population were tobacco users, while in our review, 20.8% can be considered smokers. Regarding the point of diabetes mellitus, Kelly et al. reported that below the age of 65 years, diabetes rates were estimated to be 2.7%, and above the age of 65 to be 19.8% for the injured population.[2] The 4.7% diabetes incidence in our findings can be explained by the lack of age stratifications, i.e., participants aged above 65 years were included in the analysis and increased the rate of diabetes.

The study by Pope et al.[139] indicates that approx. 3–4 million individuals aged 13–50 years in the USA are using anabolic steroids, representing 8.4% of the 155 million individuals aged 15–50 years in the USA, according to the United Nations population count.[140] Therefore, the rate of anabolic steroid usage would be approx. 1.9–2.6% in the abovementioned age groups. In our systematic review, the rate of patients who used anabolic steroids was at the upper range of normality (2.5%), according to the calculations presented above. On the other hand, the usage of anabolic steroids has a bad reputation, is connected to health risks and is often concealed by patients.

We think that risk factor analysis in distal biceps tendon ruptures is veiled due to the lower usage of arms compared to legs in daily life. Tendons of the legs are used every day to carry the whole bodyweight, while muscles of the arm are only used when carrying objects. Therefore, tendons of the lower limb are much more prone to rupture due to systemic degenerative changes of the tendons than those of the upper limb.

Pathomechanisms of relevant risk factors

Aging has a tremendous effect on all tendons in the human body. On the cellular level, the active tenoblasts transform into inactive tenocytes with a general, age-dependent decrease in the number of tendon cells. Additionally, the overall metabolic activity of tenoblasts decreases drastically with age, leading to a reduced potential to repair and heal the tendon. Furthermore, metabolic anaerobic over aerobic pathways are favored due to overall reduced metabolism.

In the extracellular matrix (ECM), qualitative and quantitative changes can be seen. Collagen content reduces slightly, proteoglycans and glycoproteins decrease more intensively, as well as elastic components. Furthermore, water and mucopolysaccharide content of the tendon is also decreased, leading to increased stiffness of the tendon and a reduced gliding ability. Overall, a reduced tendon diameter and volume can be seen. Additionally, the vascular bed of the tendon is reduced, which results in reduced oxygen and nutrition transport to the tendon. Accumulation of lipids and calcium deposits can also be observed, which disrupts the tendon matrix and weakens the tensile strength. All of these mentioned factors lead to decreased tensile strength and increased risk of tendon rupture.[141]

Tobacco smoking has a similar effect as aging. It affects the healing ability by reducing the vascular supply of oxygen and nutrients, as nicotine is a potent vasoconstrictor.[142,143]

It is hypothesized that sex is a risk factor in tendon ruptures, mainly due to hormonal differences. Estrogens are said to be tendon-protective.[144–146] The mechanism of tendon protection by estrogens can be ascribed to inhibition in lysyl oxidase, the enzyme that forms cross-links between fibers in ligaments and tendons. The enzyme inhibition results in reduced cross linkage, producing more elastic tendons, and thus decreasing the impact of an abrupt muscle contraction.[146,147] On the other hand, testosterone, by modulating androgen receptors, causes the muscle to increase its mass via protein synthesis.[148] Therefore, the combination of a deficit of female hormones and increased muscle mass and strength in men leads to an increased risk of tendon injury.

A similar mechanism of action can be ascribed to anabolic steroids since they are analogs of testosterone. Under anabolic steroids, a significant increase in muscle mass and strength can be observed, leading to increased tension on the tendon and a higher risk of rupture. Additionally, studies have shown that anabolic steroids also lead to dysplastic changes in the tendon and, therefore, decreased tensile strength.[149,150]

The BMI, usually used as a rough indicator of body fat in non-athletic people, is shown to be a risk factor for tendon ruptures. The visceral fat-releasing inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 1 beta (IL-1β), which are implicated in pathophysiological structural changes in the tendon and can increase the risk of ruptures.[151,152]

Literature has shown that glucocorticosteroids alter the mechanical tendon properties by activating the glucocorticoid receptor, which influences gene expression and transcription. A reduction in collagen fiber diameter, in cross-linkage and of collagen content can be observed, which results in decreased tensile strength. Additionally, in vitro studies showed dysfunction of tenocytes, such as decreased proliferation, reduced collagen formation and increased reactive oxygen species (ROS) formation. Glucocorticosteroids also increase visceral fat deposition, which causes an increase in inflammatory cytokines as described above.[150,151,153]

Dyslipidemia is an underestimated risk factor for tendon ruptures, as specifically cholesterol accumulates in the tendon and disrupts its integrity.[151,154] Hypercholesterolemia can be associated with weakened tendons. Statins are used to treat elevated levels of cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, yet studies have also shown that statins themselves are a risk factor for tendon ruptures. The precise mechanism of action on a molecular level is still unclear. It is hypothesized that matrix metalloproteinase (MMP) activity is increased, which mediates the catabolic state in tendons without a change in total levels of collagen, leading to a decrease in extracellular collagen strength and an increased risk of tendon rupture.[151,153,155]

Studies have shown that fluoroquinolones increase the risk of tendon ruptures. However, the mechanism of action can only be hypothesized. It is ascribed to direct toxicity as the rupture usually occurs hours after a single dose administration.[156] One of the hypotheses is that fluoroquinolones stimulate the production and accumulation of ROS and thus induce apoptosis. Another hypothesis points out the activation of MMPs and induction of prostaglandin E2, IL and cyclooxygenase 2 (COX-2) production, similar to the state observed in dyslipidemia and visceral fat accumulation.[153,157,158]

Another tendon rupture risk factor is diabetes mellitus, more commonly type 2 (T2D). It was shown that non-enzymatic cross-links can be formed through the Maillard reaction between sugars and amino acids called advanced glycation end-products (AGEs).[157] This results in the formation of stiffer tendons and a higher risk of ruptures, as an abrupt muscle contraction leads to abrupt transmission of the force to the tendon.[146,151,158] Additionally, high glucose levels modulate gene expression, such as the decreased levels of adenosine monophosphate (AMP)-activated protein kinase, leading to a decreased production of adenosine triphosphate (ATP), and therefore decreased metabolic rate of tenocytes.[159,160] In cases involving T2D, its onset is slow compared to type 1 diabetes, and therefore high glucose levels exist unrecognized for years. Furthermore, T2D is often associated with obesity, which itself is a risk factor for tendon rupture, as described above. The negative effects of glucocorticosteroids on tendons include hyperglycemia, which adds to the overall risk of steroids.

In the literature, the mechanism of rupture of the distal biceps tendon is usually described as an eccentric contraction.[161,162] This is due to biomechanical forces in different elbow positions. When the elbow is 90°-angulated in the standing position, the point of insertion at the radial tuberosity is a few centimeters anterior to the elbow joint. A lever system is created, and as soon as the arm is extended, the distance between tendon insertion and the elbow joint is reduced. This reduction in distance leads to an increase in force produced on the tendon, although the same force is being applied to the distal arm. This increase in forces leads to rupture in the weakest part of the tendon, especially when it develops abruptly or with decreased tensile strength.[163] Another biomechanical risk factor involved in the tendon rupture has been ascribed to a reduced proximal radioulnar space near the point of insertion. It has been suggested that the polymorphic traits of the radial tuberosity could lead to a decrease in the radioulnar space. The linear distance between the radius and ulnar is reduced by 45% when pronated, leaving less than 1 mm for the distal biceps tendon to slide through.[164] In cases where the radial tuberosity is more pronounced, less space is available, and impingement of the tendon is possible, causing friction, inflammation and degeneration of the tendon.[14]

Limitations

This systematic review does have its limitations. The included studies showed a wide basis of heterogeneity, which made it impossible to perform a meta-analysis. Additionally, the included studies had different interests in their outcomes, which made the collection of studies heterogenic. As already seen in the results section, many studies provided incomplete data, and precise information was limited. It is well known that fluoroquinolones are a risk factor for tendon ruptures,[7] yet only 2 studies reported information about these drugs. There is also probable reporting bias as temporary local inflammation in the area might be neglected in reporting, contrary to chronic diseases like asthma or hypertension. Therefore, hypertension and asthma might be overrepresented in this systematic review, similar to anabolic steroids, which might be purposely not reported by patients. Another limitation is that studies with level III or IV evidence were most often analyzed, and each of the included studies had their own limitations, e.g., small cohort size and incomplete data.

Conclusions

The most common and outstanding reported risk factors for distal biceps tendon ruptures were older age, male sex and sports activity. The average patient with a distal biceps tendon injury (slightly higher in the dominant arm) is a man aged 46.8 years with an active lifestyle and professional or sport activity. Steroid usage does not seem to significantly increase the risk of the distal biceps tendon rupture.

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An update on cardiovascular disorders in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a complex multifactorial etiology that develops as a result of autoimmune processes, leading to widespread inflammation and malfunction of multiple tissues and organs, and, as a consequence, triggers arterial hypertension, conduction disorders, valvular heart disease, pulmonary hypertension (PH), and venous thromboembolism events (VTE), contributing to increased mortality. Moreover, autoimmune abnormalities can accelerate atherogenesis and lead to many SLE manifestations, including coronary artery disease (CAD) and cerebrovascular events. The current review aimed to systematize existing data from the latest works and summarize published guidelines and recommendations. In particular, the prevalence of cardiovascular disorders in SLE patients, advances in diagnostics (including imaging methods and biomarker laboratory testing), the possible future direction of therapy, and the latest European Alliance of Associations for Rheumatology (EULAR) guidelines for optimal management of cardiovascular risk in SLE were overviewed.

Key words: risk, autoimmunity, cardiovascular, systemic lupus erythematosus, atherogenesis

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease associated with widespread inflammation resulting in the malfunction of multiple tissues and organs. The pathophysiology of SLE includes the production of autoantibodies to nuclear and cytoplasmic antigens and the deposition of immune complexes in tissues. Systemic lupus erythematosus is one of the most common autoimmune diseases and affects approx. 3.4 million people worldwide, including around 18,500 cases in Poland. However, epidemiological data are lacking from some regions.[1–3] In the context of this review, we consider cardiovascular complications of SLE (Fig. 1), which occur due to functional changes of the endothelium, oxidative stress increased by inflammation, and aberrations in the immune response.[4]

Systemic lupus erythematosus predominantly affects women, with a 9:1 female-to-male ratio, and patients tend to present symptoms during childbearing age.[5] Cardiovascular mortality risk in SLE is about 3 times higher compared to the general population. Antiphospholipid antibodies (aPLs) are found in about 50% of SLE patients and are a significant independent risk factor for thrombotic events.[6] Independent of aPLs, increased incidence of traditional cardiovascular and lupus-related thrombosis risk factors significantly increase the risk of premature atherosclerosis in SLE patients.[7]

Objectives

Due to the constantly expanding knowledge on the occurrence of cardiovascular complications, their pathomechanisms and their treatment methods in SLE, this review aimed to systematize data from the latest works and summarize the published guidelines and recommendations.

Materials and methods

The literature search used PubMed, Embase and Google Scholar databases, references from relevant articles, and internet sources, including the World Health Organization (WHO) and other reports. Search terms included "cardiovascular complications SLE," "arrhythmias SLE," "atherogenesis SLE," "cardiovascular complications APS," "pulmonary hypertension SLE," "thromboembolic events SLE," and "valvular disorders SLE," using SLE and antiphospholipid syndrome (APS) as abbreviations and the full name – systemic lupus erythematosus. No publication date filters were applied, with the last literature search performed on July 29, 2023. Titles and abstracts were screened by the authors to identify relevant works, and the most appropriate sources that included data on epidemiology, complications, diagnostics, and suggested treatment were summarized.

Accelerated atherogenesis

Studies unambiguously show that SLE patients suffer atherosclerosis more often and, as a result, coronary artery disease (CAD), myocardial infarction (MI) and stroke, compared to the general population. Research suggests that chronic inflammation promotes accelerated atherogenesis. Although traditional risk factors are vital in accelerated atherogenesis pathophysiology in autoimmune diseases,[8,9] the high incidence of cardiovascular disease (CVD) in SLE patients cannot be fully explained by traditional cardiovascular risk factors.[10]

Excessive immune system activity in SLE leads to oxidative stress, granulocyte activation, subsequent thrombin generation, and macrophage differentiation to foam cells, leading to accelerated lipoprotein accumulation and oxidation in the endothelium, which may stimulate plaque formation.[11] These processes are also mediated by aPLs,

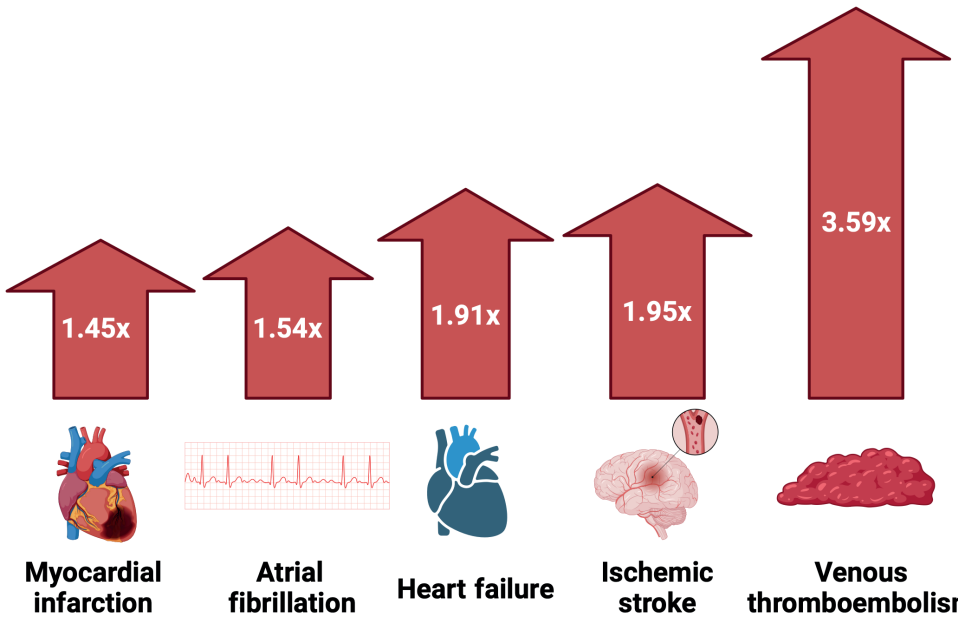

Fig. 1. Cardiovascular complications in systemic lupus erythematosus compared to a healthy population. Created with Biorender.com

which account for a higher risk of cardiovascular events in APS patients.[12] The presence of aPLs is one of the SLE diagnostic criteria, and up to 40% of SLE patients have APS.[13] Such patients are at a similar risk of new plaque formation as those with diabetes mellitus and have a 3.3 times higher risk than healthy controls.[14] The prevalence of coronary artery plaques in SLE patients was assessed by multiple studies,[15,16] and, depending on the study, plaques were found in 16.3%[17] or 42.1%[18] of patients. Patients with SLE also have a twofold higher prevalence of plaque compared to controls and a similar prevalence to patients with diabetes mellitus or rheumatoid arthritis.[19] In addition, 32% of the SLE group had atherosclerotic plaques in their carotid arteries after a 5-year follow-up, while it was 4% in healthy control.[20] Moreover, a retrospective analysis revealed that the presence of either lupus anticoagulant or anti-cardiolipin antibodies was significantly associated with cardiovascular events, whereas the presence of anti-beta-2-glycoprotein I antibodies was not, which, after confirmation in other studies, may be used as a risk assessment factor in SLE patients.[21]

The high atherosclerosis incidence in women with SLE was first observed in autopsies in 1975, with many studies confirming the association between SLE and cardiovascular diseases.[22–24] Atherosclerosis causes vessel narrowing through plaque buildup, and some plaques are prone to rupture, which may lead to MI or stroke. Generally, MI incidence is approx. 9 times greater in SLE patients than in the general population.[25] In the Framingham Offspring Study, women with SLE aged between 35 and 44 had an over 50 times higher risk of MI than similar-aged female participants.[26] A Dutch study analyzed 3,411 patients with SLE and matched control subjects and assessed their absolute 10-year MI risk at 2.17% (95% confidence intervals (95% CIs): 1.66–2.80) for SLE patients and 1.49% (95% CI: 1.26–1.75) in the healthy population. A meta-analysis by Ballocca et al. showed that 4.1% of 17,187 SLE patients presented with acute MI.[27] The cohort study published in 2021 showed that, among over 4,000 patients with SLE, the incidence of MI was 9.6 (95% CI: 8.9–10.5) events/1,000 person-year, contrary to the comparison cohort, where the incidence of MI was 4.9 (95% CI: 4.8–5.1) events/1,000 person-year.[28]

Coronary artery disease, angina pectoris and MI were found significantly more frequently in SLE patients compared to age-matched controls (15.2% compared to 3.6%, p = 0.0041).[29] The rate of ischemic stroke and MI was also up to twice as high in SLE compared with the general population.[30] Such cerebrovascular events in SLE patients may be directly attributed to the disease, as a neuropsychiatric manifestation of SLE, or the result of impaired endothelial function or accelerated clot formation resulting in ischemic incidents.[31] In a long-term follow-up study including over 17,000 patients, 3.75% (95% CI: 3.06–4.54%) of those in the SLE cohort developed ischemic stroke compared to 1.92% (95% CI: 1.66–2.20%) of control subjects.[32] Another study showed a stroke in 7.4% of SLE patients after a 3-year follow-up.[24] Stroke is responsible for 15% of SLE patient deaths.[33] Recent data from the Japan registry showed that the most common type of stroke is cerebral infarction (80%), and no significant differences were found in post-stroke prognosis and the degree of SLE activity.[34] In this regard, SLE not only influences stroke risk directly but also through the more frequent presence of comorbidities such as arterial hypertension.

Another concern is the recovery process after cardiovascular events, with analysis of patient medical history after acute MI showing that SLE subjects had higher inpatient mortality during the index hospitalization and higher 30-day hospital readmission compared to patients without SLE.[35]

A recent cross-sectional study showed a higher prevalence of hypertension, dyslipidemia and carotid atherosclerosis in women with SLE. Moreover, CC homozygosity for the *GCKR rs1260326* gene (odds ratio (OR) = 0.111, 95% CI: 0.015–0.804, p = 0.030) and an increase of 1 mmol/L in triglyceride concentrations was associated with a greater risk of carotid plaque in women with SLE (OR = 7.576, 95% CI: 2.415–23.767, p = 0.001).[36] Studying *GCKR rs1260326* variants may be useful for adjusting lipid-lowering treatment or primary prevention by prescribing statins for these patients. However, current European Alliance of Associations for Rheumatology (EULAR) guidelines only recommend statin prescriptions accompanying estimation of CVD risk from the Systematic Coronary Risk Evaluation (SCORE), not considering SLE as a unique factor, probably due to the conflicting results, especially regarding atorvastatin.[37] Studies on atorvastatin, despite good drug tolerance, failed to prove beneficial on subclinical atherosclerosis progression or disease activity in children and adult populations with SLE.[38,39] Data suggest that atorvastatin may normalize the T helper (Th)17/T regulatory (Treg) cell balance and apoptosis induction and may interact with C-reactive protein (CRP) and interleukin (IL)-6, which are elevated in SLE.[40] Thus, future studies should evaluate the direct effects in vitro and in SLE patients. Moreover, a recent study suggests that children with 2 or more copies of *C4b* genes may benefit more from atorvastatin therapy.[41] However, current guidelines recommend guiding lipid treatment in line with the general population.[42] To prevent CVD-related complications, low-aspirin treatment should be considered based on the patient's cardiovascular risk.[42]

Arterial hypertension is a frequent complication of SLE and APS, which may result from the presence of renal disease or as an unwanted treatment effect.[43] Up to 77% of SLE patients can have arterial hypertension, compared to 7.7% in the healthy population aged 22–44 years.[44] Hypertension is related to increased CVD incidence in such patients and may be associated with up to a 2 times higher risk of cognitive dysfunction or stroke.[45,46] Current guidelines suggest that a blood pressure (BP) target of <130/80 mmHg should be considered in this group of patients.[42] Moreover, patients with SLE may have a different circadian BP pattern, with the non-dipper pattern frequently presented,[47] so 24-h ambulatory BP monitoring is suggested.

Valvular disorders

Inflammation, initiated by deposits of antibodies and complement components, leads to fibrotic processes that result in valve damage. Multiple studies have shown a strong link between the presence of aPLs and valvular disorders,[48–53] which are common lupus manifestations – including leaflet thickening, vegetations, regurgitation, and stenosis (Fig. 1). Such abnormalities are reported in over 30% of SLE patients,[49,50] though most cases display no clinical symptoms concomitant to valvular disorder.[51,54] Therefore, there is a risk of overlooking this condition in a significant group of patients, which may lead to more advanced defects requiring surgical treatment.

The most affected valve is the mitral valve, followed, in order of prevalence, by tricuspid, aortic and pulmonary valves. Mitral valve abnormalities are found in over 30% of patients, and the most common valvular dysfunction is regurgitation.[48,55,56] According to a 2016 meta-analysis, patients with SLE tend to have an 11-fold increased risk of combined valvular alterations, and a 10-fold increase in mitral valve thickening, aortic valve thickening, mitral valve regurgitation, and mitral valve vegetation in comparison to healthy controls. Furthermore, SLE patients have a 5 times higher risk of tricuspid and aortic valve regurgitation and mitral valve stenosis (Fig. 2). However, there were no differences in tricuspid valve thickening or aortic valve stenosis.[57]

Valvular disorders are also associated with aPLs. After analyzing 23 studies with 1,656 SLE patients, valvular disease was found in 43% (95% CI: 40–47) of aPL-positive individuals and 22% (95% CI: 19–25) of aPL-negative patients (OR = 3.13, 95% CI: 2.31–4.24, $p < 0.00001$).[49] Meanwhile, the likelihood of developing valvular disease is estimated to be 8% for APS patients.[58] High aPL levels, defined as titers over 20, were associated with the presence of valve abnormality after adjusting for disease duration and patient

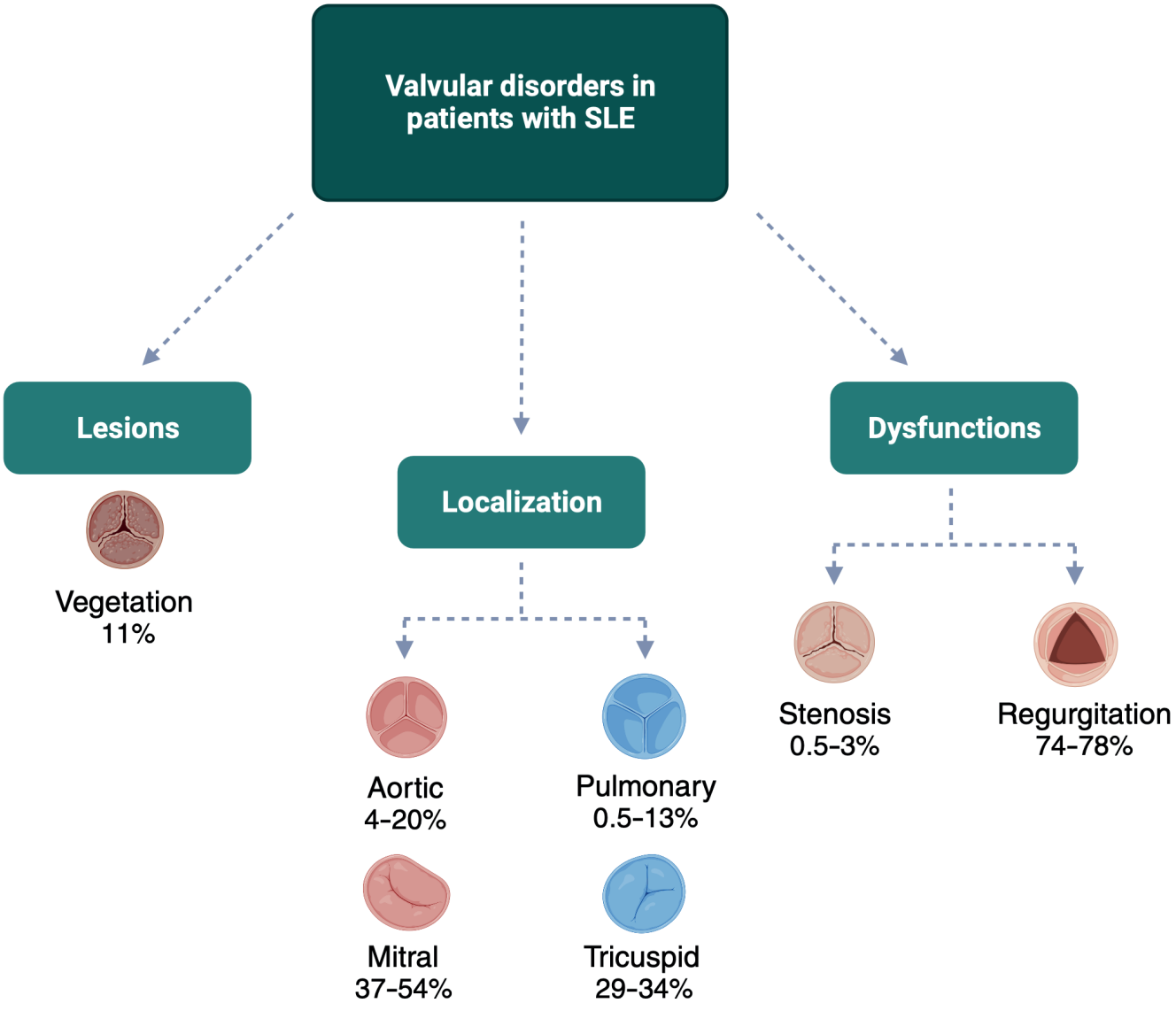

Fig. 2. Valvular disorders in patients with SLE

SLE – systemic lupus erythematosus. Created with Biorender.com

age.[48] Furthermore, there was a significant association between valvular heart disease in SLE and immunoglobulin G (IgG) anti-cardiolipin antibody positivity, suggesting that IgG anticardiolipin-positive SLE patients may profit from echocardiography screening.[59]

After discovering a valvular disorder, adequate personalized treatment should be considered. The latest reports indicate that the socio-demographic index (SDI) may be a useful predictor of cardiovascular outcomes after cardiac valve surgery.[60] However, results should be interpreted carefully due to the limited patients number. Furthermore, xenograft should be the preferred option for patients with SLE or APS, but more data should be obtained to confirm these observations.[61] Guidelines do not indicate special treatment for patients with SLE.[62]

Libman–Sacks (LS) endocarditis is a nonbacterial thrombotic endocarditis found in approx. 1 in 10 SLE patients.[51] The vegetation on valves may develop as a result of fibrin and platelet aggregation without bacterial infection present.[63] Rarely, LS is associated with valve dysfunction – usually left side valves – mitral and aortic, but the involvement of other valves is not excluded. The endothelial damage is caused by hypercoagulability, typical for SLE, APS and neoplasms,[64] so anticoagulant treatment should be considered in all patients with LS endocarditis. There is a significant association between APS and LS endocarditis. In one of the latest retrospective cohort studies, patients with LS endocarditis were more likely to have β2GP1 IgG antibodies and lupus anticoagulant.[52] In another study, after comparing SLE patients with and without LS endocarditis, the SLE group had a significantly higher prevalence of aPL and a lower prevalence of SLE-specific antibodies.[53] However, the direct mechanism of endocarditis in these patients is unknown and may be associated with immunosuppressive therapy. In practice, performing transthoracic echocardiography in SLE patients with triple aPL positivity can be useful for screening and can be confirmed by transesophageal echocardiography. Anticoagulant treatment should be considered alongside SLE treatment.[65]

Electrophysiologic dysfunctions

SLE-linked structural changes can affect the conduction system. As previously stated, patients with SLE are more likely to develop atherogenesis and, due to that, commonly suffer from ischemic cardiac events that can damage conduction pathways and lead to arrhythmogenesis.[66] The prevalence of sinus node dysfunction was reported as 4.3%.[67] Of these patients, only 3.6% had a pacemaker implanted over a 5-year period.[67] As inflammation processes progress, myocardial cells are replaced by connective tissue, disturbing repolarization and conduction.[68] Patients with SLE develop atrial fibrillation (AF) more frequently than the general population,[32] with the most common arrhythmias being tachyarrhythmias, specifically, sinus tachycardia, AF and atrial ectopies.[69]

An association exists between anti-Ro/SSA antibodies and QT prolongation.[70] Myung et al. reviewed 12-lead electrocardiogram (ECG) records of 235 SLE patients and assessed 53 patients with abnormalities that included sinus tachycardia (18% of patients), sinus bradycardia (14%), QT prolongation (17%), and tachyarrhythmias (6%) such as AF, atrial flutter, atrial tachycardia, and atrioventricular nodal reentrant tachycardia (AVNRT)/atrioventricular reentry tachycardia (AVRT).[71] Electrocardiography findings correlated positively with age, disease duration, anti-nuclear antibody (ANA) titer, and disease activity (systemic lupus erythematosus disease activity index (SLEDAI)-2K), and were associated with hypertension, positive anti-SSA and secondary Sjögren syndrome.[72] A 2018 study confirmed the independent association between SLE and AF, even after adjusting for age, sex, race, and CAD.[73] Furthermore, SLE patients have almost twice the increased risk of hospitalization due to AF.[74]

Corticosteroids and anti-malarial drugs, standard SLE treatments, may cause tachyarrhythmias and QRS prolongation, but chloroquine may have a protective effect on the conduction system.[75,76] Furthermore, hydroxychloroquine might induce QTc prolongation in SLE patients,[77] though the findings of the study published in 2022 did not confirm clinically consequential QTc prolongation in SLE patients treated with hydroxychloroquine.[78,79] Tachycardia can also be induced by other SLE therapeutics, including mycophenolate mofetil, tacrolimus and rituximab, while AF can be caused by rituximab. In the opinion of the Federal Drug Administration (FDA), azathioprine and belimumab do not affect conduction processes.[80]

Pulmonary hypertension

The link between pulmonary hypertension (PH) and SLE was first observed over 30 years ago.[81,82] However, studies assessing the prevalence of PH in SLE patients gave ambiguous results. Pulmonary hypertension is a rare but severe condition in SLE patients, and some papers report PH as the first clinical lupus manifestation,[83–85] with the prevalence of PH in SLE patients estimated at between 0.5% and 17.5%.[86] A meta-analysis of 23 cross-sectional studies showed that PH may be present in 8% of patients with SLE, though the prevalence differed depending on patient age, gender, geographical region, and the year of the study.[87] Patients with SLE may present with different types of PH resulting from interstitial lung disease, left heart disease, pulmonary arterial hypertension (PAH), and chronic thrombo-embolic complications.

According to Sun et al., 2 clinical types of PAH can be distinguished in SLE, including vasculopathic, characterized by low lupus activity, and vasculitic, with broad clinical manifestations including pericarditis, rash, arthritis, nephritis, and neuropsychiatric lupus.[88] Kaplan–Meier survival analysis showed that patients with the vasculitic type had a higher 3-year mortality rate than those

with the vasculopathic type (34.5–40.2% compared to 13.0–18.6%, p < 0.05; hazard ratio (HR) 2.84–3.15) after adjusting for treatment variations.[88] The authors suggest that 2 distinct phenotypes of PAH in SLE may have different pathophysiological processes. Moreover, the vasculitic PAH type seems more sensitive to immunosuppressive therapy. There are 2 independent predictive factors of the vasculitic subtype, which include the time interval between the diagnosis of SLE and PAH (<2 years) and an SLEDAI > 9. A weighted score ≥2, combining these 2 factors (time interval <2 years and ≥2 years were 1 and 0 points, respectively, while SLEDAI >9, 5–9 and <5 were 2, 1 and 0 points, respectively), was further developed as a prediction model of vasculitic subtype.[88–90]

The mechanism of PAH secondary to SLE was studied employing genetic analysis due to the shared and unique gene signatures of these diseases. The shared interferon (IFN)-induced genes might be crucial for identifying new biomarkers and potential therapeutic targets.[91] Meta-analysis showed that modern therapy provides a similar reduction in morbidity/mortality risk in patients with connective tissue disease-PAH compared to the PAH population overall and revealed that survival rates of SLE-PAH patients are higher than those with systemic sclerosis-PAH.[78] D-dimer levels are a known predictive factor for PAH and may act as a mediator of reduced low-density lipoprotein (LDL), although optimal cutoff points and estimations of individual pressure ranges are unknown.[92]

Patients with SLE may benefit from PH screening using transthoracic ECG, lung function tests (forced vital capacity and diffusion lung capacity for carbon monoxide) and an N-terminal pro-B-type natriuretic peptide (NT-proBNP) test, particularly in combination.[93] Moreover, genetic phenotyping may be a standard beneficial option in the future. The latest work revealed *HLA-DQA1*03:02* as a genetic variant associated with PAH in SLE patients.[94] The study included 172 patients with SLE and PAH confirmed by right heart catheterization and over 11,000 controls. Patients with PAH and the presence of *HLA-DQA1*03:02* had significantly lower rates of target role achievement (p = 0.005) and survival (p = 0.04). Therefore, genetic typing may open the way for individualized patient care, which may be particularly beneficial in autoimmune diseases since they have different patterns and manifestations in each patient.[94] Current guidelines recommend the same algorithm for treating PAH associated with connective tissue disease and idiopathic PAH, including treatment of the underlying condition.[95]

Heart failure

Pathomechanisms of heart failure (HF) include CAD, but there are also many other causes, highlighting the complexity of its development. Myocarditis and pericarditis, which are relatively common cardiac complications in SLE, may lead to HF.[96,97] Patients with SLE have up to a twofold higher risk of developing HF compared to the healthy population,[32] and the coexistence of SLE and HF contributes to increased mortality. Current HF classification based on ejection fraction includes HF with reduced ejection fraction (HFrEF), HF with mildly reduced ejection fraction (HFmrEF) and HF with preserved ejection fraction (HFpEF),[98] which is the most common type in these patients, probably due to the multifactorial nature of the disease and widespread inflammation. Complications of HF in SLE patients are similar to those in patients without autoimmune disorders and may contribute to hospitalization or death. Diagnostic pathways to confirm HF in SLE patients do not differ from those for the general population,[99] and include symptoms, laboratory and echocardiographic assessments. Interestingly, the high prevalence of subclinical HF confirmed with cardiac magnetic resonance (CMR) is observed in SLE patients.[100] A prognostic tool was developed based on CMR to enable stratification of SLE HFpEF risk, which may contribute to a more accurate diagnosis and better treatment approach. Nevertheless, this solution should be validated in future trials.[101] Current guidelines do not recommend special treatment for this group.[99]

Venous thromboembolism

The latest meta-analysis indicates a significantly higher risk of venous thromboembolism (VTE) events, including deep vein thrombosis and pulmonary embolism (risk ratio (RR) = 4.38, 95% CI: 2.63–7.29), in SLE patients compared to the general population.[7] Moreover, the absolute risk of VTE in younger patients with SLE (<40 years) is higher than in patients with SLE at the age of 41–64.[7] A retrospective study with a median follow-up period of 13 years revealed that VTE was the most frequent cardiovascular event, accounting for 50% of the total.[21]

The absolute risk of VTE events is higher in SLE patients with APS or aPL.[7] Similar to healthy populations, SLE patients with normal D-dimer levels are at low risk for thrombosis, and those with elevated D-dimer levels, without other influencing comorbidities, are at high risk for thrombosis.[102] Higher risks of VTEs and the associated risk factors among patients with SLE should be considered when optimizing treatment to balance the risks and benefits of the chosen therapy.[7]

Antiphospholipid syndrome is the primary determinant for the recurrence risk of a first SLE-associated VTE, meaning that indefinite anticoagulant therapy may be warranted in SLE patients with secondary APS. In the absence of APS treatment, decisions on SLE-associated VTE may be approached similarly to the general population. However, studies on the bleeding risk of anticoagulant therapy are needed to make an adequate benefit–risk assessment. Furthermore, active SLE at the time of a VTE may act as a transient provoking factor, but this finding needs to be confirmed in future research.[103]

Advances in diagnostics

Accurate tools can detect and monitor even subclinical changes in the cardiovascular system to prevent future cardiovascular events (Fig. 3). The detection of CAD and perfusion deficits may be supported with computed tomography (CT), single photon emission tomography (SPECT), CMR, and invasive angiography, though there are no specific recommendations for patients with SLE in the current guidelines.[104] The CAD diagnostic algorithm should be primarily based on non-invasive assessments if there are no severe symptoms refractory to medical therapy.[104] Multidetector CT (MDCT) is used for non-invasive assessment of calcium score, which reflects the extent, severity and distribution of atherosclerotic plaques in arteries.[105,106] The specificity of this method is high, so it can exclude clinically relevant stenosis, though the disease should be confirmed using coronary angiography.

CT-fractional flow reserve (FFR) can be used to coronary flow physiology, while single photon emission tomography (SPECT) is a sensitive method to evaluate myocardial perfusion defects. The extent, severity and reversibility of myocardial perfusion abnormalities can be identified at rest and under stress.[106–108] As myocardial perfusion defects are predictors of CAD in SLE patients,[109] SPECT may be used for risk stratifying purposes. Myocardial blood flow can also be measured using PET (positron emission tomography) to reflect coronary flow reserve and may be an early predictor of CAD in SLE patients.[110] Moreover, it can assess coronary microvascular dysfunction in this group.[111]

The presence of diffuse contrast enhancement in vessel walls visualized with CMR indicates inflammation and atherosclerosis in SLE patients.[112] However, such patterns of enhancement can also be found in asymptomatic patients.[113] Other pathological findings include late gadolinium enhancement (LGE) and signs of stress-perfusion deficit observed in over 40% of SLE patients without known CAD.[114] Moreover, CMR is commonly used to assess myocardial edema and ischemia and may be used to confirm myocarditis. Patients with evidence of LGE on CMR are more likely to develop CAD, atrial tachycardia, myocarditis, pericarditis, HF, and myopericarditis, suggesting that CMR should be considered as part of routine surveillance in patients with SLE for prognostic value and to guide management. Cardiac magnetic resonance can help answer the question of the substrate behind cardiac hypertrophy in SLE and what will guide the final decision for patients' risk stratification and further treatment (mainly to differentiate hypertrophic cardiomyopathy from CAD). Cardiac magnetic resonance can also identify the underlying pathophysiology of myocardial injury, such as inflammation, fibrosis, microvascular damage, or subepicardial ischemia,[115,116] which contribute to individualized treatment management. Cardiac magnetic resonance is widely used in the diagnostic pathway in patients with SLE in early and even preclinical stages of autoimmune diseases.[98,117] However, besides these benefits and general use, there are no specific recommendations for the usage of this method in SLE, probably due to poor availability and high cost.

Future perspectives include the use of artificial intelligence in accelerating the process of imaging, scar analysis with prediction of major arrhythmic events, and obtaining global circumferential strain, which may have prognostic value in patients with normal CMR.[118,119] However, it should be highlighted that, before considering introducing these solutions in patients with SLE, the algorithm ought to be optimized for this group, which is often underrepresented in the trials.

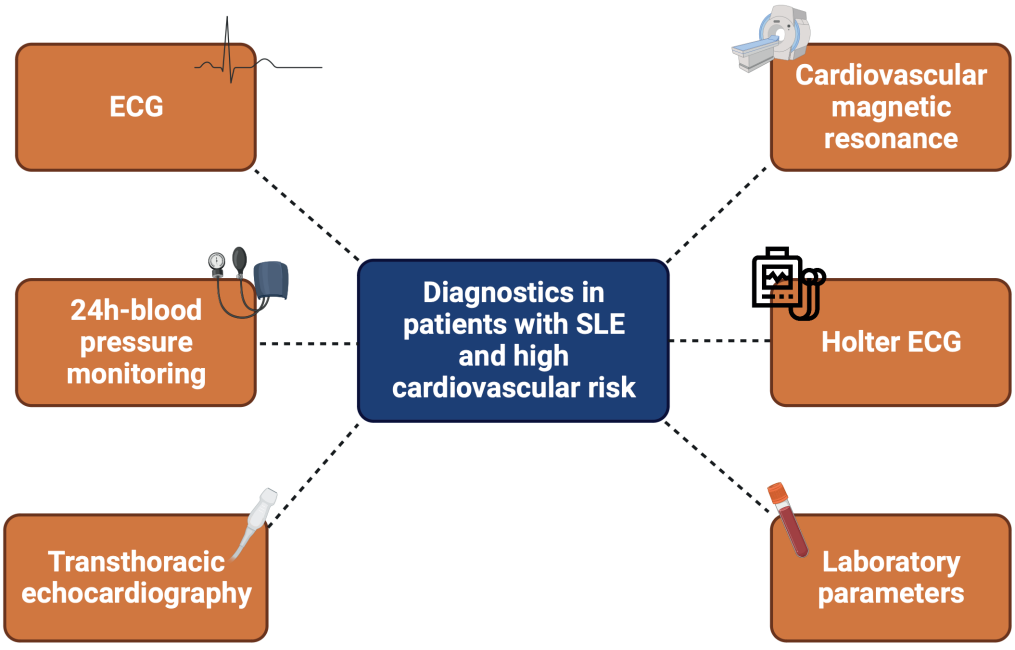

Fig. 3. Suggested diagnostic methods for patients with SLE and high cardiovascular risk

ECG – electrocardiography; SLE – systemic lupus erythematosus. Created with Biorender.com.

The role of echocardiography cannot be ignored since, as in other populations, it can assess cardiac systolic and diastolic functions, valve abnormalities and contractility disorders and estimate the pulmonary hypertension probability. There are no specific guidelines for using this method in SLE patients, though it is commonly used due to its wide availability, low cost and minimal complication risk. Libman–Sacks endocarditis is routinely detected and monitored using transthoracic echocardiography and transesophageal echocardiography, though studies reporting successful use of magnetic resonance imaging (MRI), MDCT, PET-CT, and SPECT in endocarditis diagnostics have also been published.[120,121]

Therapeutic options and future directions

EULAR recommendations were formulated for the optimal management of cardiovascular risk in SLE patients (Fig. 2). They include hyperlipidemia treatment in line with the general population and BP control with a target BP of less than 130/80 mm Hg, especially in concomitant lupus nephritis. In this case, angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blocker (ARB) therapy should be used. In SLE patients with no history of thrombosis or pregnancy complications, a prophylactic dose of aspirin (75–100 mg daily) should be recommended for those with high aPL levels and may be considered in those with low aPL.[42]

High lupus activity is associated with increased cardiovascular risk, so lupus activity should be carefully controlled.[42,122] In lupus pharmacotherapy, glucocorticoids, immunosuppressants and anti-malarials are used, though most studies suggest that a high mean daily and cumulative dose of glucocorticoid increases cardiovascular risk.[122,123] Therefore, EULAR recommends the lowest possible glucocorticoid dose.[42] In turn, hydroxychloroquine is recommended for all SLE patients without contraindications. Hydroxychloroquine use is associated with lower lupus activity, risk of atherothrombotic events and CAD.[42] Moreover, the latest meta-analysis showed that anti-malarial agents, of which the most popular was hydroxychloroquine, were associated with reduced risk of diabetes mellitus type 2 and reduced diastolic BP.[124]

There are no specific immunosuppressant recommendations, though there was no association between cardiovascular risk and the use of methotrexate, mycophenolate, cyclosporine, or rituximab, while studies testing cyclophosphamide and azathioprine gave incompatible results[42] (Fig. 4). Immunosuppressants and glucocorticoids are non-specific therapeutics with various systemic adverse effects.

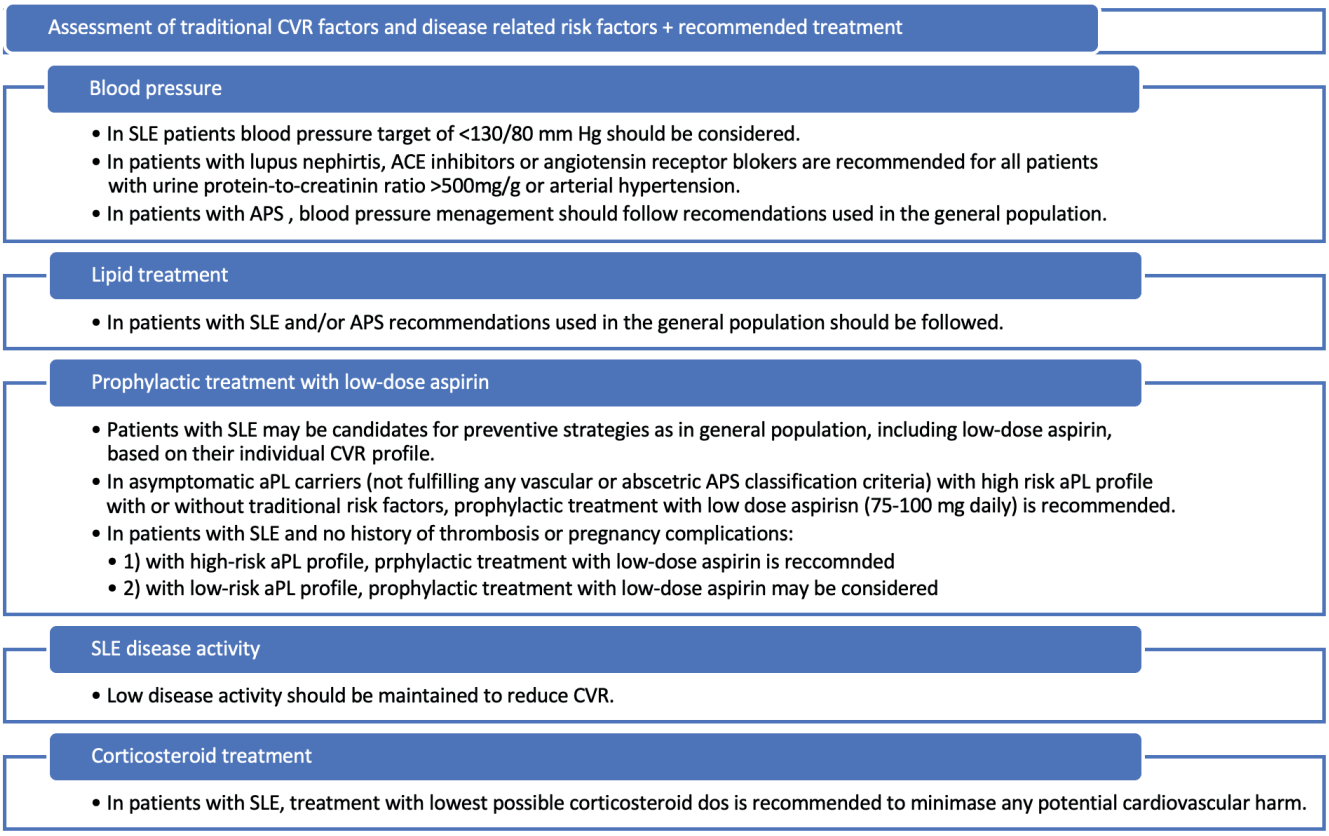

Fig. 4. European Alliance of Associations for Rheumatology Recommendations for CVR management in SLE and APS

CVR – cardiovascular risk; SLE – systemic lupus erythematosus; APS – antiphospholipid syndrome; ACE – angiotensin-converting enzyme; aPL – antiphospholipid antibody. Based on Drosos et al.[42]

Currently, we face the challenge of developing biological molecular target-specific drugs.[125] Belimumab, an antibody against B cell-activating factor (BAFF) used since 2011, was the first biologic approved for SLE patients. Studies found that belimumab modifies the course of the disease and enables the gradual lowering of glucocorticoid doses.[126] Anifrolumab, an anti-type I IFN receptor antibody, was approved in the USA in 2021.[127] Ustekinumab targets IL-12/23 (p40) and is currently in clinical trials. In a randomized study, 37 (62%) of 60 patients in the ustekinumab group and 14 (33%) of 42 patients in the placebo group achieved a SLEDAI-2K responder index-4 (SRI-4) response (percentage difference 28%, 95% CI: 10–47, p = 0.006), which meant more pronounced improvements in clinical parameters, including global SLE disease activity responder index, joint counts, mucocutaneous disease, disease flares, and laboratory parameters (C3 complement concentrations and anti-double stranded deoxyribonucleic acid (dsDNA) autoantibodies) at week 24.[128] Another drug, baricitinib, a Janus kinase 1/2 (JAK 1/2) inhibitor, mediates signal transduction for IL-6, IL-12, IL-23, and IFN.[129] Biological treatment seems to be a promising strategy in SLE treatment and may lead to the reduction of cardiovascular risk.

So far, we know that blood biomarkers such as IgG against high-density lipoprotein (HDL) and paraoxonase 1 (PON1) are increased in SLE patients (Table 1). Anti-PON1 is an indicator of decreased PON1 activity in rheumatoid arthritis and is associated with carotid intima-media thickness in SLE, while anti-HDL poses a higher risk of CVD and lower HDL serum levels at disease onset.[130,131] A study from 2020 found an elevated monocyte-to-HDL cholesterol ratio and a low-density granulocytes-to-HDL cholesterol ratio in SLE patients with traditional risk factors or subclinical atheromatosis but not in those who were CVD-free.[132] The level of dysfunctional HDLs in atherosclerosis in SLE was found to be affected by lupus therapy, which may be promising for monitoring lupus treatment.[131,133,134]

Asymmetric dimethyl arginine (ADMA) is an acute-phase plasma protein, an endogenous nitric oxide inhibitor that rises through the activation of inducible endothelial nitric oxide synthase (eNOS), and is a reliable marker of endothelial dysfunction in APS patients. High serum ADMA levels may represent a risk factor for disease activity and poor prognosis among SLE patients.[135,136] Other potentially useful biomarkers are presented in Table 1.[121,131,137–139]

Biomarkers may help to detect SLE patients at high cardiovascular risk and need further investigation to develop personalized treatment.[140] Assessing biomarker levels and genomic profiles may be the standard future strategy for early detection of CVD or estimating cardiovascular risk and monitoring therapies.

Limitations

Due to the complex characteristics of SLE, little is known about the pathomechanisms affecting the development of cardiovascular complications. As such, an explanation for their presence has not been described in full. Furthermore, the autoimmune nature of SLE means the disease course can vary greatly between patients. The studies referenced primarily refer to whole populations, though there are subgroups in which the frequency of complications may differ from those described in this work.

Table 1. Biomarkers in SLE patients related to increased CVD risk

Biomarker	Sample type	Level in patients with SLE	Clinical application
Ig-G against HDL and PON1	serum	elevated	Associated with accelerated atherosclerosis; can indicate early endothelial damage or premature atherosclerosis in SLE patients; therapeutic targets for preventing CVD in SLE patients.
LHR; MHR	serum	elevated	Indicative of CVD risk in SLE patients even at the onset of disease.
hs-cTnT	serum	elevated	Independently associated with cardiovascular events in SLE patients.
IgG-anticardiolipin antibodies; E-selectin	serum	elevated	Associated with CVD and disease activity.
Dysfunctional HDLs	serum	modified by pharmacotherapy	Useful in monitoring the treatment of SLE.
Adiponectin	serum	elevated	Useful for CVD risk stratification in accelerated carotid atherosclerosis in SLE young women.
s-LOX-1	serum	elevated	Associated with increased CVD risk in SLE patients; potential therapeutic target.
ADMA	serum	elevated	Independently associated with endothelial dysfunction in APS patients.

SLE – systemic lupus erythematosus; CVD – cardiovascular disease; PON1 – paraoxonase 1; HDL – high-density lipoprotein; MHR – monocyte-to-high-density lipoprotein cholesterol ratio; LHR – low-density granulocytes-to-high-density lipoprotein cholesterol ratio; hs-cTnT – high-senstivity cardiac troponin; ADMA – asymmetric dimethyl arginine.

Conclusions

Systemic lupus erythematosus is associated with an increased risk of cardiovascular disorders, so its management should involve early diagnosis and treatment of such complications. At present, there are no specific guidelines for cardiovascular risk prevention in SLE patients, and further research on the topic is warranted. In the treatment process of SLE patients, a personalized adaptation of EULAR and European Society of Cardiology (ESC) guidelines should be implemented to maintain low disease activity and use the lowest possible glucocorticoid dosage to avoid complications.

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From hallucinations to delusions: A narrative review of psychotic-like experiences and their implications

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Abstract

Psychotic-like experiences (PLEs) refer to sub-threshold hallucinations and delusions observed in both clinical samples and the general population. Psychotic-like experiences have far-reaching implications for an individual's coping strategies and daily functioning. They are associated with both psychotic and non-psychotic disorders. This article presents a comprehensive review of the current literature on PLEs, incorporating a detailed exploration of the definition, prevalence, risk factors, functional impairments, and comorbid psychiatric disorders. Medline/PubMed and Embase were searched to establish and identify the literature. A total of 108 studies met our inclusion criteria. The genetic and biochemical backgrounds of PLEs are discussed, focusing on gene polymorphisms, changes in brain gyrification and hypothalamic-pituitary–adrenal (HPA) axis dysfunction. Psychological factors, such as trauma exposure, emotion regulation difficulties, cognitive biases, and attachment issues, were thoroughly examined, especially in terms of their impact on the emergence of PLEs. Here, we show how important the clinical aspects of developmental PLEs are, underlining the significance of an increased risk of self-harm and suicidal behaviors in those individuals and the comorbidity of psychiatric disorders in enabling clinicians to discern specific areas to observe. Although there is limited evidence on effective protocols for PLE management, various treatment approaches are explained. Despite increased research on PLEs in recent years, further investigation is needed to fully understand the nature of PLEs and to optimize therapeutic strategies. This article consolidates the current knowledge by synthesizing information on PLEs, including risk factors, comorbidities, treatments, and their impact on individual's lives.

Key words: psychosis, delusions, hallucinations, FK506 binding protein 5, hypothalamic–pituitary–adrenal axis

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Introduction

Psychotic-like experiences (PLEs) are sub-threshold hallucinations and delusions that cannot be classified according to international diagnostic systems due to their low severity, limited duration or absence of associated functional impairment.[1,2] Positive PLEs include phenomena such as fleeting visual or auditory hallucinations, while negative PLEs include, e.g., blunted affect and physical and social anhedonia.[3] In the last decades, the view of PLEs changed from being previously dichotomously diagnosed psychotic symptoms in clinical samples to a continuum of psychotic experiences, applying also to non-help-seeking individuals from the general population.[3] The prevalence of PLEs is significantly higher among young people and more frequent among women, and those who are non-married, unemployed, have a high educational level, and low socioeconomic status.[4–7] Indirect effects from traumatic life events (TLEs), i.e., physical, sexual and emotional abuse, as well as neglect experiences and exposure to PLEs through perceived stress, dissociation, external locus of control, negative self-schemas, and negative other-schemas were found. They include phenomena such as bizarre experiences, perceptual abnormalities, persecutory ideas, and magical thinking.[8]

Psychotic-like experiences have wide implications for an individual, including maladaptive coping strategies and reduced functioning. Many studies have shown a significant association with poorer social and global functioning,[9,10] as well as impaired social and role functioning.[11] Individuals with PLEs have a lower health-related quality of life.[12] In a large study, PLEs correlated with both interpersonal violence and violence towards objects.[13,14] Childhood PLEs are linked to lower self-esteem and optimism, avoidant coping and school misconduct. They are also associated with poorer school performance, as well as language and mathematical abilities.[15] The presence of PLEs is strongly connected with poorer treatment outcomes, both with pharmacotherapy[16] and psychotherapy.[17]

Furthermore, PLEs increase the risk of conversion to a psychotic disorder, and this conversion depends on the severity or persistence of PLEs.[18] Psychotic-like experiences are associated not only with psychosis, but also with non-psychotic disorders such as depressive episodes, post-traumatic stress disorders or personality dysfunctions.[19] In a 2019 meta-analysis, childhood and adolescent PLEs were associated with over a 3-fold increased risk of affective and substance use disorders.[20] The presence of PLEs in childhood predicts future mental health service use and psychotropic pharmacological treatment.[21] Children with PLEs had higher average total healthcare costs during adolescence.[12] In a large study, PLEs were associated with an increased risk of mortality, especially due to suicide and neoplasms. For people experiencing PLEs, the predicted median lifespan was over 5 years shorter.[22]

Over the years, various terms have been used when referring to non-clinical psychotic symptoms. Firstly, the term attenuated psychotic symptoms (APS) was introduced as a part of the ultra-high risk (UHR) criteria for developing psychosis in the future.[23] The UHR criteria, including APS and brief, limited intermittent psychotic symptoms (BLIPS), are assessed through clinical interviews. Unfortunately, various modifications of UHR criteria were introduced.[24] The term "psychotic-like experiences" was first used by Strauss,[25] but the concept has changed throughout the years. In general, there is no consensus regarding the definition and conceptualization of PLEs, which may create inconsistencies in comparing and interpreting the results of different studies.[26]

Objectives

Recently, the number of publications regarding PLEs has significantly increased. This study aimed to undertake a comprehensive review with particular attention to recent advancements, notably in dynamic fields like genetics, imaging and biochemical studies. Psychological and clinical aspects, as well as the efficacy of the possible treatments are also discussed.

Genetics

There is a growing number of studies showing the association between genetic polymorphisms and prevalence of PLEs. Researchers have examined the effects of possible factors, that may increase the risk of experiencing PLEs, such as: the catechol-O-methyltransferase (COMT) rs4680, the dopamine D2 receptor (DRD2) rs6277 and the dopamine transporter (DAT1) rs28363170 variable number tandem repeat (VNTR) genes.[27] Higher severity of PLEs and a history of TLEs correlated with low levels of ASEs in *DAT1* 10R/10R homozygotes. The study implied a probability of increased PLE severity with a history of TLEs only in 10R/10R homozygotes of the *DAT1* 9R/10R *VNTR* polymorphism. The correlation between a history of TLEs and greater severity of PLEs was found among the *COMT* rs4680 Met allele carriers with high levels of cognitive biases.[28] In a study concerning the connection between *COMT* gene rs4680 polymorphism, executive dysfunction and PLEs, a recessive genetic model was found to moderate this relationship in both the overall sample and among women, but not in men.[29] The *DRD2* rs6277 C allele was associated with an increased occurrence of PLEs.[28] The relationship between zinc finger protein 804A (*ZNF804A*) polymorphisms (rs1344706 and rs7597593) and the positive dimensions of schizotypy and PLEs was investigated.[30] Results indicated a significant correlation between rs7597593 and both schizotypy and PLEs, specifically in women. Carriers of the C allele exhibited higher scores in the positive dimension of both

variables compared to TT homozygotes. Research also shows an association between the regulator of G protein signaling 4 (*RGS4*) polymorphisms and PLEs. Individuals carrying the T allele of rs951436 and/or the A allele of rs2661319 demonstrated higher scores on positive and negative PLEs.[31]

There have been numerous studies to investigate the influence of FK506 binding protein 5 (*FKBP5*) gene polymorphisms on PLEs. In a recent cross-sectional study,[32] a *FKBP5* gene was found to moderate the correlation between perceived stress and attachment style in those with PLEs. Compared to C allele carriers, there was a higher severity of PLEs in TT homozygotes with the single nucleotide polymorphism rs4713902 and a worse self-efficacy and anxious attachment style. Moreover, rs3800373 GG homozygotes demonstrated a greater incidence of PLEs, compared to T allele carriers, which corresponded with the former findings on the association between an enhanced possibility of developing schizophrenia in G allele carriers.[33] One study[34] found that individuals with the rs737054 T allele who experienced emotional neglect exhibited a more severe level of PLEs. Additionally, the rs1360780 CC (rather than the T allele carriers observed in previous studies)[35,36] and rs9296158 GG homozygotes with a history of physical abuse reported a higher severity of PLEs, which is consistent with prior findings.[37,38] The interaction between the *FKBP5* haplotype and bullying was also examined and found to be associated with positive PLEs.[37]

A mega-genome-wide association study[39] investigated the genetic overlap between PLEs in adolescents and schizophrenia, bipolar disorder and major depression in adulthood. A polygenic risk score (PRS) analysis showed that the schizophrenia PRS predicted all adolescent PLEs trait domains, while the major depression PRS predicted only 2 of them in adolescence – anhedonia and parent-rated negative symptoms.

Numerous studies have linked various genetic polymorphisms, such as those in *COMT*, *DRD2*, *DAT1*, *ZNF804A*, *RGS4*, and *FKBP5* genes, to the severity and occurrence of PLEs. These associations are often moderated by factors like TLEs and ASEs. Specifically, certain genotypes, such as *DAT1* 10R/10R homozygotes, *COMT* rs4680 Met allele carriers and *FKBP5* TT homozygotes, are associated with increased severity of PLEs, especially in the presence of TLEs. Additionally, there is evidence of genetic overlap between PLEs in adolescents and schizophrenia, bipolar disorder and major depression in adulthood, as indicated in PRS analyses.

Biology/biochemistry

There are only a few studies in the literature regarding the biochemical aspects of PLEs. These studies concentrated mainly on the hypothalamic–pituitary–adrenal (HPA) axis, as its dysregulation is thought to be associated with psychosis.[40]

A study conducted by Collip et al. investigated HPA axis functioning in people at above-average genetic risk for psychotic disorders.[41] Diurnal cortisol profiles and the association between HPA axis activity and subclinical psychotic experiences were examined in the siblings of patients with psychotic disorders and a healthy control group.

The results showed that momentary PLEs were associated with increased cortisol levels, which can be interpreted in 2 directions. One interpretation involves the impact of distress connected with psychotic experiences on increased cortisol secretion. However, after the multilevel analyses, it seemed that subclinical PLEs may have a greater contribution than negative effects on increasing cortisol levels. Another interpretation is that the increased cortisol levels are either involved in the pathogenesis of psychotic experiences or reflect secondary processes. These results could be interpreted as evidence for an association between heightened cortisol secretion, the dopaminergic system and subclinical psychotic experiences, but whether it is causal could not be established in this study.

A study by Thompson et al. examined connections between the experience of stressful events, HPA axis activity, and hippocampal and pituitary volumes in young people who met the UHR criteria of developing a psychotic disorder.[42] The criteria included the following groups: (a) experienced subthreshold positive psychotic symptoms during the past year, (b) experienced episodes of frank psychotic symptoms that have not lasted longer than a week and have been self-remitting, or (c) have a first degree relative with a psychotic disorder or the identified patient has a schizotypal personality disorder and they have experienced a significant decrease in functioning during the previous year.

The UHR participants who eventually developed psychosis had significantly lower cortisol levels at baseline than those who did not develop a psychotic episode. This confirms the fact that HPA axis dysfunction is involved in the development of psychosis. A significant correlation existed between experiencing minor stressful events and raised cortisol levels. Higher plasma cortisol levels were associated with higher levels of depression and anxiety but not with psychopathology, psychotic symptoms or general functioning. Hippocampal and pituitary volumes were not associated with either plasma cortisol levels or the number of glucocorticoid receptors.

In another study, the aspect of cortical gyrification in adolescents was investigated using magnetic resonance imaging (MRI). The results showed static gyrification changes in individuals with PLEs experiencing voice-hearing, unusual experiences of receiving messages, or persecutory ideas. The cortical gyrification was lower in the frontotemporal regions of the left hemisphere. This group also showed dynamic gyrification changes with higher gyrifications in the right parietal cortex during late adolescence.[43] A similar study was carried out by Evermann et al. in an adult sample. Psychotic-like experience distress was associated with lower gyrification of the left

precuneus. The PLE's depression dimension was correlated with lower gyrification in the right supramarginal and temporal region.[44] Another study, using deformation-based morphometry, demonstrated the role of the uncus in PLE pathogenesis. In this study, PLEs were associated with a reduced expansion of the uncus. The analysis revealed a developmental involvement of the right uncus in the cerebral basis of PLEs.[45]

A limited amount of literature exists on the biochemical aspect of PLEs with a primary focus on the HPA axis. Studies suggest associations between heightened cortisol secretion and PLEs, potentially implicating HPA axis dysfunction in the pathogenesis of psychosis. Additionally, neuroimaging studies have found structural alterations in brain regions such as the frontal-temporal cortex, precuneus and uncus among individuals experiencing PLEs, suggesting a potential neurobiological correlate to these experiences.

Psychological aspects, trauma

There have been numerous studies investigating the association between childhood trauma (CT) and PLEs. Prior research suggests that witnessing violence,[46] suffering from emotional[47,48] or sexual abuse, and having experienced 3 or more types of trauma[49] are predominantly correlated with PLEs. A recent study[50] comparing the prevalence of delusions and hallucinations in 30-year-olds who had experienced childhood maltreatment with those who had not, showed a higher likelihood of PLEs in self-reported abuse survivors.

As has been previously suggested, there is a strong connection between peer bullying and PLEs, both significantly increasing suicidality in adolescents.[51] Furthermore, research on the correlation between implicit emotion regulation, discrimination and psychopathological symptoms in children showed that greater levels of discrimination predicted a higher endorsement of PLEs.[52] The association between CT and PLEs during early adulthood was also investigated in terms of wisdom scores in college students. This study showed lower scores of wisdom in those reporting PLEs, wisdom in general was negatively correlated with CT and PLEs, while a decreased wisdom level mediated the association between CT and the occurrence of PLEs.[53]

A number of authors have recognized the relationship between family functioning (FF) and PLEs. For example, Zhan et al.[54] examined the association between PLEs, emotion-regulation (ER) strategies among adolescents and the possibility of a parental relationship impact. It indicated that lower use of reappraisal, greater use of suppression and parental conflict or divorce correlated with the number of PLEs endorsed. Moreover, these factors notably predicted a higher level of distress from PLEs. This has also been explored in terms of the COVID-19 pandemic and lockdown in a study by Wu et al.[55] that not only determined a positive association between increased stress and PLEs but also showed that better FF may alleviate the adverse influence of elevated perceived stresses on PLEs. Nevertheless, prior research[56] suggests there might be other aspects that have a mediating effect between FF and PLEs. A significant correlation was found among college students who reported, above all, trouble with sleep quality, interpersonal adaptation and loneliness. Studies concerning the relationship between PLEs and higher levels of loneliness are well documented.[57–59] However, a recent cross-lagged panel analysis indicated that, although greater feelings of loneliness and less social support predicted more PLEs over time, these variables may be implicated at earlier stages of psychosis risk.[60] Those findings are consistent with the social deafferentation hypothesis,[61] which suggests that social isolation can contribute to the development of hallucinations and delusions. Additionally, there has been an independent emotional processing pathway identified,[62] associating higher PLEs and loneliness with lower efficiency of recognizing emotion states, further mediated by higher levels of perceived rejection. On the other hand, despite existing research on the connection between the need for closure (NFC) and the occurrence of delusionality and hallucinations in patients with psychotic disorders, 1 study tested this issue on a larger sample from the general population.[63] Psychotic-like experiences, jumping to conclusions (JTC) task, and a full abridged NFC scale, consisting of "Preference for Predictability", "Discomfort with Ambiguity" and "Decisiveness", were used to assess the connection. It identified an insignificant correlation between NFC and PLEs, along with no association with JTC results, as well as a negative relationship between "Decisiveness" scores and the severity of PLEs.

Another study[64] found that experiencing distress from positive PLEs was linked to more pronounced challenges with emotion regulation, lower reappraisal self-efficacy and less habitual acceptance use. Similarly, distress from negative PLEs correlated with greater difficulties in emotion regulation and less habitual acceptance use. In a retrospective study considering affective lability (AL), the prevalence of PLE subtypes and PLE's contribution to the specific use of emotion regulation strategies in adulthood were studied.[65] The study has provided evidence that men with "hearing voices" PLEs and women with "special messages" and "bodily changes" PLEs correlated with higher levels of AL, and the use of cognitive reappraisal mediated the relationship between hearing voices during PLEs and AL. These findings suggested a significant negative correlation between the severity of paranoia symptoms and the possibility of utilizing adaptive emotion regulation strategies in men, as well as a significant positive correlation between the severity of paranoia symptoms and the frequency of using maladaptive emotion regulation strategies in women. Prior studies reported corresponding data,[66–68] indicating AL as a salient clinical characteristic for psychotic disorders. The findings of a prospective study revealed that affective dysregulation (AD) is linked

to an increased probability of developing and maintaining paranoid delusions and auditory hallucinations among adolescents and young adults.[69] Researchers also suggested a significant correlation between positive psychotic symptoms and higher levels of AL in patients diagnosed with schizophrenia.[70] Moreover, individuals with high-level PLEs were associated with greater use of emotion suppression and lesser use of acceptance,[64] which correlates with the former characteristics of psychotic disorders.[67,71] These findings implied that emotion regulation skills play a protective role in reducing the distress associated with PLEs. Furthermore, they underscore the importance of early psychotherapeutic interventions aimed at addressing emotion regulation difficulties in individuals experiencing PLEs. There is the potential efficacy of cognitive reappraisal in alleviating the impact that PLEs have on AL, which signals the necessity of further studies regarding this subject.

Psychotic symptoms and PLEs are strongly influenced by negative emotions and cognitive biases. A study investigated the moderating effects of psychosis-related cognitive biases, negative affective states and PLEs,[72] showing that emotional and cognitive processes promote the development of PLEs but do not have a cumulative effect. The study findings indicated that external attribution biases moderated the association between anxiety and positive PLEs, while attention to threat biases moderated the link between depression and positive PLEs, which is in line with former research.[73–75] Additionally, JTC biases were associated with positive PLEs and served as a moderator for the connection between anxiety and depression, as well as negative PLEs, which corresponds with a previous meta-analysis[76] and earlier findings.[74] In a cross-sectional study regarding metacognition and its role in the occurrence of PLEs,[77] results led to the assertion that metacognitive functioning, as an independent element, negatively predicts PLEs, and therefore may prevent the emergence of PLEs.

Researchers recognized possible prodromal mechanisms that may momentarily precede PLEs, such as aberrant salience (i.e., assigning excessive importance to irrelevant stimuli) and ASEs.[78] Some authors have also examined the presence of AD, including low resiliency, low reactive control and negative emotionality prior to the development of PLEs.[79] Affective dysregulation in adolescence predicted the occurrence of PLEs 3 years later. Additionally, PLEs arising during late adolescence were associated with a subsequent increase in AD in young adults.

A connection between PLEs, co-occurring distress, cognitive functioning, and early developmental delays or difficulties in adolescents reporting distressing PLEs has been investigated.[80] Compared to children without PLEs and those with non-distressing PLEs, children experiencing distressing PLEs had lower receptive language and delayed recall, and were more likely to have speech/motor developmental delays or difficulties. These findings emphasize that integrating cognitive strategies that target mechanisms underlying PLE distress may be beneficial.

In a cross-sectional study investigating the association between the 3-dimensional (cognitive, affective and reflective) wisdom levels and subclinical psychotic symptoms,[81] researchers found that high-level PLEs correlated with a lower wisdom level. Moreover, the occurrence and distress caused by PLEs were found to be negatively correlated with wisdom. Compared to affective and cognitive wisdom, reflective wisdom exhibited a negative correlation with the overall frequency and distress levels of PLEs. The findings to date suggest that individuals with PLEs may have an affective wisdom deficit, while affective wisdom is vital for establishing social connections with others. Therefore, further studies may hold the potential to prevent the progression from a high-risk state to full-blown psychosis. These results are consistent with previous research, showing an association between psychosis-proneness and lack of empathy[82] or considerable dysfunction in emotion recognition and interpersonal skills.[83]

Recently, it was examined whether daily-life executive function and attachment difficulties (avoidance and anxiety) can predict PLEs.[84] Positive PLEs were found to be predicted by greater trouble monitoring behavioral impact, less difficulty completing tasks, greater difficulty regulating emotional reactions, and greater difficulty controlling impulses. Negative PLEs were found to be predicted by greater difficulties in alternating attention, transitioning across situations and regulating emotional reactions. Higher attachment anxiety predicted both positive and negative PLEs, in contrast to individuals with schizophrenia, which was connected with attachment avoidance. This highlights the potential role of the distinction between these attachment difficulties in the maintenance of subthreshold symptoms and the risk of transitioning to a full-fledged psychotic disorder.

To gain a deeper understanding of risk factors related to suicidal ideations (SIs) and suicidal behaviors (SBs) in youths experiencing PLEs, a study[85] was conducted indicating that the occurrence of SI and SB increased as participants grew older for those with higher PLE distress. Furthermore, there was a significant association between PLEs at baseline and a progressive deterioration of both SIs and SBs, which was observed to entail a transition from SI to SB. Another study assessed the correlation between Prodromal Questionnaire-Brief Child Version (PQ-BC)[86] items and PLE distress regarding the prediction of lifetime SIs and SBs.[87] A significant association was found among items indexing thought control, auditory hallucinations, suspiciousness, and particularly nihilistic thinking/dissociative experiences, which exhibited the most substantial effect. Distress was found to be a partial mediator, as well as a moderator, between overall PLEs and PQ-BC items with SI and SB, underlining the significance of addressing it in suicide prevention endeavors.

Numerous studies have explored the association between childhood traumas, FF, emotion regulation, cognitive biases, and PLEs. Childhood trauma, peer bullying

and discrimination predict a higher endorsement of PLEs, while lower levels of wisdom and emotion regulation difficulties are associated with increased PLE distress. Neurodevelopmental delays and difficulties, as well as attachment anxiety, may contribute to the occurrence and distress of PLEs. Additionally, distress from PLEs is linked to SI and behaviors, with distress acting as both a mediator and moderator in this relationship. These findings underscore the complex interplay of various psychosocial and cognitive factors in the development and maintenance of PLEs, highlighting the importance of early intervention and holistic approaches to address these experiences.

Clinical aspects

Most people with PLEs never develop a psychotic disorder, and approx. 75–90% of developmental PLEs are transitory and benign, but these experiences may become clinically relevant, depending on the level of environmental risk.[88–90] According to various studies, 1–7% of people with PLEs develop a psychotic disorder.[91–93] The frequency of transitions depends on PLE trajectories – the group characterized by stable low levels of PLEs had lower percentages of transitions compared to the group with progressively increasing PLEs (1.28% compared to 3.39%).[93] Predictors of conversion to psychosis are regular cannabis use in adolescence and a family history of mental illnesses.[94]

What is more, PLEs are associated not only with an increased risk for psychosis but also with other, non-psychotic psychiatric disorders. In a study conducted by Bourgin et al., psychotic-like experiences were significantly correlated with 25 psychiatric disorders, such as mood disorders, anxiety disorders, post-traumatic stress disorder (PTSD), attention deficit hyperactivity disorder (ADHD), personality disorders, as well as substance use disorders and pathological gambling. The prevalence of psychiatric disorders was gradually associated with higher prevalence rates of PLEs.[4] In a study by Knight et al., patients with depression or anxiety in the presence of PLEs had more severe symptoms of these disorders at the outset than patients without PLEs. The group with PLEs had significantly lower recovery rates and required many more sessions to reach the threshold for recovery.[95] Psychotic-like experiences are associated with distress and depression, with a strength of this association depending on PLE manifestation.[96]

The meta-analysis from 2019 confirms that PLEs are correlated with a significantly increased possibility of SI, suicide attempts and suicide death.[97] In another study, the odds of suicide attempts were significantly higher in patients with an anxiety disorder, major depressive disorder or behavioral disorder who were experiencing PLEs compared with patients who did not report PLEs.[98] Systematic reviews and meta-analyses have shown that individuals with PLEs were at a 2–3-fold increased risk of self-harm and SBs.[97,99]

Children experiencing PLEs are more likely to experience mental health problems in young adulthood than children without PLEs. Psychotic-like experiences at age 12 increased the risk of low life satisfaction, loneliness, social isolation, sleep problems, overweight, tobacco dependence, parenthood, and low educational attainment. However, many of the associations between childhood PLEs and poor outcomes were explained by familial risk factors.[100]

For the time being, most studies did not differentiate between subtypes of PLEs, although they vary significantly according to distress, associated psychopathology, help-seeking and implications for mental health. Future research considering the heterogeneous character of different PLEs may help assess which PLEs are clinically relevant, improving risk screening and therapeutic strategies.[101]

Psychotic-like experiences are associated with an increased risk of various psychiatric disorders, including psychosis, mood disorders, anxiety disorders, PTSD, ADHD, and substance use disorders. Psychotic-like experiences also correlate with increased distress, depression and suicidality, with children experiencing PLEs more likely to face mental health challenges in adulthood. Differentiating between the subtypes of PLEs may aid in understanding their clinical relevance and improve risk screening and therapeutic interventions.

Psychotherapy and pharmacotherapy

High-risk criteria for psychotic disorders are based on the presence of PLEs.[102] Considerable research efforts have been invested in developing methods of delaying or preventing psychotic illness onset. Intervening earlier may offset the accumulation of damaging personal, social and economic effects.[103] Intervention during the prodromal stage and the first 3 years, defined as the critical period of illness, has the potential to reduce the ultimate severity.[104]

In a meta-analysis from 2013, cognitive behavioral therapy (CBT) had a moderate effect on reducing the transition to psychosis at 12 months. Low-quality evidence for complex psychosocial interventions also suggested that these interventions are associated with a reduced transition to psychosis. Very low-quality evidence suggests a beneficial effect of the supplementation of omega-3 fatty acids during long-term follow-up,[105] which was also confirmed in a meta-analysis from 2019.[106] The therapeutic effect of omega-3 intake may result from altered membrane fluidity and receptor responses following their incorporation into cell membranes. Omega-3 may also interact with the dopaminergic and serotonergic systems through the modulation of arachidonic acid release. Furthermore, there may be an increase in glutathione levels in the temporal lobes, which protects neurons from excitotoxicity and oxidative stress.[107] Although no other treatments have shown any clear effects, most of the studies

included in this review had several problems, mainly an unclear risk of selection bias, a high risk of detection bias, and a high risk of analytics bias owing to incomplete outcome data.

Two meta-analyses from 2018 indicate that, to date, there is no evidence that any specific intervention is particularly effective over the others in preventing the transition to psychosis. The treatments tested were needs-based interventions (NBI) and their combination with omega-3, ziprasidone, olanzapine, aripiprazole, family therapy, D-serine, CBT, and the French & Morrison protocol (CBT-F). Studies investigating integrated psychological interventions, CBT-F + risperidone + NBI and CBT-van der Gaag protocol (CBT-V) + CBT-F + NBI were also included.[108,109]

In a meta-analysis from 2019, no clear difference between the groups was found in the comparison of antipsychotic drugs (amisulpride, risperidone and olanzapine) connected with specific care packages (non-drug interventions like supportive, empathic listening and counseling) and specific care packages alone. Although a lower number of participants in the intervention group treated with olanzapine and supportive interventions transitioned to psychosis during follow-up compared to the control group (~25% compared to ~40%), these results were imprecise and did not meet levels of statistical significance. Data for the amisulpride-NFI comparison were so few and low-quality that no conclusion could be stated. There was no evidence that adding risperidone to CBT makes a difference in any outcome, including transition to psychosis. All of the studies were rated as a very low quality of evidence by the authors of the meta-analysis using the GRADE approach. The risk of bias was rated as very serious (regarding the randomization method not being described, allocation concealment method not described and high attrition rates). Furthermore, the imprecision in studies was reported. No evidence was found to support the effectiveness of NBIs, CBT, integrated psychological interventions, or family-focused therapy (FFT) in comparison with each other.[106]

The most recent meta-analysis from 2020 investigated psychological interventions and analyzed the proportion of remissions from PLEs as a primary outcome and changes in psychotic symptoms, depression, anxiety, functioning, distress, and quality of life as a secondary outcome. The findings were primarily null, except that CBT may reduce the distress associated with PLEs, but it was not effective for any other secondary outcome. The limitations included a lack of studies on this issue, a small number of participants and variable study quality. Only 2 studies provided randomized controlled trial evidence that CBT was effective in remission of PLEs. Generally, no strong evidence was found for the supremacy of any intervention. The authors suggest that, despite its limited effectiveness in preventing transitions to psychosis, CBT may be used to reduce the distress connected with PLEs. The lack of consequential evidence for clinical and functional improvements indicates a necessity for further research into psychological interventions for PLEs.[102]

Research into delaying or preventing psychotic illness onset emphasizes early intervention during the prodromal stage and the critical period of the first 3 years, potentially reducing ultimate severity. Cognitive behavioral therapy shows promise in reducing the transition to psychosis and alleviating distress associated with PLEs. Omega-3 fatty acid supplementation may have beneficial effects. However, evidence for other interventions is inconclusive, with no clear superiority of any specific treatment. Further research is needed to evaluate the efficacy of interventions for PLEs and their impact on clinical and functional outcomes.

Discussion

Psychotic-like experiences present a significant challenge for both researchers and clinicians due to their varying severity and duration and the absence of associated functional impairments. Numerous studies have connected genetic variations, such as those in *COMT*, *DRD2*, *DAT1*, *ZNF804A*, *RGS4*, and *FKBP5* genes, to the severity and occurrence of PLEs. Certain genotypes, like *DAT1* 10R/10R homozygotes and *COMT* rs4680 Met allele carriers, are linked to an increased PLE severity. Additionally, there is evidence of genetic overlap between PLEs in adolescence and later psychiatric disorders like schizophrenia and depression, as seen through PRS analyses. Research on the biochemical aspect of PLEs, mainly focusing on the HPA axis, suggests an association between heightened cortisol secretion and PLEs. Neuroimaging studies have identified structural brain alterations in individuals with PLEs, particularly in regions like the frontal-temporal cortex, precuneus and uncus.

The association between childhood trauma, FF, emotion regulation, cognitive biases, and PLEs is well documented. Childhood trauma, peer bullying and discrimination predict higher PLE endorsement, while lower wisdom and emotion regulation difficulties contribute to increased PLE distress. Additionally, PLE distress is linked to SIs and SBs, emphasizing the need for early intervention and holistic approaches. Psychotic-like experiences elevate the risk of various psychiatric disorders, including psychosis, mood disorders, anxiety disorders, PTSD, ADHD, and substance use disorders. Further research is crucial to evaluate the efficacy of psychological interventions for PLEs, although CBT and omega-3 fatty acids have demonstrated some efficacy.

This article builds upon previous research by synthesizing findings on the complex interplay of genetic, neurobiological, psychosocial, and cognitive factors in the development and implications of PLEs. It extends our understanding of how various genetic polymorphisms, trauma, family dynamics, emotion regulation, and

cognitive biases contribute to the severity and occurrence of PLEs. Highlighting the association between PLEs and a range of psychiatric disorders, distress and suicidality, it underscores the importance of early intervention and holistic approaches to address these experiences.

The article holds significant importance for both research and clinical practices. It emphasizes the need for a comprehensive understanding of PLEs. The research presented in this paper offers valuable insights into the nature and impact of PLEs on an individual's life. The findings indicate that PLEs have significant functional and psychological implications, including their association with distress, reduced quality of life, and an increased risk of various psychiatric conditions. To address these limitations and advance our understanding of PLEs, several key actions should be considered. Efforts should be made to establish a consensus on the definition and diagnostic criteria of PLEs. A standardized framework will enhance the comparability of research findings and improve the accuracy of PLE diagnoses in clinical settings. Further research is needed to explore the underlying biological mechanisms of PLEs. This may include in-depth investigations into genetic polymorphisms, biochemical pathways and neural correlates associated with PLEs. Continued interdisciplinary collaboration between geneticists, neuroscientists, psychologists, and clinicians is crucial for improving our understanding of the complex etiology and development of PLEs. Leveraging advancements in neuroimaging techniques, genetic sequencing technologies and biomarker identification tools can facilitate the elucidation of biological correlates and pathways associated with PLEs. High-quality, randomized clinical trials are pivotal in assessing the efficacy of diverse treatment approaches for managing PLEs. The inclusion of diverse populations ensures that findings are broadly applicable. Understanding the trajectory of PLEs and their predictive value in the development of psychiatric conditions is crucial. This knowledge informs early intervention strategies and enhances outcomes for individuals at risk. Implementing early intervention strategies that target individuals at risk for PLEs, such as those with a history of trauma or familial predisposition, can help alleviate the progression of symptoms and reduce the likelihood of developing psychiatric disorders. This article underlines the importance of adopting a holistic approach to intervention and prevention efforts.

Research on PLEs has expanded considerably in recent years, fueled by the growing recognition of their prevalence and clinical significance. Studies have shown various factors contributing to the occurrence and persistence of PLEs. They are relatively common in the general population; therefore, understanding them is crucial for several reasons. While most individuals who experience PLEs do not go on to develop a psychotic disorder, these experiences can still cause significant distress and impairment in daily functioning. Psychotic-like experiences can have a significant impact on the individual's quality of life, affecting various domains, including social relationships, academic or occupational functioning, and overall well-being. Research aimed at understanding and addressing PLEs can lead to an improved quality of life. Moreover, PLEs are considered to be an important risk factor for the development of psychotic disorders. Research on PLEs can help in the early detection of individuals at risk for developing psychosis. Early intervention strategies, informed by research findings, can potentially prevent or mitigate the progression of symptoms and improve long-term outcomes. Psychotic-like experiences also represent important risk factors for non-psychotic disorders, including a number of conditions such as depressive episodes, PTSDs, personality dysfunctions, and substance use disorders. Research on the co-occurrence of PLEs with non-psychotic disorders can improve the development of comprehensive treatment approaches that address both psychotic and non-psychotic symptoms simultaneously. This holistic approach can improve treatment outcomes and the overall wellbeing of individuals experiencing these comorbidities. Psychotic-like experiences have also been linked to an increased risk of SBs. By investigating the relationship between PLEs and SIs, attempts and completed suicides, researchers can identify factors that contribute to this risk and develop targeted interventions to prevent suicide and promote mental health. Furthermore, understanding the biological, psychological and social factors involved in PLEs can help reduce the stigma surrounding this mental illness. Increased awareness and knowledge can foster empathy, support and acceptance for individuals experiencing PLEs and related conditions.

Limitations

This review also has several limitations that need to be considered. One of the major limitations in studying PLEs is the heterogeneity of experiences, ranging from mild perceptual abnormalities to more distressing hallucinations and delusions. First, the lack of a consensus regarding the definition and diagnostic criteria for PLEs further complicates matters and hinders the comparability of research findings. Second, many studies in this review focused on specific subpopulations, and while these studies provide valuable insights into potential risk factors, they may not fully represent the diversity of individuals with PLEs in the general population, limiting the generalizability of the findings. Third, although the review highlights some genetic and biochemical factors associated with PLEs, the field is still in the early stages of understanding these mechanisms and further research is needed. Fourth, the paper discusses the potential effectiveness of CBT in alleviating distress related to PLEs but notes the lack of robust evidence for other treatment methods. This limitation underscores the need for further research to identify effective interventions for individuals experiencing PLEs.

Conclusions

This review highlights the complex nature of PLEs and their significant impact on individuals. Psychotic-like experiences have profound functional implications, leading to maladaptive coping strategies, impaired social and global functioning, lower health-related quality of life, and higher violence risk. They are associated with psychosis and a variety of non-psychotic psychiatric disorders. The genetic and biochemical aspects of PLEs have garnered attention, with studies revealing associations between specific gene polymorphisms and dysregulated HPA axis activities, especially elevated cortisol levels. Imaging studies have shown lower cortical gyrification in frontal-temporal regions of the left hemisphere, left precuneus, right supramarginal and temporal regions, as well as the uncus. While CBT has shown promise in managing PLEs, there is a lack of strong evidence supporting the efficacy of other procedures.

Future studies are required to better understand the trajectory of PLEs and their predictive value in developing psychotic disorders and other psychiatric conditions. Efforts should be made to establish a consensus on the definition and diagnostic criteria of PLEs. Furthermore, high-quality, randomized clinical trials are necessary to evaluate the efficacy of different treatment approaches in managing PLEs and improving outcomes. In conclusion, while this study presents several challenges and limitations, it also offers promising avenues for research and clinical practice. The continued exploration of the biological, psychological and clinical aspects of PLEs is essential for providing effective interventions and improving the lives of individuals experiencing these phenomena.

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Withania somnifera and *Trigonella foenum-graecum* as ingredients of testosterone-boosting supplements: Possible clinical implications

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Abstract

This narrative review provides an overview of scientific studies on dietary supplements that may affect circulating testosterone (T) levels to explore which substances are scientifically proven to increase T concentration. We also review the scientific literature for their potential mechanisms and laboratory test changes triggered by their use. Based on the analysis of existing data on substances used to increase endogenous T levels, especially double-blind placebo-controlled randomized clinical trials, we selected 2 herbal extracts with the best documented positive effects on T levels, *Withania somnifera* root and root extracts/leaves and seed extracts of *Trigonella foenum-graecum*. Although these substances have different postulated mechanisms of action, both significantly increase T levels in men. *Withania somnifera* may inhibit the effects of cortisol and prolactin on the hypothalamic–pituitary–gonadal axis and directly affect the hypothalamus. *Trigonella foenum-graecum* seeds contain the active substance diosgenin, which is a precursor for sex hormone synthesis in gonads.

Key words: *Trigonella foenum-graecum*, *Withania somnifera*, testosterone, testosterone boosters

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Introduction

Dietary supplement use has increased in recent years and is currently a growing global trend. According to Bailey et al., dietary supplements used in the USA between 2003 and 2006 were used by 49% of the population (44% of men and 53% of women) and as many as 70% of adults over 70.[1] The most frequently used dietary supplements (33%) were multivitamin and multi-mineral preparations, with plant supplements used by approx. 20% of adults, mainly older people.[1]

A survey conducted by the dietary supplement industry trade association, i.e., the Council for Responsible Nutrition (CRN), in a group of 2,000 people per year in 2007–2011 found that the prevalence of supplement use ranged from 64% to 69% in 2007–2011. The percentage of people regularly using dietary supplements increased from 28% to 36%, with a statistically significant increase in 2010–2011. Furthermore, supplement consumption increased with age in 2011 and was higher in women than men, with multivitamins being the most commonly used supplements.[2]

According to data from The National Health and Nutrition Survey, in 2017–2018, 57.6% of people over 20 declared dietary supplement use in the last month, which was more common in women than in men, amounting to 63.8% and 50.8%, respectively, and increased with age, with the highest percentage of people using dietary supplements in women over 60. An increase in supplement consumption was also observed compared to data from 2007–2008, as age-adjusted consumption increased by 7.7% during this period.[3]

The European Prospective Cancer and Nutrition Study on dietary supplements conducted from 1995 to 2000 on 36,034 men and women aged 35–74 found that, as in the USA, dietary supplement use was highest in the oldest age groups in most European countries.[4] According to more recent data, 18.8% of 2,359 adult respondents from European countries used at least 1 plant-based dietary supplement. Characteristics of those using dietary supplements included older age, better education, non-smoking, and self-assessment of health as "good or very good."[5]

A recent survey of 13,200 adults across 14 European Union countries found that 88% of respondents had used supplements at some point in their lives, and 93% of this group had used them in the last 12 months.[6] An online survey conducted in Poland between November 26, 2019, and March 11, 2020, on 1,560 people aged 18–90 showed that the average use of dietary supplements within the past 30 days in people surveyed before the coronavirus disease 19 (COVID-19) pandemic was 80%, and 78% during the pandemic.[7]

Research conducted on 10,520 women and men in Iran by Mahdavi-Roshan et al. indicated that 25% of people used dietary supplements, and the factors associated with their use were female gender, older age, better education, and the presence of chronic diseases, such as cardiovascular diseases, hypertension and diabetes.[8] More recent data from a cross-sectional survey of 501 individuals aged over 45 residing in Saudi Arabia in 2021 showed that the prevalence of dietary supplement use was 50.7% and was lower in those under 60 (54.9%) compared to older people (59.9%).[9]

Statistical data on the consumption of dietary supplements indicate a growing interest in products that improve health and slow the aging process, which is promoted by media marketing.[10] Some plant-based anti-aging dietary supplements may protect against age-associated health problems, such as sarcopenia and metabolic syndrome, perhaps by affecting hormone concentrations.[11] One of the potential targets of anti-aging dietary supplements may be testosterone (T) due to its complex anabolic and anti-catabolic effects and its ability to improve metabolism, vital strength and sexual function. Testosterone is responsible for the development of primary and secondary sex characteristics in men, including sexual organs, changes in voice depth and facial and body hair growth.[12] Furthermore, it is a basic anabolic steroid that influences the production of proteins in skeletal muscles, increases muscle strength, power and endurance,[13] improves bone density, mass and strength, and is active in the male sexual response.[14]

Recent research emphasized the critical role that T plays in several metabolic functions in men, with T deficiency associated with metabolic disorders such as type 2 diabetes, impaired glucose tolerance, insulin resistance, obesity, increased triglycerides, total cholesterol, and decreased high-density lipoprotein (HDL) cholesterol, contributing to cardiovascular risk. Additionally, clinical studies indicate that T replacement therapy improves insulin resistance and glucose metabolism and reduces fat mass, cholesterol and triglycerides. The mechanisms by which T influences metabolism are not fully understood, but it is suggested that it is involved in controlling the expression of proteins involved in lipid and cholesterol metabolism regulation and the glycol axis and glycogen synthesis.[15,16]

Leydig cells of the interstitium of the testes produce approx. 95% of T, which is secreted into the bloodstream and transported bound to proteins (98%), mainly sex hormone binding globulin (SHBG) and albumin. Testosterone bound to SHBG is inactive, while T unbound and bound to albumin is active. Testosterone can exert its biological effects directly by interacting with its receptor or indirectly through being metabolized to dihydrotestosterone (DHT) by the cytoplasmic enzyme 5-reductase, which is highly expressed in the skin, male reproductive organs and brain.[16] Testosterone synthesis is regulated by the hypothalamic–anterior pituitary–gonadal axis, with gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus. Under its influence, luteinizing hormone (LH) is released into the circulation from the anterior pituitary gland to stimulate Leydig cells in the testes to synthesize T. Testosterone directly and indirectly inhibits GnRH and LH release in a negative feedback loop, with GnRH release

regulated by, among others, several hypothalamic neuropeptides, including kisspeptin.[17] Testosterone exerts its direct biological effects by binding to the androgen receptor (AR), though DHT binds to the same AR with approx. 5 times greater affinity.[18] The T-AR complex binds directly to specific DNA nucleotide sequences in the cell nucleus to regulate gene transcription and act as a transcription factor.[19] In adipose tissue, T is converted to estradiol (E2) with the participation of aromatase.[18,19] There are many dietary supplements available for sale that claim to increase T concentrations.

Objectives

There is a need to identify dietary supplements that have a proven effect on T concentrations. Our study aimed to compile a scientific review of literature published between 2013 and 2023 on supplements that impact T concentrations, identify substances with the best evidence of increasing T concentration, explore the potential mechanisms of their action and highlight changes in laboratory tests caused by their use. We also aimed to determine their effectiveness, whether they have additional properties, which patients can use them (why and under what conditions), their effects on diagnostics, and if they have legal consequences for athletes.

Methods

This review aimed to assess T-booster effectiveness, summarize the evidence on this topic, and explain how they work and in which patients (why and under what conditions). Therefore, we used realist synthesis as the most appropriate method. The study followed the RAMES (Realist and Meta-narrative Evidence Syntheses: Evolving Standards) guidelines, based on which the intervention was explained as supplementation with T-boosters to increase T concentrations in men, with the intervention considered under conditions of low T concentrations. The results focused on which T-boosters had the best-documented effectiveness, how much they increased T concentrations, and what mechanism and factors influenced their effectiveness.

Publications for this review were obtained by searching Google and Medline/PubMed. Search terms included testosterone boosters, testosterone boosting supplements, *Withania somnifera*, ashwagandha, *Trigonella foenum-graecum*, and fenugreek, covering 2013 to 2023. The last search date was September 15, 2023. Only substances with the best-documented ability to increase T levels were selected for systematic review and analysis of the magnitude of effect and contributing factors in randomized controlled trials and crossover studies. Systematic reviews and animal studies were reviewed to discuss potential T mechanisms of action.

Results

The final analysis included 4 systematic reviews, with Table 1 summarizing the key findings on the effects of various ingredients found in dietary supplements on T concentrations.[20–34] *Withania somnifera* (ashwagandha) and *Trigonella foenum-graecum* (fenugreek) extracts played the most significant role in this mechanism.

Two crossover studies and 4 randomized controlled trials of ashwagandha and 5 randomized controlled trials of fenugreek were included in the analysis of the extent of their effectiveness on T levels and contributing factors, with the results presented in Table 2.

Discussion

Which ingredients of dietary supplements have scientifically proven effectiveness in increasing testosterone concentration in men?

Clemesha et al. attempted to identify dietary supplement ingredients affecting T concentration. Based on scientific research,[31] the authors selected the 50 most popular

Table 1. Key findings on the effects of various ingredients present in dietary supplements on testosterone concentrations presented in selected scientific reviews

Scientific review			
Clemesha et al.,[31] (2020)	Balasubramanian et al.,[32] (2019)	Lazarev et al.,[33] (2021)	Smith et al.,[34] (2021)
Key findings			
A significant increase in T concentration was found for the following 12 substances: *Anacyclus pyrethrum*; *Bulbine natalensis*; *Eurycoma longifolia* (Tongkat ali); *Trigonella foenum-graecum* (fenugreek); Epimedium (horny goat weed); L-arginine; L-carnitine; magnesium; *Mucuna pruriens*; pantothenic acid; selenium; and shilajit	Five out of 10 substances had scientific evidence to increase T concentration: *Eurycoma longifolia* (Tongkat Ali), *Serenoa repens*, boron, *Withania somnifera* (ashwagandha root), and *Trigonella foenum-graecum* (fenugreek)	Components with the strongest evidence of their positive effect on T concentration: *Eurycoma longifolia* (Tongkat Ali), *Withania somnifera* (ashwagandha) and *Trigonella foenum-graecum* (fenugreek)	Two herbal extracts with the best documented positive effects on T concentrations in men: *Trigonella foenum-graecum* (fenugreek) seed extracts and *Withania somnifera* (ashwagandha)

Table 2. Key findings on the efficacy of *Withania somnifera* and *Trigonella foenum-graecum* on testosterone levels from controlled trials

Study, year	Design	Subjects	Intervention	Outcome
Mahdi et al.,[20] 2009	prospective	normozoospermic heavy smokers (n = 20), normozoospermics under psychological stress (n = 20) and normozoospermics with infertility of unknown etiology (n = 20); the control group comprised of 60 age-matched healthy men who had previously initiated at least 1 pregnancy and exhibited a normal semen profile	*Withania somnifera* root powder, orally, in a single dose (5 g/day) for 3 months.	The ability of *Withania somnifera* to treat stress-related infertility; T level improved in normozoospermics by 13%, normozoospermic cigarette smokers by 10%, and infertile normozoospermics under psychological stress by 22%.
Ahmad et al.,[21] 2010	prospective	75 normal healthy fertile men (control subjects) and 75 men undergoing infertility screening	Infertile men were prescribed *Withania somnifera* root powder (5 g/day) orally for 3 months.	Treatment recovered the levels of T in normozoospermic, oligozoospermic and asthenozoospermic men significantly ($p < 0.01$).
Chauhan et al.,[22] 2022	randomized, double blind, placebo-controlled	50 participants with lower sexual desire	300 mg of ashwagandha root extract or placebo capsules twice daily.	Compared to placebo, ashwagandha root extract supplementation was associated with a statistically significant increase in serum T levels
Ambiye et al.,[23] 2013	randomized, double blind, placebo-controlled	the placebo-treated group (n = 25) and the ashwagandha-treated group (n = 21)	Study participants in the ashwagandha-treated group were administered 1 capsule (containing 225 mg of a high-concentration full-spectrum root extract of the ashwagandha plant) orally, thrice daily for a period of 12 weeks.	Serum T increased significantly by 17% following treatment with ashwagandha root extract.
Lopresti et al.,[24] 2019	randomized, double blind, placebo-controlled	50 overweight men	A placebo or an ashwagandha extract (Shoden beads, delivering 21 mg of withanolide glycosides a day) for 8 weeks.	Ashwagandha intake was associated with an 14.7% greater increase in T ($p = 0.01$).
Lopresti et al.,[25] 2019	randomized, double blind, placebo-controlled	60 healthy adults	A placebo or 240 mg of a standardized ashwagandha extract (Shoden) once daily for 60 days.	T levels increased in men ($p = 0.038$) but not in women ($p = 0.989$) over time, although this change was not statistically significant compared to the placebo ($p = 0.158$)
Wankhede et al.,[26] 2018	prospective, double-blind, randomized, placebo-controlled	60 male healthy volunteers (30 in Fenu-FG group and 30 in placebo group)	Study participants were randomized to receive 1 of the 2 treatments, namely Fenu-FG (1 capsule, 300 mg, twice a day) or matching placebo in 1:1 ratio.	On 8 weeks of treatment, the levels of free T was found to have steep (98.7%) increase from baseline ($p < 0.001$) in Fenu-FG group, whereas placebo group showed moderate (48.8%) increase from ($p < 0.01$); the increase in free testosterone from baseline was found significantly between the groups ($p < 0.05$); subjects from Fenu-FG and placebo groups showed mild but non-significant increased levels of total T as compared with corresponding baseline values; the increase in total T from baseline was also not significant between the treatment groups (Fenu-FG vs placebo).
Mokashi et al.,[27] 2014	randomized, double blind, placebo-controlled	16 healthy and non-exercising men, 2 groups (controlled and placebo; of 8 each	Single dose of 600 mg (2 capsules of 300 mg) of glycosides based standardized fenugreek seed extract (IND9).	Significant increase in T levels (free-, total- and bioavailable-T) on acute administration of IND9 supplementation as compared with placebo group.
Rao and Grant,[28] 2020	randomized, double blind, placebo-controlled	100 healthy men with symptoms of benign prostate hyperplasia	Oral dose of either 600 mg *Trigonella foenum-graceum* per day or placebo for 12 weeks.	There were no differences in the total T or free T levels in either group after treatment as measured as change from baseline ($p = 0.36$ and 0.44, respectively)
Rao et al.,[29] 2016	single-site, randomized, double-blind, placebo-controlled	120 healthy men randomly allocated either the placebo comparator group or the active intervention group; 111 completing the study (56 vs 55, respectively)	The active treatment was standardized *Trigonella foenum-graecum* seed extract at a dose of 600 mg/day for 12 weeks.	Total T levels were similar between both groups at baseline. There was a small but significant difference in the change from baseline (Δ) values between the active treatment and placebo groups for T and calculated free T at week 12
Guo et al.[30], 2018	randomized, double-blind, placebo-controlled	40 healthy male athletes	Placebo or Furosap capsules (250 mg/day) for 12 weeks.	A significant change in serum total T level was observed in the Furosap-treated subjects compared to the group receiving placebo.

T-boosters from available databases and analyzed the effectiveness of 109 ingredients contained in them. No scientific studies were found on the effects of 67, while 11 had evidence that they decreased T concentrations, and 27 demonstrated an increase in T concentrations. For most of these ingredients, single clinical studies confirmed their effectiveness. For 4 ingredients, 4 publications indicated that they increase T concentration. Only 1 substance had a beneficial effect on T concentration in 5 studies, as did another in 6 studies. Regarding 6 (of 27) substances for which an increase in T concentration was documented, conflicting data indicated a T concentration decrease. Clemesha et al.[31] found that 12 substances – fenugreek, *Anacyclus pyrethrum*, *Bulbina natalensis*, *Eurycoma longifolia* (tongkat ali), epimedium (horny goat weed), *Mucuna pruriens*, shilajit, L-arginine, L-carnitine, magnesium, pantothenic acid, and selenium – had the best data supporting increased T concentrations. Unfortunately, the authors did not provide data on which of the ingredients had the most convincing evidence of their T-boosting potency. Data for 9 supplements indicated that their use led to an increase in T concentrations or did not cause changes in T concentrations, including ashwagandha root, panax ginseng, *Lepidium meyenii* (maca), vitamin D, caffeine, resveratrol, boron, calcium, and zinc.

Another attempt to identify substances with a scientifically proven effect on T concentrations was made by Balasubramanian et al.[32] The authors analyzed 10 most popular T-boosters sold online at amazon.com, with 5 showing that they could increase T levels, including tongkat ali, ashwagandha root, fenugreek, *Serenoa repens*, and boron.

The latest systematic review of T-boosters increasing T concentration by Lazarev and Bezuglov analyzed Medline/PubMed and the Cochrane Library for scientific studies on 15 ingredients identified in the 2 reviews mentioned above.[33] The authors found studies on 10 ingredients, including fenugreek (7), L-arginine, boron (3 studies each), tongkat ali, ashwagandha root, L-carnitine, selenium (2 studies each), magnesium, shilajit, and *Serenoa repens* (1 study each). According to the authors, tongkat ali, ashwagandha root, and fenugreek had the strongest evidence of a positive effect on T concentration, while only single studies showed a positive effect of magnesium and shilajit. Meanwhile, L-arginine, L-carnitine, *Serenoa repens*, selenium, and boron data were conflicting. There were very limited data on their safety profiles.

Methodological caveats of the reviews mentioned above included the use of only 1 search term, using only the Google search engine, and testing only selected ingredients of T-boosters. Moreover, the studies only aimed to identify substances with scientifically proven effectiveness in increasing T concentrations, regardless of the methodology used. No such limitations were found in the systematic review by Smith et al.[34] Their comprehensive review only considered controlled studies conducted on the effectiveness of single herbal ingredients on T concentrations in men, apart from its fractions or binding proteins (≥18 years). The study followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines and extracted English-language publications from PubMed, Scopus, the Cochrane Library, and the Cumulative Index to Nursing and Allied Health Literature (CINAHL), and only included randomized controlled trials (including a cross-over study) in adult men (≥18 years) (or a subset of adult men). Other inclusion criteria were the effect of monotherapy with a single herb, spice, plant, or extract on T concentrations in serum, plasma or saliva, compared to a placebo or control group. After applying these criteria, only 32 of 4,384 studies published between 2001 and 2019 were included in the analysis, with 9 showing statistically significant increases in T levels. Most studies were conducted in young populations, with 16 including men <40. Based on the analyzed data, they identified fenugreek seed extracts and ashwagandha root and root/leaf extracts as 2 herbal extracts with the best documented positive effect on T concentration.

What are the characteristics of T-booster ingredients with a positive effect on testosterone concentration?

Ashwagandha

Ashwagandha, also called winter cherry or Indian ginseng, was used in Ayurveda, the traditional Indian system of medicine. The plant belongs to the *Solanaceae* family and grows in subtropical India, Egypt, Morocco, Congo, South Africa, and Jordan. Several alkaloids and steroid lactones have been isolated from its roots, aerial parts and berries.[35] Withanine is the primary alkaloid, though pseudo-withanins, somniferins, somnins, withanins, tropines, pseudo-tropines, choline, anaferins, cuscohygrins, and isopelletierins should also be mentioned. Steroid lactones include ergostane-type steroid lactones, withaferin A, withasomniferin-A, withanolides A-Y, withasomniferols A-C, withanone, and withasomidienone.[36,37]

Plant phytochemical composition can vary by location. The roots are used most often for medicinal purposes, but whole plants, leaves, stems, green berries, fruits, seeds, and bark are also applied in medicine. Ashwagandha is available as an extract, loose powder and tincture. Due to the lack of withanolide standardization in some preparations, there may be significant inconsistency in their volume between preparations. The use of ashwagandha usually does not lead to serious interactions or side effects.[38]

Sengupta et al. proposed a mechanism for the effects of ashwagandha on T concentrations and male fertility using detailed analysis of human and animal studies. According to them, ashwagandha prevents the reduction of T levels induced by stress under the influence of cortisol (C) and prolactin (PRL), leading directly to increased GnRH and

LH concentrations. In addition to normalizing T concentrations, ashwagandha improves the antioxidant potential of seminal plasma by reducing oxidative stress.[39] A research paper also showed that ashwagandha reduced nandrolone decanoate-induced hepato-renal toxicity in Wistar rats.[40]

As mentioned earlier, GnRH stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and LH to act on the gonads to regulate T production and spermatogenesis. Therefore, when hormones such as PRL and C disrupt the hypothalamic–pituitary–thalamic axis, it reduces T production and spermatogenesis. Ashwagandha root extract is believed to normalize C concentrations by lowering the stress response. Observations by Sengupta et al.[39] confirmed the results of research by Mahdi et al.,[20] who analyzed the effect of ashwagandha on infertile men with normozoospermia exposed to mental or environmental stress (challenging smokers), or whose infertility was idiopathic. The study used ashwagandha root powder at a dose of 5 g daily with skimmed milk for 3 months and found a reduction in morning and afternoon C levels and an increase in T levels by 13–22% and LH concentrations by 11–21%, depending on the experimental group. At the same time, the concentrations of FSH and PRL decreased.[20] Similarly, Ahmad et al. examined the effect of ashwagandha on T concentration in infertile men and showed that after using its powdered form (5 g daily with skimmed milk for 3 months), there was an increase in the mean T concentration by 0.85–1.43 ng/mL, which was accompanied by an increase in LH and a decrease in FSH and PRL.[21]

Chauhan et al. showed that using ashwagandha root extract at a dose of 300 mg in an aqueous solution (standardized using high-performance liquid chromatography to contain more than 5% withanolides) for 2 months in a double-blind, randomized study of adult men aged 21–45 without significant medical history caused a 17% increase in T concentration without reducing PRL.[22] Another double-blind, randomized trial using 225 mg of aqueous root extract (standardized to more than 5% of total withanolides) for 3 months in infertile men between the age of 22 and 40 demonstrated an increase in T concentration by 17% and LH by 34%.[23] Two additional double-blind, randomized studies on the effect of ashwagandha on hormone concentrations, including T, were conducted using 300 mg of ashwagandha root and leaf extract (standardized to contain 35% withanolide glycosides). Lopresti et al. showed that men aged 40–70 with clinical symptoms of mild-to-moderate fatigue and reduced vitality had significant increases in T (≥45.58 pmol/L) and dehydroepiandrosterone sulfate (DHEA-S) (≥1.49 nmol/L) without a substantial reduction in C concentrations after 4 months of treatment.[24] Lopresti et al. examined the effect of the same preparation at a dose of 240 mg for 2 months on T and DHEA-S concentrations in adult men and women aged 18–65, demonstrating a reduction in C concentration by 23% and DHEA-S by 8%, as well as a statistically significant increase in T concentrations by 11% if not divided by gender and an 11.4% increase in men. A non-significant 0.2% reduction in T concentrations was observed in women.[25]

Kataria et al. studied the effects of ashwagandha on the hypothalamus using the clonal GnRH cell line from the rat hypothalamus cells.[41] The authors assessed GnRH expression and release in response to ashwagandha, and showed that it stimulated GnRH neuronal activity and increased GnRH release.

Fenugreek

Fenugreek, a herb belonging to the legume family, grows in India and North Africa. The plant is used in the cooking and the food industry as the seeds can be eaten raw or cooked, are used as a spice due to their characteristic bitter taste, and have a high fiber content. Fenugreek is characterized by a high water-holding capacity and is used to produce jellies and spreads and as a thickener for soups, drinks and sauces. The plant is also sometimes added to flour to increase the fiber content of bread and to baked goods such as muffins and cakes due to its maple syrup-like aroma, while the leaves are used as a green leafy vegetable.

Fenugreek seeds are used in traditional Indian medicine to treat anorexia, in the postpartum period to increase lactation, and as a gastric stimulant, while Persians and Arabs traditionally used them to increase lean muscle mass in women.[42] Fenugreek is available as a standardized extract or tea for medical purposes, with its medicinal properties derived from high concentrations of glycosides and saponins, including diosgenin, tigogenin, gitogenin, neotigogens, and yamogenin. Diosgenin, the primary sapogenin, is a precursor for sex hormone synthesis and is believed to be the main mechanism responsible for fenugreek increasing T concentrations.[42] Since pharmaceutical T is obtained by chemical conversion of diosgenin (Fig. 1), regular intake of diosgenin is believed to increase T concentrations and enhance its effects.[43,44]

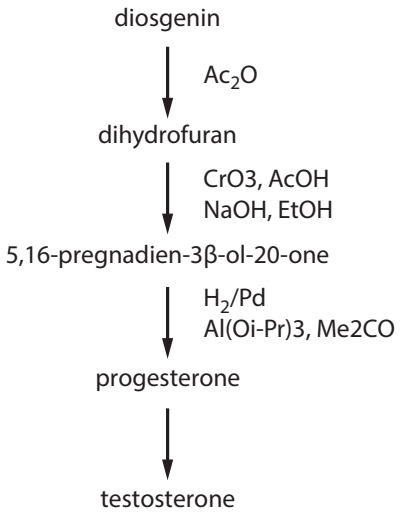

Fig. 1. Chemical conversion of diosgenin to testosterone

Diosgenin is also a precursor for the synthesis of other steroid hormones and metabolites, so its biological effect is not limited to increased T synthesis. Indeed, diosgenin and its metabolites may influence numerous physiological processes (having hypocholesterolemic, gastro- and hepato-protective, antioxidant, anti-inflammatory, and anti-diabetic properties) and diseases, including inhibiting the development of cancer (including prostate cancer, oral squamous cell carcinoma, laryngeal cancer, esophageal cancer, liver cancer, gastric cancer, lung cancer, cervical cancer, glioma, and leukemia), neurodegenerative and cardiovascular diseases, and many others.[45–49] The beneficial effects of the diosgenin found in fenugreek on metabolism and cancers common in men means they are indicated in situations where these conditions coexist with male hypogonadism.

The effect of fenugreek on T concentration in men has been studied in several randomized clinical trials. Wankhede et al. studied healthy men aged 18–35 administered 300 mg of fenugreek seed extract (Fenu-FG, a patented composition; details not shown) twice daily for 8 weeks alongside resistance training. The regimen increased free T concentration (from 17.76 ng/dL to 35.29 ng/dL).[26] Mokashi et al. studied the effects of fenugreek on T concentrations in healthy sedentary men aged 18–41 receiving a single dose (600 mg) of standardized fenugreek seed extract (IND9, a patented composition; details not shown) during two 10-h intervals. Despite the short duration, the results showed an increase in the total (from 405.5 ng/dL to 519.0 ng/mL) and free T (from 11.7 ng/dL to 13.5 pg/mL).[27] Rao and Grant assessed the effects of Fenu-FG on benign prostatic hyperplasia (BPH) symptoms in men aged 45–80 taking 300 mg of the extract twice a day for 3 months.[28] The results showed that fenugreek did not reduce BPH symptoms, and the concentrations of T, free T, SHBG, and prostate-specific antigen did not change significantly and remained within normal limits.[28] Another double-blind, randomized study by Rao et al. assessed the effects of Fenu-FG on androgen concentrations in healthy men aged 43–70 given 600 mg/day for 12 weeks. Total and free T concentrations increased significantly (1.3 nmol/L and 33 pmol/L, respectively), though no significant changes in DHEA-S, androstenedione, estradiol, SHBG, or PRL were observed in any group.[29]

Guo et al., in their double-blind placebo-controlled randomized clinical trial, investigated the effects of fenugreek extract enriched with 20% protodioscin (Furosap, patented, so composition not shown) in healthy male athletes aged 20–28 at a dose of 250 mg/day for 12 weeks. In the group receiving Furosap, a significant increase in total serum T concentration was observed (by 124 ng/dL).[30] The results of animal studies indicate that fenugreek extract caused degenerative changes in the structure of the testes, sperm parameters and concentrations of sialic acid in the epididymis and fructose in the seminal vesicle, and negatively affected the oxidative state in the testicles.[50]

Limitations

In our work, we used realist synthesis as the best method. However, this was a limitation. Although there are reporting standards for realist synthesis, there are no specific standards for conducting a realist inquiry or protocol development frameworks. This allows for flexibility and inclusivity, but there is a risk of suboptimal data analyses due to a lack of prescriptive guidance. However, since the results of the synthesis focus on explaining to the reader why and how a specific supplement works and enable a conscious choice of its further use, this is the only method that allowed the implementation of the objectives of the work, despite its limitations.

Conclusions

Based on the analysis of existing data on T-booster ingredients used to increase endogenous T concentrations, especially double-blind placebo-controlled randomized clinical trials, 2 herbal extracts with the best documented positive effects on T levels in men were selected, ashwagandha root and root extracts/leaves and fenugreek seed extracts. Both can significantly raise T concentrations in men, which may be useful in clinical practice. On the other hand, they may change the results of laboratory tests if they are not declared by the patients. Moreover, athletes need to be aware that taking these products can lead to positive results in doping control because of adulteration or unintentional contamination of commercial products with prohibited substances.[51,52.]

Ashwagandha and fenugreek show differences in their postulated mechanisms of action, with the literature demonstrating that ashwagandha extract inhibits C and PRL effects on the hypothalamic–pituitary–gonadal axis and perhaps directly affects the hypothalamus. Additionally, it improves the antioxidant potential of semen plasma by reducing oxidative stress. Fenugreek seeds increase T levels thanks to the active ingredient diosgenin, which is a precursor for sex hormone synthesis. Data from animal studies indicate that *Trigonella foenum-graecum* seed extract treatment caused degenerative changes in rodent testes and had a negative effect on rodent sperm parameters. The different mechanism of action determines other possible clinical indications for ashwagandha and fenugreek, with their influence on semen parameters one of the most critical factors to consider. In addition, clinical trials on ashwagandha used a standardized extract with a known composition, while the research on fenugreek employed patented preparations of unknown composition. For this reason, using fenugreek preparations other than those used in clinical trials is difficult as we do not know how the extracts were standardized.

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