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It takes a society: A biopsychosocial approach to women's brain health

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Abstract

Only recently has there been a push for increased funding in women's health, an area that has historically been neglected and underfunded. In this editorial, we argue that the higher risk of poor brain health faced by women can be usefully understood from a biopsychosocial perspective. In addition to biological differences, psychosocial stressors contribute to poorer health outcomes over time. Therefore, we advocate for a broad biopsychosocial approach to health, encompassing biological, psychological, and social factors, to better understand and promote women's brain health and to inform ambitious public health policy and research in this area.

Key words: social determinants of health, health equity, dementia risk, women's brain health, biopsychosocial approach

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Highlights

- Women face a disproportionately higher risk of poor brain health due to interacting biological, psychological, and social determinants, highlighting the need for a biopsychosocial approach.
- Addressing women's brain health requires increased research investment, public health prioritization, and policies that consider both biological sex differences and psychosocial factors.
- A comprehensive biopsychosocial framework can improve prevention, understanding, and long-term outcomes for women's brain health across the lifespan.

Introduction: The growing interest in women's brain health

Brain health is gaining momentum as a research and policy priority worldwide. The goal of the World Health Organization (WHO) Intersectoral Global Action Plan on Epilepsy and Other Neurological Disorders 2022–2031 (IGAP), applicable in all 194 Member States, is to elevate brain health to the status of a public health priority.¹ Several countries now have national brain health plans^{2,3} and dementia prevention plans,⁴ which have previously been analyzed from public health⁵ and ethical⁶ perspectives.

Here, we focus on women's brain health. Women are at higher risk of poor brain health than men. A striking statistic supporting this claim is that 2 out of 3 cases of dementia worldwide occur in women; although women live longer than men, greater longevity does not fully explain their higher risk.^{7–9} Yet, women's health has historically been under-researched and underfunded, although there is now growing interest in women's brain health across science and society.¹⁰ We focus on cisgender women; for an analysis of the brain health of sex and gender minorities, see Huo et al.¹¹ We argue for greater action and awareness regarding women's brain health. First, we define brain health from a biopsychosocial, rights-based perspective, before discussing the research and policy priorities that emerge from this position.

Defining a biopsychosocial, rights-based approach to women's brain health

There is a clear need to define concepts carefully and use precise language when addressing women's brain health. It is often unclear what the term “brain health” refers to. Several definitions have been proposed in recent decades, but no consensus has been reached.¹² The concept of women's brain health is frequently used to refer interchangeably to the risk of developing diseases such as dementia and to mental health conditions.

The concept of menopause and its treatment provides a cautionary example. Calvo and Einstein¹³ highlight

the problem of “poor evidence” that results when neither the type of menopause nor the type of hormone therapy is specified. This lack of clarity has practical consequences. In November 2025, the U.S. Food and Drug Administration (FDA) reversed a black box warning for estrogen treatments for menopause that had been in place for more than 20 years.¹⁴ Widespread misunderstanding of the types of menopause and their treatment has significant consequences, not only for the management of menopausal symptoms but also for brain health and dementia prevention.

Here, we approach women's brain health from a biopsychosocial, rights-based perspective¹⁵ across the life course, from birth through menopause and into older age. Given the wide range of factors that contribute to poorer brain health among women over time, we define women's brain health using a biopsychosocial approach; that is, we understand it as embedded in biological, psychological, and broader social factors. The central idea behind our definition of women's brain health is the need to expand our understanding of the factors that determine health, allowing us to critically examine the influence of psychosocial factors and social determinants of health.¹⁶ We position this definition within a life-course perspective,¹⁷ in which good brain health is understood as being sustained by social conditions, including access to adequate education, freedom from violence and deprivation, and supportive, high-quality relationship.

The biopsychosocial model was first introduced by the psychiatrist Engel in 1977,¹⁸ who argued that the biomedical model was overly reductionist and could not account for the psychosocial and behavioral factors that affect the experience of health and disease. Because the way we define health affects how we promote it,¹⁹ we resist the narrow biomedicalization of women's brain health by adopting a biopsychosocial definition as a starting point. The practical purpose of our biopsychosocial definition of women's brain health is to support equitable brain health promotion at the policy level. In this paper, we discuss the determinants of brain health in women as a “multi-tiered system,” that is, from “molecule to network to society.”²⁰

We consider mental health and brain health to overlap fundamentally.²¹ To help clarify this position, we draw on a recent example that shows the essential relationship

between girls' and women's rights, mental health, and brain health. The United Nations Children's Fund (UNICEF) report *Girl Goals: What Has Changed for Girls? Adolescent Girls' Rights over 30 Years*²² includes an extensive focus on mental health, providing a lens through which to understand certain patterns of gender inequality. For example, the report found that almost 4 out of 10 adolescent girls worldwide do not complete upper secondary school, particularly those in poor, rural, and marginalized populations. This schooling gap can be understood in light of gender-based violence and socialization, particularly as they occur during puberty, a critical window for longer-term health.

Psychosocial stressors, such as gender-based violence and expectations linked to gender norms, including those related to physical appearance, contribute to poorer mental health, reduced access to education and healthcare, and poorer mental and physical health, creating a vicious cycle. Depression is the leading cause of disability among girls aged 15–19 years worldwide, and other common responses to psychosocial stressors include eating disorders and a disease-agnostic reduction in wellbeing. Meta-analyses from the Lancet Commission on Dementia have consistently identified low educational attainment as the most robust modifiable risk factor for dementia.²³

Moreover, the Global Burden of Disease Study suggests that the regions in which dementia cases are expected to increase the most this century (the Middle East and sub-Saharan Africa) overlap with those where girls' right to education is least respected, creating conditions that may further undermine women's brain health in these regions.^{24,25}

These structural challenges facing girls early in life can help us understand brain health later in life.²⁶ The high exposure of girls to psychosocial stressors poses 2 problems: first, these stressors undermine the universal right to health¹⁵; second, they lead to downstream losses for broader society because of their impact on girls' flourishing as full participants in society.¹⁰ Although women may indeed be more predisposed to dementia because of hormonal changes, neurobiological mechanisms, and genetic factors,^{27,28} we argue that biopsychosocial mechanisms, such as structural barriers to education early in life, are also likely to be important contributors. Moreover, exposure to psychosocial stressors persists across the life course, through the many roles and multiple responsibilities that women are expected to perform,²⁹ including childbearing, caregiving, additional professional duties,³⁰ and domestic responsibilities.³¹ Women's sleep is often more disrupted because of caregiving duties,^{32,33} and this chronic sleep disturbance contributes to increased stress and poorer brain health outcomes.^{34–36} Although women may learn to cope with and manage these multiple roles,³⁷ these demands take a toll on physical and mental health across the lifespan.^{38–40}

In summary, a rights-based approach to women's brain health first entails certain duties, including those

of governments, to respect girls' inalienable rights to education and health. Second, it entails further duties to reduce the impact of structural factors that increase psychosocial stress among girls and women across the life course. Third, it means that women are entitled to participate in, shape, and benefit from prevention strategies and programs that affect them,^{41–43} to have their lived experiences inform priorities, and to make autonomous, informed decisions about interventions that may affect their cognitive futures.^{44,45}

The consequences of a biopsychosocial approach: broad policy inputs

There are many different biopsychosocial mechanisms through which brain health inequalities arise and are reinforced worldwide, and these mechanisms do not act in isolation. Broadly speaking, health policy must address inequality if it is to be both ethical and effective.^{46,47} Here, we focus on 3 important mechanisms: 1) structural inequalities that shape women's exposure to risk and access to care; 2) stress-related and physiological pathways, such as chronic sleep disruption and hormonal transitions; and 3) behavioral mediators that protect brain health.

Structural inequalities

Responsibility for maintaining brain health cannot be placed solely on individuals. Adequate health-promotion policy should address the health-related burdens faced by girls and women.⁴⁸ Thus, adopting a biopsychosocial approach to women's brain health requires changing modifiable unhealthy environments while supporting girls and women within their existing daily environments⁴⁹ through actions that span social, psychological, and biological domains. Inequalities in brain health mirror broader patterns of health inequality.

In the UK, life expectancy among women living in the most deprived 10% of areas fell between 2010–2012 and 2016–2018.⁵⁰ This suggests that brain health among the most deprived populations may also be declining. However, the downward trend extends beyond people living in deprived neighborhoods. An April 2026 report from the Health Foundation in the UK⁵¹ found that, over the decade from 2012–2014 to 2022–2024, healthy life expectancy across the UK fell by approx. 2 years, to 60.7 years for men and 60.9 years for women, with a more rapid decline among women. The authors of the report stress that these reductions are not inevitable. They reflect policy choices that have increased health inequalities⁵² and reinforce the need for a life-course approach to understanding how health inequalities contribute to poorer brain health.⁵³

These policy considerations also apply to individual-level preventive and therapeutic interventions. In this context,

it is necessary to consider not only biological risk factors but also the support that people need to maintain brain health,⁵⁴ as well as the timing and manner in which interventions are implemented.

Stress-related and physiological pathways

A central pathway linking these inequalities to brain health is chronic stress, particularly the burden of caregiving and the sleep disruption it produces. Improving sleep should be prioritized because it is vital for cognition and brain health.^{55,56} Women may experience chronic sleep disturbances for extended periods because of caregiving responsibilities, which creates an under-discussed health burden.^{32–36} Establishing supportive systems is essential for maintaining women's health and wellbeing.

In research, another important consideration is the recognition of sex differences.^{57–62} These include biological differences as well as psychosocial factors. Hormonal fluctuations, including those that occur during menopause, pregnancy, and the postpartum period,⁶³ can significantly affect brain health.⁶⁴ Women should be informed about these changes early, and interventions should proactively address their impact.

The adage “it takes a village” is particularly relevant for women raising children: It is important to consider how a woman can maintain her own health when she lacks support or bears the full burden of caregiving. This is particularly important for mothers with preexisting mental⁶⁵ and/or physical health concerns.⁶⁶

Behavioral mediators

Primary care physicians and general practitioners are well positioned to have supportive conversations with new mothers and new parents about expectations and possible interventions for sleep disturbances that may increase mothers' risk of postpartum mental health challenges.^{58–60} For instance, if a woman has childcare responsibilities, an intervention should be feasible within her working life, allowing her to balance motherhood, career, and personal identity.⁶¹ This is, however, no small task.

For example, when women earn less than men, they often have fewer resources to invest in mental and physical activities that promote brain health. A recent study suggests that women's brain health suffers as a result of pay gaps; therefore, closing pay gaps should be a priority action for brain health promotion and preventive care.⁶² Furthermore, societal expectations and caregiving responsibilities exacerbate the barriers women face in obtaining adequate care. Interventions must address these disparities if they are to be fair and far-reaching.

At present, women continue to be underrepresented in research and clinical trials,^{67–69} and this is not limited to Alzheimer's disease⁶⁹; it is particularly pronounced among ethnic minority populations.^{70–72} Women are still underrepresented in Alzheimer's disease clinical trials relative to the overall proportion of women diagnosed with Alzheimer's disease.^{73,74} New antibodies for Alzheimer's disease appear to be less effective in women,^{75,76} which makes it increasingly urgent to investigate the mechanisms

Table 1. Policy and research priorities for a biopsychosocial approach to women's brain health

Women's brain health: A biopsychological definition		
We define women's brain health as lifelong functionality and wellbeing from birth through puberty to different types of menopause and beyond, that depends upon access to adequate education, the absence of violence and privation, and the presence of quality, supportive relationships.		
Improving women's brain health through public policy		
Social	Psychological	Biological
Guaranteeing the right to education for all girls. ²⁵ Changing laws that perpetuate violence against girls and women. ²² Increasing online safety by better regulation of social media platforms. ^{78,79} Supporting caregiving needs, e.g., through the use of sleep diplomacy, ⁸⁰ and better access to primary and secondary care pathways through integrated care for women and parents in the postpartum period. ⁸¹ Increasing labor force participation for women, including better access to maternity leave and postnatal return to work. ^{82–84} Early-life safety nets, improved social mobility, and robust pension schemes. ^{85,86}	Cost-effective school-based, public health, and mental health interventions to improve health and wellbeing from childhood, with benefits extending into later life. ^{87–90} Campaigns to reduce gender-based violence, including intimate partner and family violence, as well as adolescent bullying, as risk factors for later-life mental and physical health challenges. ^{91,92}	Improving recognition and awareness of sex-specific hormones (e.g., estrogen, progesterone, testosterone) ^{5,93,94} and their impact on brain health, mental health, and dementia. Support women through their reproductive life transitions, ⁹⁵ from puberty to pregnancy, postpartum, perimenopause and menopause, allowing women the right to safe and personal choices. Improving research and funding on the role of inflammation and neuroimmune processes and dementia risk, ⁹⁶ as well as gut–brain axis and stress inflammation hypothesis, ^{97,98} as women are at a higher risk of developing autoimmune diseases. ⁹⁹ Increasing awareness of the role of cardiometabolic risk factors in dementia. ^{100,101}
Research priorities		
Research initiatives should focus on: 1. Improving conceptual clarity to avoid “muddy research” ¹¹³ on women's brain health, including further study of the different types of menopause, specific treatments, and their role in promoting brain health. 2. Considering sex-specific risk factors for dementia, e.g., in future reports of the Lancet Commission on Dementia. ^{8,73,93} 3. Investigating how sex and gender influence wellbeing, e.g., in future reports of the Lancet Commission on Gender and Global Health. ¹⁰² 4. Studying sex differences in brain ageing within exposome studies. ¹⁰³		

underlying this difference, as well as other factors that may hinder women's access to clinical trials and reduce the effectiveness of available therapies.⁷⁷

It is essential that researchers do not reinforce the erroneous notion that individuals should bear responsibility for maintaining their own brain health.⁵⁴ Effective interventions to support women's brain health and cognitive function must be responsive to these challenges and to the distinct characteristics of different life stages.⁵⁷ It is also important to ensure that public health and community-based approaches are not only available but also accessible to women, and that they are integrated into existing programs or policies to make them more affordable and effective within established care pathways.

Conclusion: Tackling health inequalities is a policy priority for women's brain health

Promoting and safeguarding women's brain health across the life course requires society-wide action to support well-being and neurological function (see Table 1^{13,22,25,73,78–103}). A biopsychosocial approach emphasizes the importance of factors beyond the narrow biomedical boundaries of medicine and the need for public policy to pursue brain health equity ambitiously.^{47,104} We also need to consider the economic benefits of optimizing women's brain health. Supporting women's brain health can drive economic growth,¹⁰ especially as women now make up around 42% of the global workforce.¹⁰⁵ Retaining talented women for as long as they choose to work, and for as long as their health allows, is essential.

By supporting women throughout the reproductive life course¹⁰⁶ and beyond, ensuring access to education for women and girls, and reducing structural burdens on women across the life course, we can maximize women's brain health as both an intrinsic and an instrumental good,¹⁵ while making a significant positive impact on the global economy.^{10,107} Further research could focus on the brain health of subpopulations, including sex and gender minorities.^{11,108}

Use of AI and AI-assisted technologies

Not applicable.

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EQ-5D-5L quality of life scores predict long-term outcomes in kidney transplant recipients

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Abstract

Background. Kidney transplantation is the optimal method of renal replacement therapy. The EQ-5D-5L is a widely used instrument for assessing health-related quality of life (HRQoL) across different populations.

Objectives. This study aimed to assess HRQoL in kidney transplant recipients (KTRs) compared with the general population and to identify factors associated with impaired HRQoL.

Materials and methods. A total of 156 KTRs (58 women and 98 men) participated in this prospective observational study. All participants completed the EQ-5D-5L questionnaire. Demographic and laboratory data were collected, and physical performance tests were performed. Statistical analyses were conducted using Statistica.

Results. Of the 156 participants, 54 (34.62%) had an EQ-5D-5L health state of 11111, whereas 102 (65.38%) reported impairment in HRQoL in at least 1 domain. No significant differences in sex, age, or Charlson Comorbidity Index (CCI) were observed between the analyzed groups. Compared with Polish population reference values for the EQ-5D-5L, KTRs had similar HRQoL, with a median utility score of 0.970 [Q1–Q3: 0.916–1.000]. In the multivariable Cox regression model, the EQ-5D-5L utility score independently predicted mortality in KTRs (hazard ratio (HR) = 0.007; 95% confidence interval (95% CI): 0.000–0.134; *p* = 0.001). The EuroQol Visual Analogue Scale (EQ VAS) score also independently predicted adverse outcomes (HR = 0.966; 95% CI: 0.935–0.997; p = 0.030).

Conclusions. Assessment of HRQoL should be an integral component of the holistic management of KTRs. HRQoL assessment is associated with long-term outcomes in KTRs. Self-rated HRQoL measured on a 0–100 VAS may help identify patients at increased risk of adverse outcomes.

Key words: kidney transplantation, quality of life, EQ-5D-5L survey, long-term outcomes, health-related quality of life (HRQoL)

Highlights

- The assessment of health-related quality of life using the EQ-5D-5L survey is associated with long-term outcomes in kidney transplant recipients.
- Kidney transplant recipients (KTRs) in Poland report similar health-related quality of life to the general Polish population.
- Self-assessed health-related quality of life on a scale of 0–100 can identify patients at risk of adverse outcomes.

Background

The concept of health-related quality of life (HRQoL) emerged in the 1960s and is now considered an important endpoint in clinical trials and medical research. Consequently, improving patients' quality of life has become one of the principal therapeutic goals. To assess HRQoL, a number of validated instruments have been developed, including the EQ-5D-5L questionnaire.¹ It is one of the most widely used instruments for evaluating quality of life across 5 dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, each rated on a 5-level scale: 1 (no problems), 2 (slight problems), 3 (moderate problems), 4 (severe problems), and 5 (extreme problems).^{2,3} The questionnaire also includes the EuroQol Visual Analogue Scale (EQ VAS), a vertical, thermometer-like self-assessment scale that provides a subjective measure of HRQoL. The results can be used to compare HRQoL across different patient populations.^{4–6}

The prevalence of chronic kidney disease (CKD) exceeds 9% worldwide. In some patients, CKD progresses to irreversible end-stage renal disease (ESRD), requiring renal replacement therapy (RRT). Kidney transplantation (KTx) is considered the optimal form of RRT.⁷ It is well established that KTx improves not only clinical outcomes but also patients' quality of life. According to the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, living-donor preemptive kidney transplantation should be considered in adults with an estimated glomerular filtration rate (eGFR) <20 mL/min/1.73 m² and evidence of progressive, irreversible CKD over the preceding 6–12 months.⁸ The management of kidney transplant recipients (KTRs) remains challenging because they have a higher risk of mortality than individuals without kidney disease.⁹ Chronic immunosuppressive therapy increases susceptibility to infections and elevates the risk of cardiovascular disease (CVD), diabetes, and malignancy.^{10,11} In addition, chronic allograft injury remains a major long-term complication.¹² Clinicians must also decide whether and when to ligate a patent arteriovenous fistula (AVF) or remove a Tenckhoff catheter after KTx.^{13,14} Furthermore, KTRs are predisposed to hypertension, post-transplant diabetes mellitus, obesity, muscle wasting, sarcopenia, and frailty.^{15–17} All of these conditions are strongly associated with poorer clinical outcomes and reduced HRQoL.

According to the European Working Group on Sarcopenia in Older People (EWGSOP), sarcopenia is defined as a progressive and generalized skeletal muscle disorder associated with an increased likelihood of adverse outcomes, including falls, fractures, physical disability, and mortality.¹⁸ Sarcopenia is now recognized primarily as a disorder of muscle function rather than merely a change in body composition. Kidney transplant recipients appear to be particularly susceptible to muscle dysfunction and sarcopenia because of the cumulative effects of dialysis, chronic immunosuppressive therapy, low-protein intake during the predialysis stage of CKD, and reduced appetite. The diagnosis of sarcopenia may be supported by physical performance tests,^{19,20} including the 30-Second Sit-to-Stand (STS) test, which assesses lower-extremity muscle function.²¹ Previous studies have shown that sarcopenia adversely affects both clinical outcomes and HRQoL.^{18,22}

Objectives

The aim of this study was to assess HRQoL in KTRs and compare it with that of the general Polish population. The secondary objectives were to identify differences between patients with good and impaired HRQoL, determine predictors of impaired HRQoL, and identify predictors of adverse outcomes in KTRs.

Materials and methods

Study procedures and obtaining ethical approvals

The study was designed as a prospective observational study. Written informed consent was obtained from all participants. The study was approved by the Institutional Ethics Committee of Wrocław Medical University (approval No. KB-43/2020) and registered at ClinicalTrials.gov (NCT04478968). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The study flowchart and patient cohort have been described previously.^{23,24} The inclusion criteria were: age >18 years, KTRs, >12 months after KTx,

stable graft function, and provision of written informed consent. The exclusion criteria were: $\text{EGFR} < 15 \text{ mL/min/1.73 m}^2$, mL/min/1.73 m^2 , severe infection within 3 months before study assessment, an increase in serum creatinine concentration $>0.5 \text{ mg/dL}$ within 3 months before study assessment, active malignancy, and severe heart failure (New York Heart Association Functional Classification (NYHA) Class IV).

Study procedures

At baseline, 156 KTRs were recruited from the Transplant Outpatient Clinic of the University Clinical Hospital of Wrocław Medical University (Fig. 1). All study visits and assessments were conducted by the same trained investigator. Basic demographic and anthropometric data were collected, together with medical history, including the cause of CKD, Charlson Comorbidity Index (CCI), time since KTx, the presence of a functioning patent AVF, AVF duration (months), and current pharmacological treatment. All participants completed the EQ-5D-5L questionnaire independently in Polish. Responses across the 5 dimensions were combined into a 5-digit health-state code ranging from 11111 (no health problems) to 55555 (extreme problems in all 5 dimensions). Utility values (EQ-5D-5L index) were calculated using the EuroQol Group's standard valuation protocol, which assigns population-based preference weights to each health state according to time trade-off (TTO) valuations derived

from a representative sample of the general population. The utility scores in our study were calculated using the Polish EQ-5D-5L value set developed by Golicki et al.²⁵ Each domain contributes differently to the scoring algorithm. Utility scores were in the range of -0.523 to 1.000 .²⁶ The utility value for each health state was calculated as a weighted combination of the 5 dimensions according to the published coefficients of the Polish value set. At the end of the questionnaire, participants assessed their overall health using the EQ VAS by marking a value from 0 (the worst health imaginable) to 100 (the best health imaginable).²⁷ The EQ VAS reflects respondents' overall perception of their health on the day of assessment. Higher EQ-5D-5L index and EQ VAS scores indicate better health status.

Blood samples were collected, routine laboratory tests and urinalysis were performed, and the eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) equation. Handgrip strength was measured in both hands using a Saehan Smedley Spring Hand Dynamometer (model SH5002; Saehan Corporation, Seoul, South Korea). Subsequently, the 3-Meter Stand and Go (3m SAG) test and the 30s STS test were performed. Finally, lung ultrasound (LUS) was conducted using the 28-zone scanning protocol, and the total number of B-lines was recorded.²⁸ Sarcopenia was defined by the presence of muscle weakness, with cutoff values of fewer than 15 repetitions for women and fewer than 17 repetitions for men on the 30s STS test.²¹

Utility scores were calculated from the EQ-5D-5L results and compared with those of the general Polish population.^{25,29} Patients were divided into 2 groups according to their EQ-5D-5L results: those reporting no limitations in mobility, self-care, usual activities, pain/discomfort, or anxiety/depression (health state 11111), and those reporting limitations in at least 1 domain. A 3-year follow-up period was planned. Adverse outcomes included return to dialysis, loss to follow-up, and death.

Statistical analyses

Statistical analyses were performed using Statistica v. 13.3 (StatSoft, Tulsa, USA) with the Plus 5.0 package. Differences between the study groups were analyzed. Data distribution was assessed using the Lilliefors test. Because the data were not normally distributed, continuous variables are presented as the median [Q1–Q3], whereas categorical variables are presented as frequencies and percentages, as appropriate. Quantitative variables were compared using the two-tailed Mann–Whitney U test, and categorical variables were analyzed using the χ^2 test. A $p < 0.05$ was considered statistically significant.

To identify predictors of adverse outcomes, a multivariable logistic regression model was constructed. The assumption of linearity between continuous predictors and

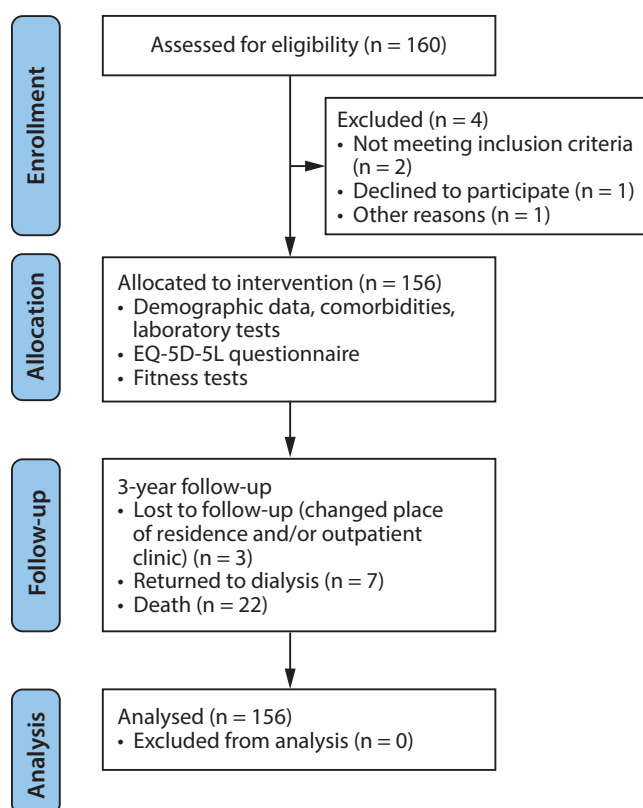


Fig. 1. Flow diagram of study recruitment and study procedures

Table 1. EQ-5D-5L results in KTRs

Variable	Anxiety/depression	Pain/discomfort	Usual activities	Self-care	Mobility
No problems, n (%)	91 (58.34)	77 (49.36)	113 (72.44)	143 (91.67)	101 (64.74)
Slight problems, n (%)	52 (33.33)	51 (32.70)	25 (16.03)	9 (5.77)	31 (19.87)
Moderate problems, n (%)	11 (7.05)	20 (12.82)	17 (10.89)	3 (1.92)	15 (9.62)
Severe problems, n (%)	2 (1.28)	8 (5.12)	0 (0)	1 (0.64)	9 (5.77)
Extreme problems, n (%)	0 (0)	0 (0)	1 (0.64)	0 (0)	0 (0)

KTRs – kidney transplant recipients.

the logit of the dependent variable was evaluated using visual inspection of plots and the likelihood ratio (LR) test. Multicollinearity was assessed using a correlation matrix. Influential observations were identified using Cook's distance. For survival analysis, Cox proportional hazards regression models were fitted, and Kaplan–Meier survival curves were generated. Before constructing the Cox model, multicollinearity among predictors was assessed using the covariance matrix and the variance inflation factor (VIF). The proportional hazards (PH) assumption was evaluated using Schoenfeld residual-based tests, whereas Martingale residuals were used to assess the linearity of continuous variables. For binary variables, the less prevalent category was used as the reference category. Details of all statistical assumptions are provided in the supplementary data.

Results

Quality of life in KTRs

The results of the EQ-5D-5L questionnaire are presented in Table 1. Overall, 58.34% of patients reported no problems with anxiety/depression, 49.36% reported no pain/discomfort, and 91.67% reported no problems with self-care. Compared with the Polish population reference values for the EQ-5D-5L, KTRs had similar HRQoL, with a median utility score of 0.970 [Q1–Q3: 0.916–1.000], whereas the corresponding value for the Polish reference population was 0.970 [Q1–Q3: 0.922–1.000] (Table 2).^{25,29} EQ VAS scores in KTRs were also comparable to the Polish population reference values.³⁰

Table 2. Utility score and EQ-VAS results in KTRs compared to the Polish EQ-5D-5L reference values^{25,26,29}

Variable		KTRs	Polish EQ-5D-5L value set
Mean value (SD)		0.933 (0.103)	0.922 (0.002) ²⁹
Median value [Q1–Q3]		0.970 [0.916–1.00]	0.970 [0.922–1.000] ²⁶
EQ VAS	mean (SD)	73.2 (15.7)	73.7 (0.3) ²⁵
	median [Q1–Q3]	75 [60–85]	–

KTRs – kidney transplant recipients; SD – standard deviation; EQ-VAS – EuroQoL Visual Analogue Scale; Q1–Q3 – 1st quartile–3rd quartile.

Group characteristics

For the purposes of this study, patients were divided into 2 groups according to their EQ-5D-5L results: those reporting no health limitations (health state 11111) and those reporting limitations in at least 1 domain (any health state other than 11111). Of the 156 participants, 54 (34.62%) had the health state 11111, whereas 102 (65.38%) reported limitations in at least 1 domain. The characteristics of these 2 groups are presented in Table 3. No significant differences were observed with respect to age, sex, CKD etiology, comorbidities, or laboratory findings. However, a significant difference was found in the use of antihypertensive medications. Specifically, 95.1% of patients with impaired HRQoL were receiving antihypertensive treatment, compared with 81.48% of patients with good HRQoL ($p = 0.006$).

Patients with impaired HRQoL had poorer performance on the 30s STS test, as shown in Table 3. In addition, KTRs with impaired HRQoL had significantly lower right-hand grip strength than those with good HRQoL (35 kg [Q1–Q3: 27–44] vs 40 kg [Q1–Q3: 28–51], $p = 0.028$).

Survival analysis

In the multivariable Cox regression model, the EQ-5D-5L utility score independently predicted mortality in KTRs (hazard ratio (HR) = 0.007; 95% confidence interval (95% CI): 0.000–0.134; $p = 0.001$) (Table 4). Age was also independently associated with mortality (HR = 1.093; 95% CI: 1.033–1.157; $p = 0.002$) (Table 4).

Because heart disease status violated the PH assumption, the Cox model was stratified by this variable. Stratification allowed separate baseline hazard functions to be estimated for each stratum while preserving the estimation of HRs for all other covariates. The 3-year overall survival and event-free survival probabilities of KTRs are presented in Fig. 2 and Fig. 3. Patients with impaired HRQoL had significantly lower overall survival and event-free survival probabilities than those reporting no limitations in HRQoL according to the EQ-5D-5L (Fig. 2, 3).

Regression model

The multivariable logistic regression model revealed that heart disease and the EQ VAS score were independently

Table 3. Study group characteristics, comparison of patients with impaired HRQoL and patients reporting no limitations in HRQoL assessed with the EQ-5D-5L. Continuous variables were compared using the Mann–Whitney U test, whereas categorical variables were compared using the χ^2 test

Group profile	Patients reporting limitations on the EQ-5D-5L (n = 102)	Patients reporting no limitations on the EQ-5D-5L (n = 54)	p-value
Age [years], [Q1–Q3]	57 [46–64]	57.5 [49–64]	0.556 $z = -0.59$
Gender (males/females) and percentage of men	60/42 58.8%	38/16 70.37%	0.156 df = 1 $\chi^2 = 2.02$
Cause of chronic kidney disease (CKD), n (%)			
Glomerulonephritis	58 (56.8)	34 (63)	0.461 df = 1 $\chi^2 = 0.54$
Autosomal dominant polycystic kidney disease	16 (15.7)	9 (16.7)	0.874 df = 1 $\chi^2 = 0.03$
Renovascular hypertension	14 (13.7)	4 (7.4)	0.240 df = 1 $\chi^2 = 1.38$
Interstitial nephritis	7 (6.9)	2 (3.7)	0.421 df = 1 $\chi^2 = 0.65$
Diabetes mellitus	5 (4.9)	3 (5.5)	0.860 df = 1 $\chi^2 = 0.03$
Other	2 (2)	2 (3.7)	0.512 df = 1 $\chi^2 = 0.43$
Comorbidities, physical features and laboratory findings			
Patent arteriovenous fistula, n (%)	53 (51.96)	29 (53.7)	0.836 df = 1 $\chi^2 = 0.04$
Body mass index (BMI) [kg/m ²], [Q1–Q3]	25.96 [23.83–29.32]	25.84 [23.67–28.73]	0.958 $z = 0.05$
Body surface area [m ²], [Q1–Q3]	1.90 [1.77–2.01]	1.94 [1.79–2.03]	0.182 $z = -1.34$
Heart disease, n (%)	38 (37.25)	14 (25.9)	0.153 df = 1 $\chi^2 = 2.04$
Diabetes mellitus, n (%)	23 (22.55)	11 (20.37)	0.754 df = 1 $\chi^2 = 0.1$
Charlson Comorbidity Index (CCI), [Q1–Q3]	4 [3–5]	4 [3–5]	0.624 $z = -0.49$
Time since kidney transplantation (KTx) [months], [Q1–Q3]	89 [60–141]	102 [64–159]	0.284 $z = -1.07$
EuroQoL Visual Analogue Scale (EQ VAS), [Q1–Q3]	70 [60–81]	80 [70–90]	0.002 $z = -3.09$
Creatinine concentration [mg/dL], [Q1–Q3]	1.35 [1.18–1.63]	1.35 [1.08–1.71]	0.882 $z = -0.15$
Estimated glomerular filtration rate (eGFR) [mL/min/1.73 m ²], [Q1–Q3]	53.5 [44–64]	55 [42–65]	0.868 $z = -0.16$
Albumin concentration [mg/dL], [Q1–Q3]	4.3 [4.1–4.4]	4.3 [4.1–4.5]	0.358 $z = -0.92$
Triglyceride concentration [mg/dL], [Q1–Q3]	137 [108–183]	117.5 [99–189]	0.324 $z = 0.99$
Total cholesterol concentration [mg/dL], [Q1–Q3]	215 [184–249]	210.5 [181–243]	0.896 $z = 0.13$
Urine specific gravity [g/mL], [Q1–Q3]	1.011 [1.008–1.014]	1.01 [1.008–1.015]	0.531 $z = -0.63$
C-reactive protein (CRP) concentration [mg/dL], [Q1–Q3]	1.99 [1.09–3.95]	1.73 [0.86–5.87]	0.982 $z = -0.02$
Hemoglobin concentration [g/dL], [Q1–Q3]	14.3 [13.1–15.3]	15 [13.7–15.7]	0.058 $z = -1.89$

Table 3. Study group characteristics, comparison of patients with impaired HRQoL and patients reporting no limitations in HRQoL assessed with the EQ-5D-5L. Continuous variables were compared using the Mann–Whitney U test, whereas categorical variables were compared using the χ^2 test – cont.

Group profile	Patients reporting limitations on the EQ-5D-5L (n = 102)	Patients reporting no limitations on the EQ-5D-5L (n = 54)	p-value
Medication use, n (%)			
Steroids	97 (95.1%)	51 (94.4%)	0.860 df = 1 $\chi^2 = 0.03$
Antihypertensive medications	97 (95.1%)	44 (81.48%)	0.006 df = 1 $\chi^2 = 7.53$
Tacrolimus	86 (78.43%)	42 (88.89%)	0.312 df = 1 $\chi^2 = 1.02$
Mycophenolate mofetil	84 (82.35%)	43 (79.63%)	0.677 df = 1 $\chi^2 = 0.17$
Antidiabetic medications	22 (21.56%)	11 (20.37%)	0.862 df = 1 $\chi^2 = 0.03$
Statin	36 (35.3%)	23 (42.6%)	0.371 df = 1 $\chi^2 = 0.80$
Antiplatelet/anticoagulant therapy	26 (20.58%)	12 (31.48%)	0.651 df = 1 $\chi^2 = 0.2$
Results of functional, physical and ultrasound examination			
3-Meter Stand and Go [s], [Q1–Q3]	7.28 [6.35–8.52]	7.12 [6.36–8.23]	0.739 z = 0.33
30-Second Sit-to-Stand (30s STS) test (repetitions), [Q1–Q3]	16 [13–19]	17 [15–21]	0.043 z = –2.02
Right-hand grip strength [kg]	35 [27–44]	40 [28–51]	0.028 z = –2.19
Left-hand grip strength [kg]	32 [24–42]	35 [26–46]	0.127 z = –1.53
Peripheral edema / crackles on auscultation, n (%)	17 (16.67%)	7 (12.96%)	0.542 df = 1 $\chi^2 = 0.37$
Number of B-lines in lung ultrasound, [Q1–Q3]	3 [2–7]	4 [2–9]	0.413 z = –0.82
Sarcopenia, n (%)	48 (47.05%)	18 (33.33%)	0.098 df = 1 $\chi^2 = 2.73$

associated with adverse outcomes in KTRs (Table 5). Age was excluded from the model because of collinearity with the EQ VAS score. The EQ VAS score independently predicted adverse outcomes (odds ratio (OR) = 0.966; 95% CI: 0.935–0.997; $p = 0.030$) (Table 5).

Discussion

Our study showed that KTRs in Poland reported HRQoL comparable to that of the general Polish population. We also identified differences between KTRs reporting no limitations on the EQ-5D-5L and those reporting limitations in at least 1 domain. Patients with impaired HRQoL had lower overall survival and event-free survival

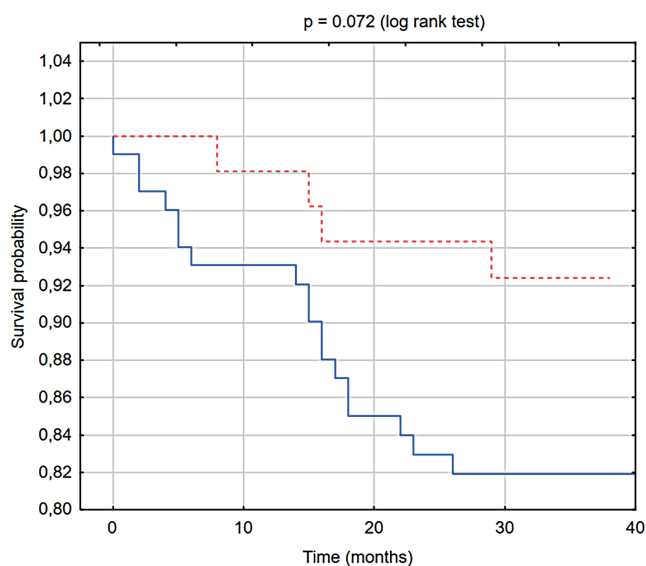
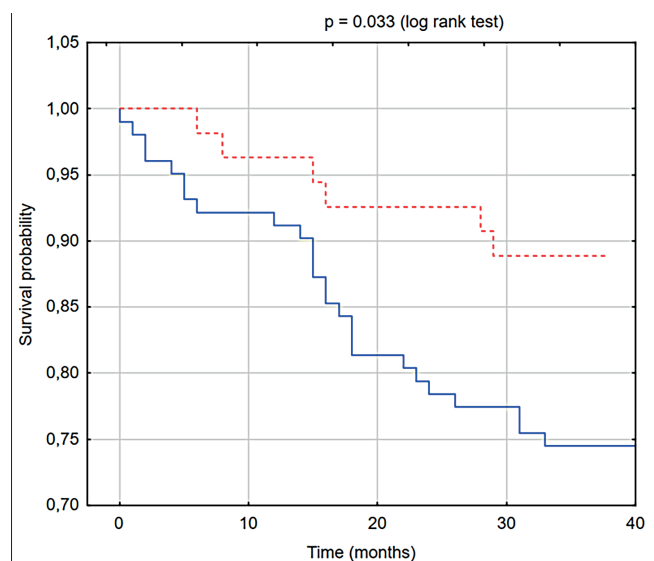
probabilities in the Kaplan–Meier analyses. In addition, both the EQ-5D-5L utility score and the EQ VAS score were identified as predictors of adverse outcomes in KTRs.

The comparable HRQoL observed among Polish KTRs may, in part, be explained by the relatively young age of the study population (median age: 57 years; [Q1–Q3: 47.5–64.0]). Younger age has consistently been associated with better HRQoL.³¹ It is also possible that self-reported HRQoL is overestimated by KTRs because of their previous experience with the burden of dialysis. Furthermore, KTRs in our cohort reported substantially better HRQoL than patients receiving hemodialysis in a recent UK study (mean utility score: 0.57 ± 0.30).³² Similarly, patients undergoing dialysis in Australia reported lower utility scores (mean: 0.719, SD = 0.267) than those observed in our cohort.³³

Table 4. Multivariable Cox proportional hazards regression analysis of predictors of mortality in kidney transplant recipients (KTRs). The Cox regression model was stratified by heart disease status because this variable violated the proportional hazards (PH) assumption

Variable	B	95% CI lower	95% CI upper	HR (95% lower-upper CI)	p-value
Age	0.089	0.032	0.146	1.093 (1.033–1.157)	0.002
Gender (M)	−0.821	−1.774	0.131	0.440 (0.170–1.140)	0.091
BMI	0.039	−0.044	0.122	1.039 (0.956–1.130)	0.361
Creatinine concentration	0.675	−0.163	1.513	1.965 (0.850–4.542)	0.114
Utility score	−4.993	−7.973	−2.01	0.007 (0.000–0.134)	0.001

BMI – body mass index; HR – hazard ratio; 95% CI – 95% confidence interval.

**Fig. 2.** Kaplan–Meier survival curves showing the overall survival probability of kidney transplant recipients (KTRs). The blue curve represents patients reporting limitations in at least one EQ-5D-5L domain, whereas the red curve represents patients reporting no limitations on the EQ-5D-5L (health state 11111)**Fig. 3.** Kaplan–Meier event-free survival curves for kidney transplant recipients (KTRs). The blue curve represents patients reporting limitations in at least one EQ-5D-5L domain, whereas the red curve represents patients reporting no limitations on the EQ-5D-5L (health state 11111)**Table 5.** Multivariable logistic regression analysis of predictors of adverse outcomes (mortality, return to dialysis, and graft loss). For this analysis, the EQ-5D-5L domains were dichotomized into 2 categories: no reported limitations and any reported limitations. *EQ-5D-5L domains were dichotomized for the need of this analysis into 2 groups – reporting no limitations and reporting any limitations

Variable	B	Lower 95% CI	Upper 95% CI	OR	Lower 95% CI	Upper 95% CI	p-value
Gender (M)	−0.354	−1.370	0.662	0.702	0.254	1.939	0.495
Patent arteriovenous fistula	0.201	−0.713	1.115	1.223	0.490	3.050	0.666
Heart disease	1.436	0.499	2.372	4.203	1.647	10.723	0.003
BMI	0.059	−0.027	0.144	1.060	0.974	1.155	0.179
Creatinine concentration	0.745	−0.189	1.679	2.106	0.827	5.362	0.118
EQ VAS	−0.035	−0.067	−0.003	0.966	0.935	0.997	0.030
EQ-5D-5L – mobility*	0.095	−0.975	1.164	1.099	0.377	3.203	0.862
EQ-5D-5L – self-care*	0.800	−0.843	2.479	2.226	0.430	11.934	0.335
EQ-5D-5L – usual activities*	−0.429	−1.641	0.783	0.651	0.194	2.188	0.488
EQ-5D-5L – pain/discomfort*	−0.726	−1.851	0.399	0.484	0.157	1.491	0.206
EQ-5D-5L – anxiety/depression*	0.113	−0.943	1.169	1.120	0.390	3.218	0.834

BMI – body mass index; OR – odds ratio; 95% CI – 95% confidence interval; EQ VAS – EuroQol Visual Analogue Scale.

These findings suggest that KTx is not only the optimal treatment for CKD in terms of survival but also provides substantial benefits with respect to HRQoL, likely because recipients can directly compare their quality of life before

and after transplantation. Consistent with our findings, Kostro et al. reported that KTx improves HRQoL regardless of the renal replacement therapy modality used before transplantation.³⁴

In our study, KTRs most frequently reported problems related to pain/discomfort, followed by anxiety/depression. Self-care was the domain in which the fewest problems were reported. These observations are consistent with previous studies of the Polish general population, in which pain/discomfort and anxiety/depression were also the most frequently affected EQ-5D-5L domains.^{30,35} Likewise, self-care was the least frequently reported problem in the Polish population.³⁵

In our study, patients with impaired HRQoL had poorer results on physical performance and functional tests, suggesting greater muscle dysfunction. Sarcopenia was originally defined primarily by a reduction in muscle mass. However, the concept has evolved, and muscle function, particularly muscle strength and power, is now considered a more important determinant because it is more strongly associated with mortality.³⁶ Furthermore, muscle power has been shown to decline before measurable loss of muscle mass in individuals who develop sarcopenia.³⁷ According to Alcázar et al., the 30s STS test provides a valid measure of bilateral lower-extremity muscle power and is more strongly associated with physical performance than maximal handgrip strength.²⁰ The association between physical performance and HRQoL has previously been demonstrated in several patient populations, including KTRs.^{38,39} Our findings further emphasize the importance of early recognition of muscle dysfunction because it is associated with both HRQoL and clinical outcomes.⁴⁰ Therefore, rehabilitation programs aimed at improving physical performance should be considered for KTRs with developing sarcopenia.

Another important finding of our study was that patients with impaired HRQoL had worse outcomes, including higher overall mortality, than patients reporting no HRQoL limitations according to the EQ-5D-5L. An association between HRQoL and mortality has previously been demonstrated in patients receiving hemodialysis.⁴¹ According to Liebman et al., lower HRQoL measured using the Short Form-36 (SF-36) questionnaire is associated with increased mortality among patients undergoing maintenance dialysis.⁴² In KTRs, HRQoL assessed using the Kidney Disease Quality of Life Short Form (KDQOL-SF) has also been shown to be independently associated with an increased risk of mortality.⁴³ Our findings confirm the association between HRQoL and clinical outcomes in KTRs using the EQ-5D-5L. Moreover, we demonstrate that the EQ VAS is a simple, rapid, and noninvasive tool for identifying patients at increased risk of adverse outcomes. These findings support the use of EQ-5D-5L as part of the routine assessment of KTRs because HRQoL is associated with long-term clinical outcomes.

Limitations of the study

The main limitation of this study is the relatively small sample size, which may have affected the statistical power

of some analyses, particularly the comparison of HRQoL with that of other populations. In addition, sarcopenia was diagnosed solely on the basis of physical performance tests.

Conclusions

Assessment of HRQoL should be an integral part of the holistic management of KTRs. The EQ VAS is a simple, rapid, and noninvasive tool for identifying patients at increased risk of adverse outcomes. Assessment of HRQoL using the EQ-5D-5L is associated with long-term outcomes in KTRs.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.17671687>. The package contains the following files:

Supplementary Materials A. Logistic regression model assumptions.

Supplementary Materials B. Cox regression.

Supplementary Materials C. Lilliefors test.

Data Availability Statement

The datasets supporting the findings of the current study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17669305>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technology

Not applicable.

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Prospective analysis of risk factors in pediatric tic disorders: A clinical observational study

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Conflict of interest

None declared

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Abstract

Background. Tic disorders (TD) are common neuropsychiatric conditions in children, often accompanied by anxiety and depression. Understanding the associated risk factors and their relationship with TD severity is crucial for effective management.

Objectives. This study investigated risk factors for TD in children and examined the relationship between anxiety, depression, and TD severity using the Screen for Child Anxiety Related Emotional Disorders (SCARED) and the Depression Self-Rating Scale for Children (DSRSC).

Materials and methods. A prospective observational study was conducted in 113 children diagnosed with TD at our hospital between January 2021 and January 2024. Tic disorder severity was evaluated using the Yale Global Tic Severity Scale (YGTSS). Anxiety and depression were assessed using SCARED and DSRSC, respectively. Demographic and clinical characteristics, as well as comorbidities, were analyzed using IBM SPSS v. 25.0.

Results. Children with TD had significantly higher rates of febrile seizures, family history of TD, recurrent respiratory infections, and comorbid attention-deficit/hyperactivity disorder (ADHD). Patients with TD exhibited elevated SCARED and DSRSC scores, which were significantly higher in moderate-to-severe cases than in mild cases. Yale Global Tic Severity Scale scores were positively correlated with both SCARED and DSRSC scores. Key risk factors associated with TD and with moderate-to-severe disease included a family history of TD, higher DSRSC scores, and higher SCARED scores.

Conclusions. This study highlights a strong association between anxiety, depression, and TD severity, with higher SCARED and DSRSC scores observed in moderate-to-severe cases. A family history of TD, along with elevated anxiety and depression scores, were identified as significant associated factors. These findings underscore the importance of psychological assessment and tailored interventions for children with TD.

Key words: risk factors, tic disorders, Yale Global Tic Severity Scale (YGTSS), Screen for Child Anxiety Related Emotional Disorders (SCARED), Depression Self-Rating Scale for Children (DSRSC)

Highlights

- Anxiety and depression are significantly elevated in children with tic disorders (TD) and strongly correlate with tic severity, as measured using YGTSS, SCARED, and DSRSC scores.
- Family history of TD, comorbid ADHD, febrile seizures, and recurrent respiratory infections emerge as key risk factors for pediatric TD.
- Children with moderate-to-severe TD show markedly higher anxiety and depression scores compared with those with mild symptoms, highlighting a severity-dependent psychological burden.
- Routine psychological screening and early mental health interventions are essential for effective management of pediatric TD, particularly in high-risk children.

Background

Tic disorders (TD) are neurodevelopmental conditions characterized by involuntary, repetitive motor and vocal tics. These disorders typically emerge in childhood and can significantly affect a child's daily functioning and quality of life.^{1,2} Tic disorders usually manifest between the ages of 3 and 8 years (mean age at onset: 6–7 years), with 93% of patients experiencing symptom onset by the age of 10 years.³ Tic disorders affect approx. 6% of children, making them relatively common in pediatric populations.⁴ Understanding the prevalence of TD and the factors associated with its development is crucial for identifying vulnerable populations and developing effective intervention strategies.

Children with TD often experience anxiety and depressive symptoms, which may exacerbate sleep disturbances and impair daily functioning.^{5–7} Another study reported that 1 in 10 adolescents with chronic TD experienced suicidal thoughts and/or behaviors, which were associated with more complex clinical presentations and frequently occurred during periods of anger and sadness.⁸ Version 2.0 of the European Tourette Syndrome Guidelines recommends psychoeducation as a first-line intervention across all levels of symptom severity.⁹ Anxiety and depressive symptoms may further increase the burden experienced by children with TD; therefore, investigating these psychological profiles may contribute to improved clinical management.

Objectives

This study aimed to examine risk factors for TD and to explore how anxiety and depression scores (Screen for Child Anxiety Related Emotional Disorders (SCARED) and the Depression Self-Rating Scale for Children (DSRSC)) relate to TD severity. Through this research, we hope to gain a deeper understanding of the etiology and development of TD.

Materials and methods

Study population

This prospective observational study included 113 children with TD who sought medical care at our hospital between January 2021 and January 2024. All patients with TD (aged 3–18 years) met the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5),¹⁰ criteria for TD. Exclusion criteria were as follows: 1) transient tic disorder (TTD); 2) comorbid neurological or psychiatric disorders (e.g., Huntington's disease, epilepsy, intellectual disability, or schizophrenia); 3) severe infections, hematological disorders, malignancies, or abnormal cardiac, hepatic, or renal function; and 4) comorbid autoimmune diseases.

The exclusion of children with autoimmune diseases was intended to minimize potential confounding, as conditions such as pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) have been linked to neuropsychiatric symptoms, including tics, and could introduce additional variability into the results. By excluding these cases, we aimed to ensure a more homogeneous study population and to focus on the clinical and psychological factors associated with TD.

Additionally, 100 healthy children were included as controls, matched to the TD group by age and sex. The control group had normal liver and renal function, and chest X-ray findings were within the normal range. None of the control children had neurological or psychiatric disorders. Informed consent was obtained from the parents or legal guardians of all participants prior to enrollment. The study was approved by the Ethics Committee of The First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine (Harbin, China; approval No. 2021034).

Assessment measures

The severity of TD in all children was assessed using the Yale Global Tic Severity Scale (YGTSS). We used only the 2nd section of the YGTSS (motor/vocal tic severity,

score range: 0–50) to quantify tic severity, classifying scores of 0–20 as mild and 21–50 as moderate-to-severe.¹¹ We selected this section because it provides a standardized quantitative assessment of tic severity, whereas the 1st section serves primarily as a symptom checklist without contributing to severity scoring, and the 3rd section reflects overall functional impairment, which may be influenced by subjective factors such as individual coping strategies and environmental support.

Additionally, SCARED was used to assess anxiety symptoms in all participants,¹² and DSRSC was used to assess depressive symptoms.¹³ SCARED is a 41-item questionnaire designed to assess anxiety symptoms in children. Each item is rated on a 3-point Likert scale (0 = not true or hardly ever true; 1 = somewhat true or sometimes true; 2 = very true or often true), yielding a total score ranging from 0 to 82. Higher total scores indicate greater anxiety symptom severity. The DSRSC consists of 18 items, each scored as 0 (no), 1 (sometimes), or 2 (yes), with total scores ranging from 0 to 36; higher scores indicate greater depressive symptom severity. The Chinese versions of the YGTSS, SCARED, and DSRSC have been validated in previous studies, demonstrating good internal consistency, test–retest reliability, and construct validity in Chinese pediatric populations.^{14–16}

Clinical data collection

In addition to the psychological scales, demographic and clinical data were collected for all study participants. Variables included age, body mass index (BMI), sex, tic subtype (Tourette syndrome (TS), chronic tic disorder (CTD), and chronic vocal tic disorder), preterm birth, cesarean delivery, primiparity, history of febrile seizures, allergies, asthma, sleep disorders, attention-deficit/hyperactivity disorder (ADHD), family history of TD, and recurrent respiratory infections.

Statistical analyses

Data analysis was performed using IBM SPSS v. 25.0 (IBM Corp., Armonk, USA). The normality of data distribution was assessed using the Kolmogorov–Smirnov test. Normally distributed data are presented as mean $\pm$ standard deviation (SD), whereas non-normally distributed data are presented as median (range). Group comparisons were performed using Student's *t*-test or the Mann–Whitney *U* test, as appropriate. Differences in proportions were assessed using the χ^2 test. Pearson correlation analysis was used to examine associations between YGTSS scores and SCARED/DSRSC scores. Heteroskedasticity was assessed using the Breusch–Pagan test. All clinically relevant variables were included in the logistic regression models based on theoretical considerations to comprehensively evaluate their associations with TD and its severity. A *p* < 0.05 was considered statistically significant.

Results

Basic characteristics of study participants

This prospective observational study included 113 children with TD (TD group) and 100 healthy controls (HC group). The TD group was categorized as follows: TS (*n* = 65, 57.5%), chronic motor tic disorder (*n* = 32, 28.3%), and chronic vocal tic disorder (*n* = 16, 14.2%). In the TD group, the median age was 10 years (range: 3–18), 59.3% were boys, and the mean BMI was 18.29 ± 2.30 kg/m². In the HC group, the median age was 9 years (range: 3–18), 59.0% were boys, and the mean BMI was 18.10 ± 2.12 kg/m². There were no significant differences between the 2 groups in terms of age, sex, or BMI. However, comparison of demographic and clinical characteristics revealed that the TD group had significantly higher proportions of children with a history of febrile seizures, a family history of TD, recurrent respiratory infections, and comorbid ADHD (Table 1).

Higher SCARED and DSRSC scores indicating increased anxiety- and depression-related symptoms in TD children

Subsequently, we compared SCARED and DSRSC scores between the 2 groups. As shown in Fig. 1, children in the TD group had significantly higher SCARED and DSRSC scores than those in the HC group. These findings suggest that children with TD are more likely to exhibit anxiety- and depression-related symptoms.

Comparison of clinical characteristics in tic disorder children with different severity

Children with TD were stratified into a mild group (YGTSS score: 0–20, *n* = 61) and a moderate-to-severe group (YGTSS score: 21–50, *n* = 52) based on YGTSS scores. Comparison of the clinical characteristics between the 2 groups revealed that the moderate-to-severe group had a significantly higher proportion of children with a family history of TD than the mild group (Table 2). The Breusch–Pagan test indicated no evidence of heteroskedasticity. Pearson's correlation analysis demonstrated positive correlations between YGTSS scores and both DSRSC and SCARED scores (Fig. 2).

Binary logistic regression analysis in tic disorder children

To explore factors associated with TD in children, we performed a multivariable binary logistic regression analysis. As shown in Table 3, the Hosmer–Lemeshow test (*p* = 0.864) and Nagelkerke *R*² (0.671) indicated good model fit. Logistic regression analysis showed that a history of febrile seizures, a family history of TD, higher DSRSC scores, and higher SCARED scores were independently associated with TD.

Table 1. Basic characteristics of study participants

Variable	TD, n = 113	HC, n = 100	Z/t (df)/ χ^2 (df)	p-value
Age [years]	10 (3–18)	9 (3–18)	–0.183	0.855
Sex, male (%)	67 (59.3)	59 (59.0)	0.002 (1)	0.966
BMI [kg/m ²]	18.29 $\pm$ 2.30	18.10 $\pm$ 2.12	0.876 (211)	0.197
Tic types				
Tourette syndrome, n (%)	65 (57.5)	–	–	–
Chronic motor tic disorder, n (%)	32 (28.3)			
Chronic vocal tic disorder, n (%)	16 (14.2)			
Premature, n (%)	15 (13.3)	13 (13.0)	0.004 (1)	0.950
Cesarean section delivery, n (%)	61 (53.9)	50 (50.0)	0.305 (1)	0.581
Second child and above, n (%)	18 (15.9)	14 (14.0)	0.142 (1)	0.706
Medical history				
Febrile seizures, n (%)	46 (40.7)	11 (11.0)	20.482 (1)	<0.001
Allergies, n (%)	27 (23.9)	19 (19.0)	0.713 (1)	0.399
Asthma, n (%)	18 (15.9)	14 (14.0)	0.142 (1)	0.706
Sleep disorders, n (%)	30 (26.5)	26 (26.0)	0.006 (1)	0.936
ADHD, n (%)	27 (23.9)	5 (5.0)	14.448 (1)	<0.001
Tic disorders, n (%)	40 (35.4)	4 (4.0)	31.164 (1)	<0.001
Recurrent respiratory infections, n (%)	32 (28.3)	9 (9.0)	12.276 (1)	<0.001

TD – tic disorders; BMI – body mass index; ADHD – attention deficit hyperactivity disorder; df – degrees of freedom; HC – healthy controls. Continuous data presented non-normal distribution and expressed by median (minimum to maximum) and analyzed using Mann–Whitney U test. Continuous data presented normal distribution and were expressed by mean $\pm$ standard deviation (SD) and analyzed with Student's t test; χ^2 test was used for rates.

Table 2. Clinical characteristics in tic disorder (TD) children with different severity

Variable	YGTSS score 0–20, n = 61	YGTSS score 21–50, n = 52	Z/t (df)/ χ^2 (df)	p-value
Age [years]	10 (3–18)	9.5 (3–18)	–1.115	0.265
Sex, male (%)	36 (59.0)	31 (59.6)	0.007 (1)	0.931
BMI [kg/m ²]	18.03 $\pm$ 2.29	18.59 $\pm$ 2.28	–1.297 (111)	0.197
Premature, n (%)	7 (11.5)	8 (15.4)	0.653 (1)	0.419
Cesarean section delivery, n (%)	33 (54.1)	28 (48.1)	0.720 (1)	0.396
Second child and above, n (%)	9 (14.8)	9 (17.3)	0.232 (1)	0.630
Medical history				
Febrile seizures, n (%)	21 (34.4)	25 (48.1)	3.328 (1)	0.061
Allergies, n (%)	12 (19.7)	15 (28.8)	2.254 (1)	0.133
Asthma, n (%)	8 (13.1)	10 (19.2)	1.374 (1)	0.241
Sleep disorders, n (%)	14 (23.0)	16 (30.8)	1.547 (1)	0.214
ADHD, n (%)	12 (19.7)	15 (28.8)	2.254 (1)	0.133
Tic disorders, n (%)	13 (21.3)	27 (51.9)	19.922 (1)	<0.001
Recurrent respiratory infections, n (%)	15 (24.6)	17 (32.3)	1.501 (1)	0.221

BMI – body mass index; ADHD – attention deficit hyperactivity disorder; YGTSS – Yale Global Tic Severity Scale. Continuous data presented non-normal distribution and were expressed by median (minimum to maximum) and analyzed using Mann–Whitney U test. Continuous data presented normal distribution and were expressed by mean $\pm$ standard deviation (SD) and analyzed with Student's t test; χ^2 test was used for rates.

Binary logistic regression analysis in moderate/severe tic disorder children

Lastly, we performed a multivariable binary logistic regression analysis to investigate factors associated with moderate-to-severe TD compared with

mild TD. As shown in Table 4, the Hosmer–Lemeshow test ($p = 0.519$) and Nagelkerke R^2 (0.419) indicated good model fit. Logistic regression analysis showed that a family history of TD, higher DSRSC scores, and higher SCARED scores were independently associated with moderate-to-severe TD.

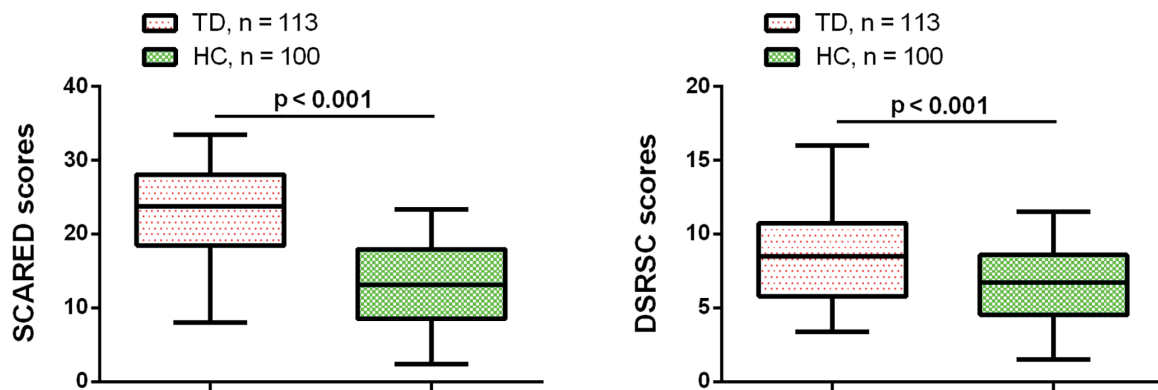


Fig. 1. Screen for Child Anxiety Related Emotional Disorders (SCARED) and Depression Self-Rating Scale for Children (DSRSC) scores in all participants. In the boxplots, data are presented as median (minimum to maximum). Comparisons between the 2 groups were performed using Student's t-test. All data were normally distributed

TD – tic disorders; HC – healthy controls.

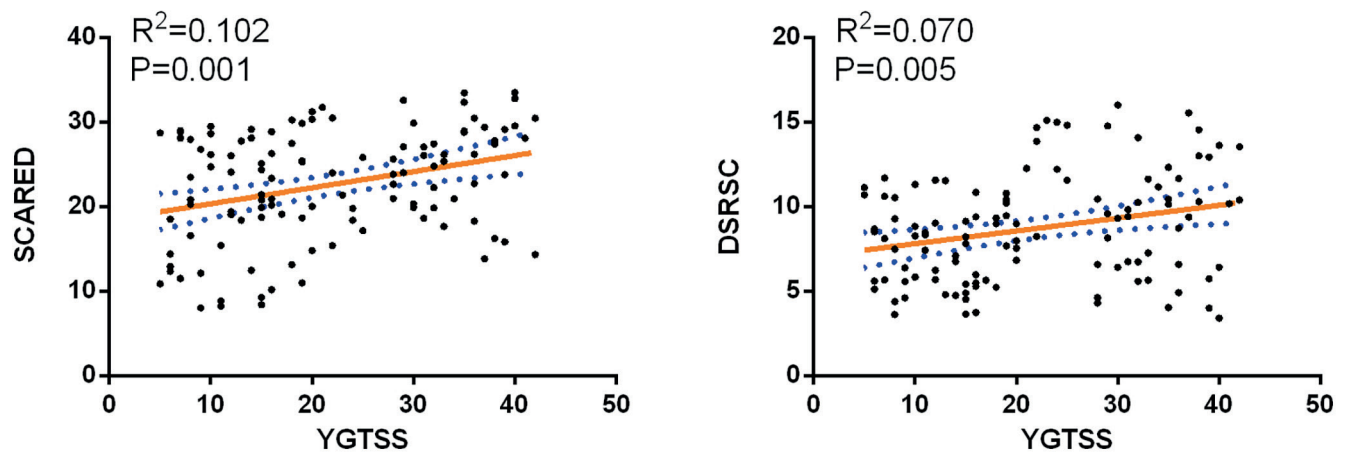


Fig. 2. Scatterplots of Yale Global Tic Severity Scale (YGTSS) scores vs Depression Self-Rating Scale for Children (DSRSC) scores and Screen for Child Anxiety Related Emotional Disorders (SCARED) scores. Both scatterplots include regression lines fitted to the data (orange lines) with corresponding confidence intervals (blue dots)

Table 3. Logistic regression of risk factors for tic disorder (TD) children

Variables	Wald	OR	95% CI	p-value
Age (per 1-year increase)	0.276	0.975	0.889–1.070	0.600
Sex	0.285	0.785	0.322–1.910	0.593
BMI (per 1-unit increase)	0.554	0.928	0.764–1.129	0.457
Premature	0.669	0.581	0.158–2.135	0.413
Cesarean section delivery	0.288	0.794	0.341–1.847	0.592
Second child and above	0.486	0.673	0.221–2.050	0.486
Febrile seizures	10.533	0.147	0.046–0.468	0.001
Allergies	0.208	1.287	0.436–3.801	0.648
Asthma	0.001	0.983	0.236–4.093	0.981
Sleep disorders	0.012	1.061	0.370–3.040	0.912
ADHD	2.169	0.305	0.063–1.481	0.141
Tic disorders	8.440	0.125	0.031–0.508	0.004
Recurrent respiratory infections	3.827	0.302	0.091–1.002	0.050
SCARED scores (per 1-point increase)	34.566	0.789	0.729–0.854	<0.001
DSRSC scores (per 1-point increase)	6.775	0.797	0.671–0.945	0.009

BMI – body mass index; ADHD – attention deficit hyperactivity disorder; OR – odds ratio; 95% CI – 95% confidence interval; SCARED – Screen for Child Anxiety Related Emotional Disorders; DSRSC – Depression Self-rating Scale for Children.

Table 4. Logistic regression of risk factors for moderate/severe tic disorder (TD) children

Variables	Wald	OR	95% CI	p-value
Age (per 1-year increase)	1.042	0.946	0.849–1.053	0.307
Sex	0.628	1.527	0.536–4.347	0.428
BMI (per 1-unit increase)	2.344	1.182	0.954–1.465	0.126
Premature	0.036	0.859	0.179–4.118	0.850
Cesarean section delivery	0.029	0.918	0.342–2.464	0.866
Second child and above	0.917	1.867	0.520–6.702	0.338
Febrile seizures	0.811	1.581	0.584–4.281	0.368
Allergies	0.001	1.001	0.324–3.095	0.999
Asthma	0.005	0.948	0.225–3.994	0.942
Sleep disorders	0.066	0.855	0.259–2.823	0.798
ADHD	0.022	1.093	0.343–3.477	0.881
Tic disorders	9.305	5.302	1.815–15.454	0.002
Recurrent respiratory infections	0.429	1.429	0.419–4.166	0.513
SCARED scores (per 1-point increase)	7.636	1.118	1.033–1.211	0.006
DSRSC scores (per 1-point increase)	9.383	1.313	1.103–1.562	0.002

BMI – body mass index; ADHD – attention deficit hyperactivity disorder; OR – odds ratio; 95% CI – 95% confidence interval; SCARED – Screen for Child Anxiety Related Emotional Disorders; DSRSC – Depression Self-rating Scale for Children.

Discussion

Patients with TD often experience subjective discomfort, social challenges, and emotional difficulties. These problems are particularly prominent in moderate-to-severe TD.^{17,18} Therefore, in this study, we investigated factors associated with TD and with moderate-to-severe TD. Logistic regression analysis showed that a family history of TD, higher DSRSC scores, and higher SCARED scores were independently associated with both TD and moderate-to-severe TD.

Previous studies have explored differences in clinical characteristics between patients with TD and typically developing children. Hesapçioğlu et al. reported that higher parental education was associated with a lower likelihood of TD, whereas a family history of psychiatric disorders was associated with a higher likelihood.¹⁹ Brander et al. reported a significant association between TS and CTD and metabolic and cardiovascular diseases.²⁰ Mataix-Cols et al. suggested that comorbid psychiatric disorders and a family history of psychiatric disorders during childhood were major factors associated with the persistence of TD into adulthood.²¹

Studies investigating the mechanisms underlying TD have suggested that regulation of gene expression through non-coding variants, particularly in cortico-striatal circuits, may represent a fundamental biological mechanism underlying TDs,²² supporting the hypothesis that genetic factors play an important role in TD. Consistent with these findings, our study identified a family history of TD as a factor independently associated with both TD and moderate-to-severe TD, in agreement with the clinical findings reported by Ueda et al.¹ and Abdulkadir et al.²³

In addition to traditional clinical factors, we found that DSRSC and SCARED scores were significantly higher in patients with TD, with further increases observed in those with moderate-to-severe TD. These findings suggest that patients with TD are more likely to exhibit anxiety- and depression-related symptoms, which are also associated with tic severity. Research has shown that, compared with control groups, adolescents with TD have higher scores on the Multidimensional Anxiety Scale for Children (MASC).⁵ Another study reported that, in adolescents with CTD, suicidal thoughts and/or behaviors were significantly associated with tic severity, and Child Behavior Checklist (CBCL) anxiety/depression scores mediated the relationship between tic severity and suicidal thoughts and behaviors.⁸ A recent study on 85 children and adolescents with CTD also found that those with comorbid obsessive-compulsive disorder (OCD) and/or ADHD had a higher prevalence of depressive symptoms compared to those without comorbidities, and that depression played an important moderating role in the relationship between tic severity and functional impairment.¹¹ Furthermore, a review by Hibberd et al. reported that patients with TS or CTD had a higher prevalence of sleep difficulties, with the highest risk observed in those with comorbid anxiety disorders.²⁴ In our study, multivariable logistic regression analysis also showed that higher DSRSC and SCARED scores were independently associated with both TD and moderate-to-severe TD.

While our study identified significant correlations between tic severity (YGTSS scores) and anxiety/depression severity (SCARED and DSRSC scores), it is important to consider the potential bidirectional nature of these relationships. On the one hand, the chronic and often socially

stigmatizing nature of tics may lead to increased anxiety and depression in children with TD, particularly as they face challenges in social interactions and daily functioning. On the other hand, pre-existing anxiety and depression may exacerbate tic severity by increasing stress levels, which are known to worsen tic symptoms.²⁵ Although our study design does not allow causal inferences, future longitudinal studies are needed to determine whether anxiety and depression are consequences of tic severity or factors contributing to tic severity. Understanding the direction of these relationships will be crucial for developing targeted interventions addressing both TD and associated psychological symptoms.

Given the high rates of anxiety and depression observed in children with TD, clinicians should adopt a multidisciplinary treatment approach that integrates both medical and mental health support. Early identification of psychiatric comorbidities, such as anxiety and depression, is important for effective management. Clinicians should consider regular psychological screening for children with TD and provide targeted interventions, including cognitive-behavioral therapy (CBT), to address mental health concerns. Additionally, clinicians should be mindful of the potential psychiatric side effects of medications prescribed for TD, as these may contribute to or exacerbate psychological symptoms. By adopting a holistic and proactive approach, clinicians may better support the mental wellbeing of children with TD and improve their overall quality of life.

Limitations of the study

There are several limitations to this study. A major limitation is the potential confounding effect of psychotropic medications commonly used in TD management, as these may independently influence anxiety and depression scores. Medications prescribed to manage TD symptoms, particularly psychotropic drugs such as dopamine antagonists, may affect psychiatric comorbidities, including anxiety and depression, and these effects were not fully accounted for in our analysis. Second, this was a single-center study with a relatively small sample size, which may limit the generalizability of our findings. Third, we did not perform subgroup analyses to further classify patients with TD, which might have provided additional insights into specific clinical patterns or associated factors. Future research should aim to control for these potential confounders and further explore the mechanisms contributing to psychological distress in children with TD.

Conclusions

Our study demonstrated significantly higher DSRSC and SCARED scores in patients with TD, with further increases observed in those with moderate-to-severe TD.

Additionally, a family history of TD, higher DSRSC scores, and higher SCARED scores were identified as factors independently associated with both TD and moderate-to-severe TD.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.18236518>. The package contains the following files:

Supplementary Table 1. Normality tests for the data presented in Table 1, Table 2, and Fig. 1.

Supplementary Table 2. Breusch–Pagan test results for the data in Fig. 2.

Supplementary Table 3. Assumption tests for the logistic regression analyses in Table 3 and Table 4.

Data Availability Statement

The datasets supporting the findings of the current study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.14505290>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Clinical characteristics and risk factors for carbapenem-resistant *Pseudomonas aeruginosa* infection

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Abstract

Background. *Pseudomonas aeruginosa* (PA) is a major opportunistic pathogen responsible for nosocomial infections, particularly those affecting the lower respiratory tract. The emergence of carbapenem-resistant PA (CRPA) poses a significant global therapeutic challenge, as increasing resistance rates complicate clinical management.

Objectives. To investigate the clinical characteristics and antimicrobial resistance profiles of CRPA infections, identify associated risk factors among patients at The Affiliated Hospital of North Sichuan Medical College, and provide evidence for the development of effective prevention and control strategies.

Materials and methods. A retrospective case–control study was conducted involving 94 patients with CRPA infection (study group) and 50 patients with CSPA infection (control group) between January 2021 and December 2022. Clinical characteristics and risk factors were analyzed using univariate analyses and multivariable logistic regression. Variable selection for the primary model was performed using best-subset selection based on the Bayesian information criterion (BIC).

Results. Carbapenem-resistant PA isolates were primarily recovered from lower respiratory tract secretions (77.66%) and were most frequently identified in the intensive care unit (ICU) (41.49%). Compared with CSPA strains, CRPA exhibited significantly higher resistance rates to aztreonam, ciprofloxacin, levofloxacin, and cephalosporins ($p < 0.05$). Multivariable logistic regression using best-subset selection identified prior exposure to carbapenems (adjusted odds ratio (aOR) = 6.914, 95% confidence interval (95% CI): 2.765–17.290, $p < 0.001$) and aminoglycosides (aOR = 6.209, 95% CI: 1.701–22.668, $p = 0.006$) as independent risk factors for CRPA infection.

Conclusions. Carbapenem-resistant PA infection imposes a considerable burden in our hospital, particularly among ICU patients. Prior exposure to carbapenems and aminoglycosides significantly increases the risk of CRPA infection. The BIC-selected model identified the same main risk factors as the fully adjusted sensitivity analysis, supporting the consistency of the findings. Strengthening antimicrobial stewardship and tailoring empirical therapy according to local antimicrobial susceptibility patterns are essential to curb resistance and improve clinical outcomes.

Key words: risk factors, clinical features, respiratory infection, resistance characteristics, carbapenem-resistant *Pseudomonas aeruginosa*

Highlights

- High ICU incidence: Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) infections were most common among ICU patients and predominantly originated from lower respiratory tract specimens.
- Major risk factors identified: Use of aminoglycosides (adjusted odds ratio (aOR) = 6.209) and carbapenems (aOR = 6.914) were identified as independent risk factors for CRPA infection.
- Elevated antibiotic resistance: CRPA strains showed significantly higher resistance to aztreonam, ciprofloxacin, levofloxacin, and cephalosporins compared with carbapenem-sensitive strains.
- Invasive procedures increase risk: Use of invasive mechanical ventilation, urinary catheters, and ICU admission was significantly associated with CRPA infection.
- Clinical implication: Rational antibiotic stewardship and minimization of unnecessary invasive procedures are essential for controlling CRPA infections and improving patient outcomes.

Background

Pseudomonas aeruginosa (PA) is an opportunistic pathogen widely distributed in nature. Although the isolation rate of PA has steadily decreased in recent years, it remains a major pathogen causing lower respiratory tract infections in healthcare institutions. Carbapenems are broad-spectrum antibiotics with potent antibacterial activity and are characterized by stability against β -lactamases and low toxicity. They are among the most important antibacterial agents used in the clinical treatment of serious bacterial infections. With the increasing misuse of antibiotics, the prevalence of carbapenem-resistant PA, multidrug-resistant PA (MDRPA), and even extensively drug-resistant PA (XDRPA) has gradually increased. According to the China Antibacterial Monitoring Network (CHINET; 2022 Annual Bacterial Resistance Monitoring Report),¹ the resistance rates of PA to meropenem and imipenem in 2022 were 22.1% and 17.6%, reference. The high morbidity, mortality, and substantial resistance rates associated with these infections pose major challenges to clinical prevention and control.^{2–4} The resistance mechanisms of PA are complex and multifactorial, including the production of β -lactamases (such as carbapenemases), overexpression of efflux pumps that actively expel antibiotics, modification of antibiotic target sites, and reduced outer membrane permeability, all of which limit antibiotic penetration and efficacy. The spread of such resistant strains poses a significant threat to public health, resulting in limited treatment options, prolonged hospital stays, increased healthcare costs, and higher patient mortality.^{5,6} Despite numerous studies investigating the epidemiology of PA, the incidence of CRPA remains high, and effective management strategies are still lacking.

Objectives

The primary objective of this study was to analyze and compare the clinical characteristics and antimicrobial resistance profiles of CRPA and carbapenem-susceptible PA

(CSPA) infections among patients treated at the Affiliated Hospital of North Sichuan Medical College (Nanchong, China). Furthermore, this study aimed to identify specific risk factors associated with CRPA infection. The findings are intended to provide an evidence base for optimizing clinical management, guiding antimicrobial stewardship, and developing effective strategies for the prevention and control of CRPA.

Materials and methods

Definitions

Multidrug resistance refers to acquired non-susceptibility to at least one agent in 3 or more antimicrobial categories.⁷ Extensively drug-resistant infection is a more severe form of resistance, defined as non-susceptibility to at least one agent in all but 2 or fewer antimicrobial categories. The latter often develops from multidrug resistance and implies resistance not only to conventional antibiotics but also to certain “last-line” antibiotics. “Last-line antibiotics” are reserve agents used when first- and second-line therapies have failed and typically include drugs such as colistin, polymyxin B, and newer combination agents, including ceftazidime-avibactam, which are critical for the treatment of severe infections caused by resistant pathogens.⁸

Study design and population

A retrospective case-control study was conducted at The Affiliated Hospital of North Sichuan Medical College, a major tertiary-care referral center with more than 2,000 beds. The hospital serves a diverse patient population from both urban and rural areas, which may influence the epidemiological and antimicrobial resistance characteristics observed in this study. The study population included patients with culture-confirmed PA infections

identified between January 2021 and December 2022. The case group comprised 94 patients with CRPA infections. The control group consisted of 50 patients with CSPA infections, selected from the same period and matched to the cases according to admission to the same ward and similar age to ensure comparability.

Ethical approval

This study was approved by the Institutional Review Board and Ethics Committee of The Affiliated Hospital of North Sichuan Medical College (approval No. 2023ER346-1, issued on January 3, 2023). The requirement for individual informed consent was waived by the committee due to the retrospective and anonymized nature of the data analysis.

Inclusion and exclusion criteria

Inclusion criteria: 1. Patients aged ≥ 18 years who were admitted to our hospital for treatment; 2. Patients with a first-time isolation of CRPA or CSPA and corresponding clinical evidence of infection. Duplicate isolates were excluded. Exclusion criteria: 1. Cases in which CRPA or CSPA was isolated without signs of infection; 2. Patients with incomplete clinical data, incomplete medical records, or age < 18 years.

Bacterial isolation and drug susceptibility testing

Data on the isolation, culture, and identification of pathogenic bacteria were retrieved from the hospital laboratory information system. These procedures were originally performed in accordance with the National Operating Procedures for Clinical Examination.⁹ *Pseudomonas aeruginosa* was identified using the VITEK® 2 Compact automated system (bioMérieux, Marcy-l'Étoile, France). Antimicrobial susceptibility testing was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).¹⁰ An isolate was classified as CRPA if it demonstrated resistance to one or more carbapenem agents (e.g., imipenem or meropenem). For analytical purposes, isolates with intermediate susceptibility were categorized as resistant. Molecular analysis of specific carbapenemase genes was not performed as part of this study.

Methods

A retrospective case-control design was used to collect relevant variables from the 2 study groups, including: 1) Basic data: age, sex, presence of comorbidities (such as chronic obstructive pulmonary disease (COPD), diabetes, hypertension, and coronary heart disease), surgery, ICU admission, and specimen type; 2) Antibacterial therapy:

use of restricted antibiotics, use of antifungal drugs, and combination antibiotic therapy; 3) Invasive procedures: use of invasive mechanical ventilation (tracheotomy or endotracheal intubation), urinary catheterization, placement of a central venous catheter, placement of a gastric tube, and other related procedures. The data from the 2 groups of patients were compiled, and a logistic regression model was constructed. Risk factors for CRPA infection were identified by comparing the variables collected from both groups.

Statistical analyses

This retrospective case-control study was analyzed using logistic regression and evaluated for model adequacy. This design was chosen because it directly tests whether prior exposures are associated with CRPA infection while controlling for potential confounding factors.

Best-subset selection based on the Bayesian information criterion (BIC) was applied to identify the most parsimonious primary model, whereas a fully adjusted model including all candidate covariates was evaluated as a sensitivity analysis to confirm robustness.

Analyses were conducted using SPSS v. 26.0 (IBM Corp., Armonk, USA) and R v. 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria). Categorical variables were summarized as n (%) and compared using Pearson's χ^2 test or Fisher's exact test, as appropriate. Continuous variables were summarized as the median (interquartile range (IQR)) and compared using the Mann-Whitney U test. For multiple comparisons of antibiotic susceptibility, Benjamini-Hochberg false discovery rate control was applied to the p -values obtained from the overall tests.

To identify risk factors for CRPA, a multivariable logistic regression model was fitted for the binary outcome (coded as 1 for CRPA and 0 for CSPA). Best-subset selection based on the BIC was used for variable reduction to obtain the primary model, and a fully adjusted model including all candidate covariates was additionally analyzed as a sensitivity analysis to confirm robustness. Linearity of the logit for age was assessed using the Box-Tidwell test ($p = 0.889$). Influence diagnostics included Cook's distance (threshold $> 4/n$), leverage and studentized residuals. The model was refitted after removing the 1–3 observations with the greatest influence. Student's t -test was not used.

Results

Strain department distribution and specimen type

Among the 94 CRPA strains collected in this study, isolates were primarily recovered from the respiratory department, ICUs, and surgical departments. The ICU

group (including the ICU, emergency intensive care unit (EICU), and other intensive care units) accounted for 41.49% of isolates, the respiratory department for 14.89%, and the surgical departments for 11.70%. Specimens were primarily obtained from lower respiratory tract secretions, midstream urine, wound secretions, whole blood, drainage fluid, tissues, and other clinical samples. In both the CRPA and CSPA groups, lower respiratory tract secretions were the predominant specimen type, accounting for 77.66% and 76.00% of isolates, respectively.

Comparison of drug resistance rate

IBM SPSS v. 26.0 software was used to compare and analyze the resistance and susceptibility profiles of the 2 groups of strains to commonly used antimicrobial agents. As shown in Table 1, resistance rates to aztreonam, piperacillin/tazobactam, cefepime, ceftazidime, levofloxacin, and ciprofloxacin were significantly higher in the CRPA group than in the CSPA group ($p < 0.05$), whereas resistance rates to amikacin and gentamicin did not differ significantly between the 2 groups ($p > 0.05$). In the comparison of susceptible strains, susceptibility

rates to aztreonam, piperacillin/tazobactam, cefepime, ceftazidime, levofloxacin, and ciprofloxacin were significantly higher in the CSPA group than in the CRPA group ($p < 0.05$), whereas susceptibility to amikacin and gentamicin did not differ significantly between the 2 groups ($p > 0.05$).

CRPA univariate analysis

A total of 144 patients were enrolled in this study, including 50 patients in the CSPA group and 94 patients in the CRPA group. As detailed in Table 2, comparisons between the groups showed that the use of invasive mechanical ventilation, urinary catheters, and other invasive procedures differed significantly between the 2 groups ($p < 0.05$). In addition, significant differences were observed between the 2 groups regarding the use of aminoglycosides, fluoroquinolones, carbapenems, tetracyclines, restricted antibiotics (including vancomycin, linezolid, and tigecycline), antifungal drugs, and combination antimicrobial therapy ($p < 0.05$). The rate of ICU admission in the CRPA group was also significantly higher than that in the CSPA group ($p = 0.002$).

Table 1. Comparison of antimicrobial susceptibility profiles between CSPA and CRPA isolates

Antibacterial drug	Susceptibility category	CSPA	CRPA	Adjusted p-value
Aztreonam	intermediate	4 (8.0)	26 (27.7)	<0.001
	resistant	3 (6.0)	32 (34.0)	
	susceptible	43 (86.0)	36 (38.3)	
Piperacillin/tazobactam	intermediate	2 (4.0)	25 (26.6)	0.002
	resistant	2 (4.0)	8 (8.5)	
	susceptible	46 (92.0)	61 (64.9)	
Cefepime	intermediate	3 (6.0)	10 (10.6)	0.009
	resistant	2 (4.0)	21 (22.3)	
	susceptible	45 (90.0)	63 (67.0)	
Ceftazidime	intermediate	2 (4.0)	15 (16.0)	0.002
	resistant	2 (4.0)	20 (21.3)	
	susceptible	46 (92.0)	59 (62.8)	
Levofloxacin	intermediate	6 (12.0)	30 (31.9)	<0.001
	resistant	1 (2.0)	28 (29.8)	
	susceptible	43 (86.0)	36 (38.3)	
Ciprofloxacin	intermediate	2 (4.0)	22 (23.4)	<0.001
	resistant	1 (2.0)	32 (34.0)	
	susceptible	47 (94.0)	40 (42.6)	
Amikacin	intermediate	1 (2.0)	2 (2.1)	0.799*
	resistant	0 (0.0)	3 (3.2)	
	susceptible	49 (98.0)	89 (94.7)	
Gentamicin	intermediate	1 (2.0)	5 (5.3)	0.395*
	resistant	0 (0.0)	3 (3.2)	
	susceptible	49 (98.0)	86 (91.5)	

All reported p-values were adjusted using the Benjamini–Hochberg method and refer to the overall comparison of susceptibility-category distributions for each antibiotic; *Fisher's exact test; CRPA – carbapenem-resistant *Pseudomonas aeruginosa*; CSPA – carbapenem-susceptible *Pseudomonas aeruginosa*.

Table 2. Univariate analysis of factors associated with CRPA infection

Variable		CSPA	CRPA	Z/ χ^2	p-value
Age		66.00 (54.75, 72.75)	65.00 (53.00, 73.75)	−0.474	0.635
Sex	Male	32 (64.0)	66 (70.2)	0.579	0.447
	Female	18 (36.0)	28 (29.8)		
Hospital department	Department of surgery	17 (34.0)	42 (44.7)	1.540	0.215
	Internal medicine	33 (66.0)	52 (55.3)		
Underlying diseases	COPD	9 (18.0)	17 (18.1)	0.00	0.990
	Coronary heart disease	3 (6.0)	9 (9.6)	0.178	0.673
	Hypertension	17 (34.0)	25 (26.6)	0.886	0.352
	Diabetes	7 (14.0)	18 (19.1)	0.603	0.437
	Malignant tumor	10 (20.0)	10 (10.8)	2.311	0.128
Ventilation	Invasive mechanical ventilation	10 (20.0)	47 (50.0)	12.283	<0.001
	Noninvasive ventilation	2 (4.0)	13 (13.8)	3.38	0.066
Smoking (yes)		19 (38.0)	39 (41.5)	0.165	0.684
Urinary catheterization (yes)		16 (32.0)	62 (66.0)	15.16	<0.001
Surgery (yes)		14 (28.0)	31 (33.0)	0.377	0.539
Other invasive procedures (yes)		30 (60.0)	79 (84.0)	10.255	0.001
Antibiotic exposure	Penicillins	18 (36.0)	40 (42.6)	0.583	0.445
	Cephalosporins	37 (74.0)	75 (79.8)	0.632	0.426
	Aminoglycosides	3 (6.0)	27 (28.7)	10.218	0.001
	Fluoroquinolones	10 (20.0)	39 (41.5)	6.714	0.010
	Carbapenems	7 (14.0)	50 (53.2)	20.963	<0.001
	Beta-lactams	2 (4.0)	7 (7.4)	0.204	0.651
	Tetracyclines	2 (4.0)	18 (19.1)	6.263	0.012
	Restricted antibiotics	7 (14.0)	31 (33.0)	6.052	0.014
Antifungal drugs		1 (2.0)	13 (13.8)	3.944	0.047
Combination antibiotic therapy		16 (32.0)	56 (59.6)	9.927	0.002
ICU admission (yes)		8 (16.0)	39 (41.5)	9.645	0.002

Age is presented as median (interquartile range (IQR)) and compared using the Mann–Whitney U test. Categorical variables are presented as n (%). COPD – chronic obstructive pulmonary disease; ICU – intensive care unit; CRPA – carbapenem-resistant *Pseudomonas aeruginosa* (CRPA); CSPA – carbapenem-susceptible *Pseudomonas aeruginosa*.

Multivariate logistic regression analysis of CRPA infection risk factors

For robustness, we also fitted a fully adjusted model. Using best-subset selection based on the BIC, the final multivariable logistic regression model retained carbapenem and aminoglycoside exposure as predictors. Both exposures were associated with higher odds of CRPA infection: carbapenems (adjusted odds ratio (aOR) = 6.914; 95% confidence interval (95% CI): 2.765–17.290; $p < 0.001$) and aminoglycosides (aOR = 6.209; 95% CI: 1.701–22.668; $p = 0.006$). Linearity of age in the logit was confirmed (Box–Tidwell test: $p = 0.889$). Influence diagnostics identified a small number of high-influence observations (Cook's distance $>4/n \approx 0.028$); refitting the model after removing the top 1–3 observations produced no material changes in the estimates.

As a sensitivity analysis to confirm the robustness of our findings, a fully adjusted model including all candidate

covariates was also fitted, and the results are summarized in Table 3. In this model, after adjustment for potential confounders, prior exposure to aminoglycosides (adjusted odds ratio (aOR) = 8.90, 95% CI: 1.96–40.46, $p = 0.005$) and carbapenems (aOR = 6.26, 95% CI: 1.68–23.28, $p = 0.006$) remained independent risk factors significantly associated with CRPA infection.

Discussion

Pseudomonas aeruginosa is a significant cause of nosocomial (hospital-acquired) infections, particularly among patients with compromised immune function. In addition, it is one of the leading causes of ventilator-associated pneumonia (VAP).¹¹ Carbapenem antibiotics, such as imipenem, meropenem, and doripenem, have historically played an important role in the treatment of infections

Table 3. Univariate and multivariable logistic regression analyses of risk factors for CRPA infection

Variable	Univariate		Multivariable	
	OR (95% CI)	p-value	aOR (95%CI)	p-value
Age	0.99 (0.97–1.02)	0.536	–	–
Female sex	0.77 (0.37–1.60)	0.472	–	–
Surgical department	1.60 (0.79–3.31)	0.198	–	–
COPD (yes)	1.02 (0.42–2.58)	0.967	–	–
Coronary heart disease (yes)	1.68 (0.47–7.84)	0.454	–	–
Hypertension (yes)	0.71 (0.34–1.51)	0.374	–	–
Diabetes mellitus (yes)	1.47 (0.59–4.05)	0.423	–	–
Malignant tumor (yes)	0.48 (0.18–1.26)	0.134	–	–
Invasive mechanical ventilation (yes)	3.91 (1.81–9.12)	0.001	1.89 (0.29–12.38)	0.508
Noninvasive ventilation (yes)	3.90 (1.02–25.63)	0.081	–	–
Smoking (yes)	1.18 (0.59–2.41)	0.648	–	–
Urinary catheterization (yes)	4.05 (1.98–8.60)	<0.001	2.20 (0.69–7.04)	0.185
Surgery (yes)	1.22 (0.58–2.66)	0.599	–	–
Other invasive procedures (yes)	3.47 (1.58–7.76)	0.002	1.29 (0.45–3.75)	0.638
Penicillins (yes)	1.34 (0.66–2.76)	0.416	–	–
Cephalosporins (yes)	1.37 (0.60–3.06)	0.447	–	–
Aminoglycosides (yes)	6.41 (2.11–27.94)	0.004	8.90 (1.96–40.46)	0.005
Fluoroquinolones (yes)	2.76 (1.27–6.45)	0.014	2.81 (0.88–8.92)	0.080
Carbapenems (yes)	6.84 (2.94–18.05)	<0.001	6.26 (1.68–23.28)	0.006
β -lactams (yes)	1.95 (0.45–13.46)	0.415	–	–
Tetracyclines (yes)	5.76 (1.57–37.27)	0.023	1.91 (0.33–11.02)	0.467
Restricted antibiotics (yes)	2.93 (1.23–7.79)	0.021	0.99 (0.22–4.53)	0.986
Antifungal drugs (yes)	7.26 (1.37–134.28)	0.061	–	–
Combination antibiotic therapy (yes)	3.08 (1.51–6.47)	0.002	0.42 (0.11–1.59)	0.199
ICU admission (yes)	3.63 (1.60–9.11)	0.003	0.39 (0.06–2.59)	0.326

Fully adjusted logistic regression model including all candidate covariates; the primary estimates are derived from the best-subset logistic regression model selected according to the Bayesian information criterion (BIC) reported in the Results section; COPD – chronic obstructive pulmonary disease; ICU – intensive care unit; CRPA – carbapenem-resistant *Pseudomonas aeruginosa*; CSPA – carbapenem-susceptible *Pseudomonas aeruginosa*.

caused by PA, especially in cases in which the bacterium exhibits resistance to other classes of antibiotics. However, the overuse and misuse of carbapenems in both healthcare and agricultural settings have led to the emergence and spread of CRPA. Multidrug-resistant PA (MDRPA) and extensively drug-resistant PA aeruginosa (XDRPA) strains have become increasingly common in healthcare settings worldwide.¹² These strains are resistant not only to carbapenems but also to multiple other classes of antibiotics, making them difficult to treat and posing a significant threat to patients, particularly those who are immunocompromised or critically ill. The problem of antibiotic resistance has become a global crisis. Despite the introduction of several new antibiotics in recent years, including ceftolozane-tazobactam, ceftazidime-avibactam, and imipenem-relebactam, which are recommended by international guidelines for the treatment of severe difficult-to-treat resistant PA (DTRPA) infections,⁸ antimicrobial resistance to these agents has also begun to emerge. This study identified risk factors associated with CRPA

infection through multivariable logistic regression analysis, with the aim of providing evidence to support CRPA intervention and control strategies.

Our analysis of antibiotic susceptibility patterns revealed a concerning trend of co-resistance among CRPA isolates. These strains demonstrated markedly higher resistance rates to aztreonam, cephalosporins, and fluoroquinolones than their carbapenem-susceptible counterparts, thereby exhibiting a multidrug-resistant profile that substantially limits therapeutic options and complicates clinical management. In contrast, the relatively low resistance rates observed for aminoglycosides, including amikacin and gentamicin, suggest that these agents may retain some clinical value against CRPA in our setting. Nevertheless, given their identification as risk factors for CRPA, their use should be approached with caution. A previous study demonstrated that PA is highly susceptible to lipopeptides and aminoglycosides but highly resistant to cephalosporins, fluoroquinolones, and aztreonam.¹³ These findings indicate that CRPA isolates exhibit high levels of resistance to multiple

antibiotics, and the presence of such resistance may increase the difficulty of treatment, particularly when agents such as aztreonam, ciprofloxacin, and levofloxacin are required. For piperacillin/tazobactam, CRPA exhibited significantly higher resistance than CSPA, whereas differences for amikacin and gentamicin were not statistically significant, which has important implications for the development of treatment protocols and preventive measures. Our findings are consistent with national surveillance data, which have reported persistently high resistance rates of CRPA to fluoroquinolones and resistance to aztreonam, reflecting a similar antimicrobial resistance profile across multiple tertiary hospitals in China.¹ International studies have likewise identified prior exposure to carbapenems and aminoglycosides as important risk factors for CRPA infection, lending further support to our findings.¹³ Interestingly, the aminoglycoside resistance rate observed in our study (3.19%) was notably lower than those reported in both national and international datasets, possibly reflecting differences in local antibiotic stewardship strategies, prescribing practices, and infection control measures. In clinical practice, physicians should select appropriate antimicrobial agents based on these data and closely monitor patients' responses to treatment to ensure optimal therapeutic outcomes.

The BIC-selected model identified prior exposure to carbapenems (aOR = 6.914) and aminoglycosides (aOR = 6.209) as independent risk factors; the fully adjusted sensitivity model produced estimates in the same direction (Table 3). In univariate analyses, invasive mechanical ventilation, invasive procedures and ICU admission were also associated with CRPA infection, but these variables were not retained as independent predictors after adjustment. Prior exposure to carbapenems emerged as the most statistically significant predictor (aOR = 6.914, 95% CI: 2.765–17.290, $p < 0.001$), which is consistent with the well-established principle of antibiotic selection pressure. This finding strongly reinforces the need for stringent carbapenem stewardship. In addition, prior exposure to aminoglycosides was also confirmed as a significant risk factor (aOR = 6.209, 95% CI: 1.701–22.668, $p = 0.006$). The wide confidence intervals indicate that the magnitude of these associations should be confirmed in larger multicenter studies.

The predictably higher resistance rates of CRPA strains to multiple antibiotics compared with CSPA strains, as shown in Table 1, are consistent with the definition of carbapenem resistance, which often co-occurs with resistance to other antimicrobial classes. A comparison with national surveillance data from CHINET highlights the severity of resistance in this subgroup. The 2022 CHINET report indicated that the overall resistance rate of PA to ciprofloxacin was 15.3%.¹ This contrasts sharply with the 34.0% ciprofloxacin resistance rate observed among the CRPA isolates in our study. This substantial difference underscores that CRPA strains are not only resistant to carbapenems but also exhibit markedly higher rates of co-resistance to other critical antibiotics, such

as fluoroquinolones, compared with the general PA population. Furthermore, this study was conducted between 2021 and 2022, during the COVID-19 pandemic. This period was characterized by a global increase in the use of broad-spectrum antibiotics, including carbapenems, to treat or prevent secondary bacterial infections in critically ill patients with COVID-19. This intensified antibiotic pressure likely accelerated the selection and spread of resistant pathogens such as CRPA, which may have influenced the incidence and resistance patterns observed in our cohort. However, our institutional COVID-19 treatment protocol did not routinely include carbapenems as first-line therapy, which may have mitigated this effect. Although this retrospective study could not formally compare antibiotic consumption before and during the pandemic, the potential increase in antibiotic pressure during this global health crisis remains an important consideration when interpreting our findings and represents a notable limitation of the study.

A meta-analysis by Raman et al.¹⁴ summarized the risk factors associated with the acquisition of MDRPA, XDRPA, and other DRPA strains. Compared with patients infected with drug-susceptible or less-resistant PA strains, hospitalized patients and those admitted to the ICU exhibited higher rates of CRPA, and the duration of ICU stay was significantly associated with the acquisition of MDRPA or XDRPA, suggesting that ICU stay is a risk factor for CRPA infection.

In a 6-month prospective study conducted in 1997,¹⁵ CRPA isolates in the medical ICU accounted for 22.1% of all isolates, compared with 13.7% in the surgical ICU. These findings suggest that CRPA is more prevalent in medical ICUs. Consistent with these observations, the incidence of CRPA in medical wards was higher than that in surgical wards in our study, which may be attributable to higher rates of antibiotic prescribing in medical departments.

Teelucksingh et al.¹⁶ underscored the importance of invasive procedures, such as central venous catheter insertion and mechanical ventilation, in increasing the risk of PA infection and influencing patient outcomes. In many previous case-control studies,^{17–19} carbapenems, including imipenem and meropenem, have frequently been identified as risk factors for the development of antibiotic resistance in various pathogens, including PA.

Given the established association between carbapenem use and the development of resistance, antimicrobial stewardship programs advocate the prudent use of these antibiotics to preserve their effectiveness and minimize the emergence of resistant strains. This includes appropriate antibiotic prescribing practices, infection control measures, and surveillance of antibiotic-resistant pathogens to guide treatment decisions and mitigate the spread of resistance.

Previous patient-level case-control analyses have reported that prior use of fluoroquinolones²⁰ or amikacin²¹ is associated with the isolation of imipenem-resistant PA. While these observational findings suggest a link between

prior antibiotic exposure and the development of resistance, causality cannot be directly inferred. Consistent with these reports, our multivariable analysis confirmed that prior aminoglycoside exposure is an independent risk factor for CRPA infection. However, further research is needed to establish direct causality and elucidate the underlying mechanisms.

In addition, factors such as antimicrobial stewardship, infection control practices, and bacterial resistance mechanisms should be considered when interpreting these findings and implementing interventions aimed at mitigating the spread of antibiotic-resistant pathogens such as CRPA.

Indeed, retrospective observational studies often have inherent limitations. To reduce their impact, we used a multivariable logistic regression model to adjust for baseline patient characteristics. Second, the relatively small sample size and the single-center nature of the study may have resulted in insufficient matching between cases and controls, potentially affecting the accuracy and reliability of the findings and limiting their generalizability to other settings. Finally, the study may not have included all relevant factors influencing CRPA infection, which may have affected a comprehensive understanding of the infection. Despite these limitations, the study has important clinical implications. By improving our understanding of the resistance patterns and risk factors associated with CRPA, appropriate measures can be implemented to prevent its spread, and more rational antimicrobial stewardship strategies can be developed to improve treatment outcomes. This is important for preventing hospital outbreaks and reducing the occurrence of MDR and pandrug-resistant (PDR) infections. Overall, despite its limitations, this study provides valuable information, offers guidance for the prevention and treatment of CRPA infections, and highlights the importance of effective management of these infections.

A strength of this study is the use of model diagnostics and sensitivity analyses. The final multivariable model was developed using best-subset selection based on the BIC, which helps to avoid overfitting. Furthermore, rigorous model diagnostics were performed, confirming the linearity assumption for continuous variables (Box–Tidwell test, $p = 0.889$) and including influence diagnostics. Sensitivity analysis demonstrated that the removal of high-influence observations did not materially alter the risk estimates, supporting the consistency of the findings.

Limitations of the study

However, several limitations must be acknowledged. First, the retrospective single-center design is susceptible to selection and information bias and limits the generalizability of our findings. Second, the relatively small sample size may have limited the statistical power to detect additional potential risk factors and contributed to the wide CIs observed for the primary findings. A post hoc power analysis was not conducted.

Third, our analysis did not account for the potential clustering of patients within specific wards (e.g., the ICU), which could influence the risk factor estimates; future studies may benefit from the use of multilevel modeling to address this issue. Fourth, there is a risk of omitted-variable bias, as we were unable to account for all potential confounders, such as measures of underlying disease severity.

In addition, although the primary model was selected using the BIC and a fully adjusted model was fitted as a sensitivity analysis, model development may still be affected by the moderate sample size and the limited number of events. Some clinically relevant confounders may therefore have been excluded, and residual model-selection bias cannot be ruled out. Post hoc diagnostics showed acceptable multicollinearity (all variance inflation factor (VIF) values <10) and adequate model fit according to the Hosmer–Lemeshow test. Nevertheless, the potential for selection bias inherent to this approach remains a limitation of the study. Finally, the study period coincided with the COVID-19 pandemic, which may have altered antibiotic prescribing practices and infection control measures, thereby potentially influencing the results. The absence of molecular typing of CRPA strains also precludes further insight into the genetic mechanisms underlying resistance.

Conclusions

This study demonstrates that CRPA infections are particularly prevalent in ICU settings and that prior exposure to carbapenems and aminoglycosides is an independent risk factor. These findings provide important guidance for antimicrobial stewardship programs (ASPs). First, ASPs should incorporate prospective audits with real-time feedback to clinicians regarding the appropriateness of carbapenem and aminoglycoside prescriptions, thereby reducing unnecessary exposure. Second, formulary restrictions or pre-authorization policies for last-line agents should be enforced to preserve their efficacy. Third, cumulative local antibiogram data should be routinely analyzed to develop and update institutional empirical therapy guidelines, thereby supporting more rational initial antibiotic selection and timely de-escalation once susceptibility results become available. Finally, minimizing avoidable invasive procedures and reinforcing infection control bundles are essential strategies for reducing the overall risk of CRPA infection. Collectively, these measures can optimize antibiotic use, limit the development of resistance, and improve patient outcomes.

Data Availability Statement

Data sharing is not applicable to this article, as all data are already included in the manuscript.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.17698555>. The package contains the following files:

Supplementary Table 1. Comparison of commonly used antibiotics in CSPA and CRPA (unadjusted p-value).

Supplementary Table 2. Post hoc tests and p-value adjustment.

Supplementary Table 3. Shapiro–Wilk normality test.

Supplementary Table 4. Multicollinearity diagnostics for variables included in the multivariate model.

Supplementary Table 5. Hosmer–Lemeshow goodness-of-fit test for binary logistic model.

Supplementary Fig. 1. Q–Q plot of age distribution in the CSPA group. This Q–Q plot evaluates whether age in the CSPA group follows a normal distribution. The observed values (x-axis) were plotted against expected normal quantiles (y-axis). Data points align closely with the reference line, indicating no major deviation from normality.

Supplementary Fig. 2. Q–Q plot of age distribution in the CRPA group. This Q–Q plot assesses the normality of age distribution in the CRPA group. Although slight deviations are observed at the lower and upper tails, most points lie close to the diagonal reference line, suggesting acceptable normality for subsequent statistical modeling.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Changes in hip joint anatomical shape as a predictor of osteoarthritis

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Abstract

Background. Hip osteoarthritis (HOA) is one of the leading causes of musculoskeletal disability worldwide, yet its etiology remains incompletely understood. Its pathogenesis is multifactorial, involving systemic factors and biomechanical alterations of the hip joint that trigger degenerative changes.

Objectives. The aim of this study was to identify the most informative radiographic views and the key acetabular and proximal femoral parameters for detecting structural abnormalities of the hip joint that may contribute to the development of HOA.

Materials and methods. This observational case-control study included 156 patients older than 55 years who underwent total hip arthroplasty for HOA (osteoarthritis (OA) group) and 61 age-matched controls without clinical HOA, recruited from a single center between 2019 and 2022. Patient demographics, clinical history, and hip-specific functional scores were collected. Standardized anteroposterior (AP), frog-leg lateral (LL), and Dunn 45° radiographs were obtained, and multiple radiographic parameters were measured.

Results. Asymptomatic contralateral hips in patients with OA had significantly more cam-type deformities (higher alpha angles, lower head–neck offset ratios, and a higher prevalence of pistol-grip deformity) than those in the control group ($p < 0.001$). In contrast, pincer-type features were more common in the control group. Logistic regression analysis identified higher alpha angles, greater femoral neck diameter, and lower head–neck offset ratios as predictors of OA, whereas a larger femoral head diameter was identified as a protective factor (model area under the curve (AUC) = 0.90). Bone quality was superior in the control group, with longer femoral neck axis lengths and higher Singh indices ($p < 0.001$).

Conclusions. Radiographic markers of cam-type femoroacetabular impingement (FAI) are significantly more prevalent in patients with HOA and may serve as early indicators of disease risk. Dunn and LL views are optimal for detecting these abnormalities. A larger femoral head diameter, as measured on both AP and LL radiographic views, appears to be a protective factor against the development of HOA. Understanding these morphological differences may facilitate earlier diagnosis and support preventive strategies for HOA.

Key words: osteoarthritis, impingement, hip joint shape, radiographic measurements, Dunn view

Cite as

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Highlights

- Cam-type femoroacetabular impingement (FAI) markers, including higher alpha angles and lower head–neck offset ratios, were more common in patients with hip osteoarthritis (HOA) and were associated with HOA group status.
- The largest between-group differences in alpha angle were observed on Dunn 45° and frog-leg lateral (LL) radiographs, suggesting that these views may be particularly informative for assessing cam-type morphology.
- In multivariable analysis, a higher LL alpha angle, greater anteroposterior (AP) femoral neck diameter, and a Dunn head–neck offset ratio <0.17 were associated with higher odds of HOA, whereas a larger LL femoral head diameter was associated with lower odds of HOA (AUC = 0.90).
- Controls had longer femoral neck axis lengths and higher Singh indices, whereas the Cortical Thickness Index was slightly higher in the HOA group, indicating differences in proximal femoral morphology and radiographic bone characteristics between the groups.

Background

Hip osteoarthritis (HOA) is a degenerative joint disease and one of the leading causes of musculoskeletal disability in developed countries. Although osteoarthritis (OA) can affect any joint in the body, it most commonly affects the knee and hip joints.¹ Hip osteoarthritis has a variable prevalence worldwide, ranging from 1.20% in Africa to 12.59% in Europe.² Currently, the onset of HOA cannot be reliably predicted, nor can its progression be completely prevented.

Hip osteoarthritis is classified as either primary (idiopathic), accounting for approx. 80% of all HOA cases, or secondary, resulting from conditions such as inflammatory, infectious, or metabolic diseases, trauma, osteonecrosis, or developmental abnormalities of the hip.³ The pathogenesis of HOA is not yet fully understood. It is widely accepted that HOA is a multifactorial disease resulting from the interaction of systemic risk factors, such as age, ethnicity, sex, heredity, and obesity, with local factors related to hip joint biomechanics. Systemic factors increase susceptibility to OA, whereas local biomechanical alterations of the hip joint may trigger a cascade of pathological changes that ultimately lead to the development of HOA.^{4,5}

Since the pathological process in patients with HOA usually does not involve other joints, it was hypothesized as early as the 1970s that many cases previously considered primary OA are, in fact, secondary to subtle morphological or structural abnormalities of the proximal femur and/or the acetabulum.^{6,7} Femoroacetabular impingement (FAI) is now increasingly recognized as a major contributor to HOA.^{7,8} It is classified into cam, pincer, and mixed types. Cam-type FAI is characterized by an aspherical femoral head that abrades the acetabular cartilage and labrum, resulting in cartilage damage, labral tears, chronic inflammation at the site of injury, and ultimately the development of HOA.⁹ In pincer-type FAI, the femoral head is excessively covered by the acetabular rim, resulting in abnormal contact between the acetabular rim and the femoral head–neck junction. This leads to labral tears, progressive ossification, cartilage degeneration, and ultimately OA.¹⁰

Murray's epidemiological study suggested that 25–40% of HOA cases are associated with mild acetabular dysplasia.¹¹ In dysplasia, a shallow and more vertically oriented acetabulum reduces femoral head coverage and increases mechanical stress on the cartilage and labrum, thereby promoting degeneration and the development of OA.¹²

Early detection of these morphological abnormalities is essential for timely preventive or surgical intervention. Despite the availability of advanced imaging modalities, such as magnetic resonance imaging (MRI) and computed tomography (CT), conventional pelvic and hip radiographs remain the primary diagnostic tool for detecting HOA.¹³ Several radiographic measurements have been proposed to identify structural abnormalities; however, their reliability remains a matter of debate.¹⁴

Objectives

The aim of this study was to determine which radiographic views and specific acetabular and proximal femoral parameters are most effective for detecting structural hip abnormalities associated with the development of HOA.

Materials and methods

This observational case-control study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the National Central Medical Ethics Committee of Latvia (Riga, Latvia; approval No. 4/21-02-17). All participants provided written informed consent before enrollment. Patients who underwent total hip arthroplasty (THA) for HOA between 2019 and 2022 were included in the OA group, whereas volunteers or patients hospitalized for nonarthritic conditions at the same center were included in the control group. The following data were collected for each participant: sex, age, body mass index (BMI), history of sports participation, smoking status, family history of HOA, duration of hip pain (in years), pain

intensity assessed using the Numeric Rating Scale (at rest, during walking, and after walking 500 m), and hip function assessed using the American Academy of Orthopedic Surgeons (AAOS) Hip and Knee Questionnaire.

All participants underwent calibrated radiographic imaging in 3 projections: anteroposterior (AP) pelvis, frog-leg lateral (LL), and Dunn 45° views. The severity of OA was assessed using the Tönnis classification: Grade 0 – no radiographic signs of OA; Grade I – mild joint space narrowing, marginal osteophyte formation, and slight subchondral sclerosis; Grade II – subchondral cysts, further joint space narrowing, and moderate loss of femoral head sphericity; and Grade III – large subchondral cysts, obliteration of the joint space, severe deformity, and avascular necrosis.¹⁵

The more widely used Kellgren–Lawrence classification was originally developed to grade the severity of knee OA and was subsequently applied to other joints. In contrast, the Tönnis classification was specifically designed for the hip, aligns with hip morphology, and more accurately reflects the radiographic features of hip osteoarthritis on AP pelvic radiographs. Therefore, given our focus on hip pathomorphology, we used the Tönnis classification in this study.

For the OA group, the nonoperated hip was evaluated clinically and radiographically as a surrogate for the predeformity status of the operated hip. Inclusion criteria for the OA group were: 1) planned unilateral THA for primary osteoarthritis; 2) Tönnis grade II or lower OA in the contralateral study hip; 3) no evidence of secondary causes of OA (e.g., posttraumatic osteoarthritis, rheumatic disease, avascular necrosis of the femoral head, or developmental hip dysplasia according to the Crowe classification¹⁶); and 4) age older than 55 years. Patients with Tönnis grade III OA of the nonoperated hip were excluded because severe OA is associated with marked joint deformity, making reliable radiographic assessment of morphological parameters impossible. Inclusion criteria for the control group were: 1) no history of hip pain; 2) no radiographic signs of HOA on pelvic radiographs; and 3) age older than 55 years. The age criterion was based on epidemiological data indicating that the incidence of HOA increases after 55 years of age, with a decline after 65 years.¹⁷ Individuals younger than 55 years without symptoms or radiographic signs of HOA are less likely to develop the disease.

In total, 156 patients were included in the OA group after the exclusion of 32 patients with bilateral Tönnis grade III HOA, and 61 participants were included in the control group after the exclusion of 7 individuals with radiographic evidence of HOA.

To evaluate hip joint anatomy in multiple planes, pelvic and hip radiographs were obtained in 3 projections. An AP pelvic radiograph was acquired with the patient in the supine position and both legs internally rotated by 15°. For the lateral projection, the LL view was obtained with the patient in the supine position, the knee flexed to 30°, and the hip abducted to 45°, with the heel resting against

the medial aspect of the contralateral knee.¹⁸ The Dunn 45° view was obtained with the patient in the supine position, the affected hip flexed to 45°, abducted by 20°, and maintained in a neutral rotational position.⁴ In the OA group, radiographic parameters were analyzed in the nonoperated hip because it was considered to best reflect the morphology of the operated hip before the development of advanced osteoarthritic changes. In the control group, the hip selected for analysis was chosen by randomization. All radiographs were independently evaluated by 2 specialists (a radiologist and an orthopedic surgeon). In cases of disagreement or uncertainty (e.g., regarding the presence of a radiographic feature), the images were reviewed jointly until a consensus was reached. This procedure ensured consistency and minimized measurement variability. Using AGFA IMPAX 6 PACS Client v. 6.6 software (Agfa HealthCare, Mortsel, Belgium), the following parameters and radiographic features were assessed on AP pelvic radiographs: hip axis length, femoral neck axis length, femoral head diameter, femoral neck diameter, neck–shaft angle (NSA), femoral shaft width, acetabular depth, acetabular index (AI), acetabular morphology (normal, coxa profunda, or acetabular protrusion), center-edge angle, crossover sign, alpha (α) angle, pistol-grip deformity, and bone quality assessed using the Singh Index and Cortical Thickness Index (CTI). On Dunn and LL radiographs, femoral head diameter, femoral neck diameter, alpha angle, and the head–neck offset ratio were measured. Statistical analyses were performed using Jamovi software v. 2.5 (<https://www.jamovi.org>).

Radiological parameters

Hip axis and femoral neck axis length

Hip axis length was measured on AP radiographs as the distance from the inner pelvic brim to the lateral edge of the greater trochanter, measured along the femoral neck axis.¹⁹ Femoral neck axis length was defined as the distance from the lateral base of the greater trochanter to the apex of the femoral head on AP radiographs (Fig. 1).

Femoral head and neck diameters

Femoral head and neck diameters were measured on AP, LL, and Dunn 45° radiographs to compare proximal femoral morphology between the study groups (Fig. 2,3). Femoral neck diameter was defined as the minimum width of the femoral neck.

Alpha angle and head–neck offset ratio

The alpha angle was measured on all 3 radiographic views using a best-fit circle of the femoral head. It was defined as the angle between the femoral neck axis and a line extending from the center of the femoral head to the point where the femoral head–neck junction extends beyond

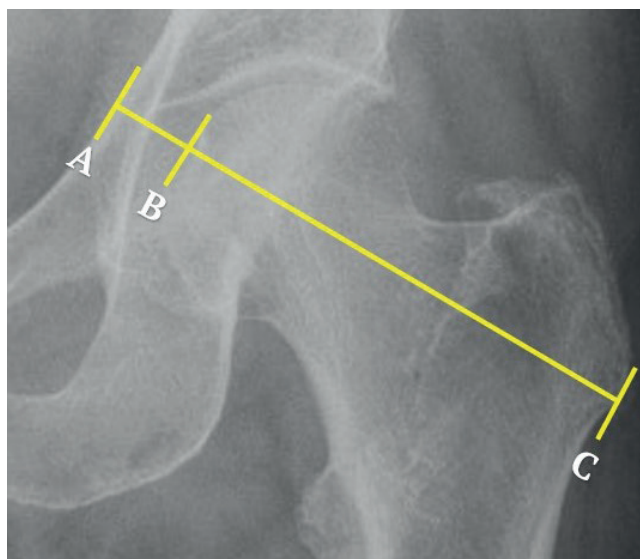


Fig. 1. Hip axis length (A–C) and femoral neck axis length (B,C)

the contour of the best-fit circle¹¹ (Fig. 4). An alpha angle greater than 50° was considered abnormal.

Femoral head–neck offset ratio

The femoral head–neck offset ratio was measured on LL and Dunn 45° radiographs using 3 parallel lines¹¹

(Fig. 5). The first line was drawn longitudinally along the axis of the femoral neck. The 2nd and 3rd lines were drawn parallel to the 1st line: one tangential to the anterior (or posterior) aspect of the femoral neck and the other tangential to the corresponding aspect of the femoral head. The offset was defined as the perpendicular distance between the 2nd and 3rd lines. The head–neck offset ratio was calculated by dividing this distance by the femoral head diameter. This measurement reflects the anterior concavity of the femoral head–neck junction and helps identify cam-type deformities.

Pistol-grip deformity

The presence of a pistol-grip deformity on AP radiographs indicates an abnormal morphology of the proximal femur, in which the femoral head–neck junction loses its normal concavity and assumes the appearance of a pistol grip.²⁰ (Fig. 6).

Acetabular morphology

On an AP pelvic radiograph, acetabular morphology was classified by evaluating the relationship between the floor of the acetabular fossa and the ilioischial line.¹³ A normal acetabulum was defined when the acetabular fossa was located lateral to the ilioischial line; coxa profunda was defined when the acetabular fossa was in contact with the ilioischial line;

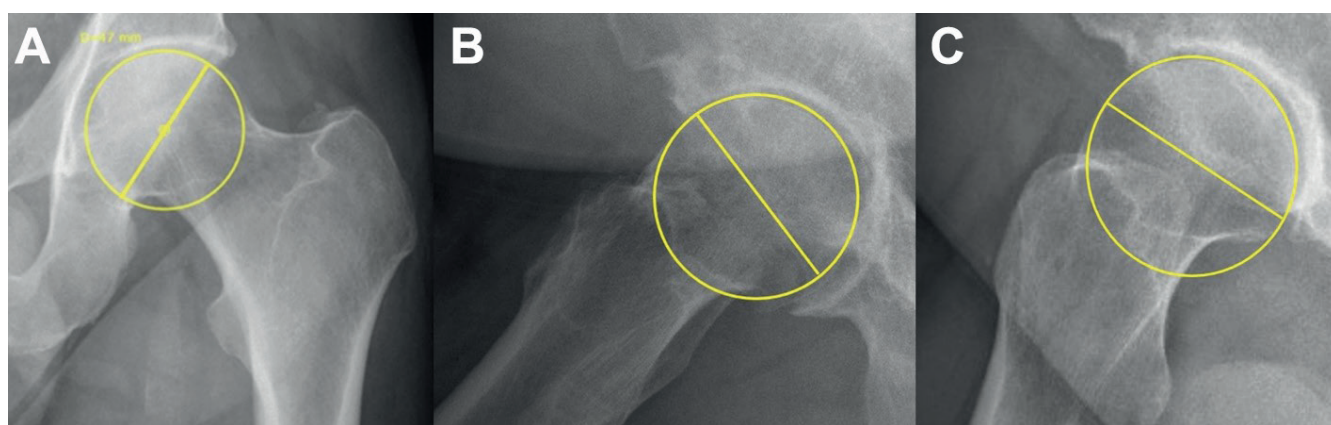


Fig. 2. Femoral head diameter measurement in hip anteroposterior (AP) (A), Dunn (B), and lateral (C) projections

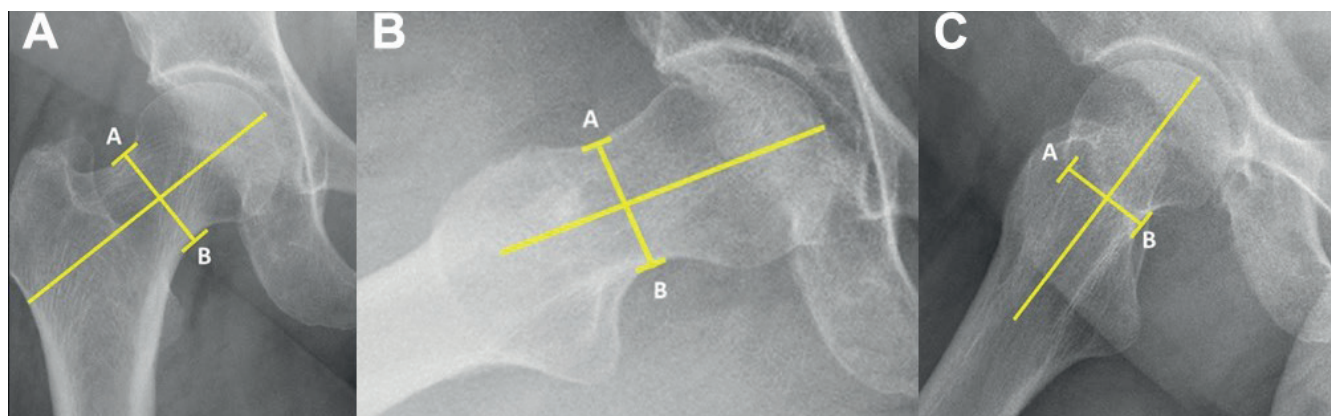


Fig. 3. Femoral neck diameter measurement in hip anteroposterior (AP) (A), Dunn (B), and lateral (C) projections

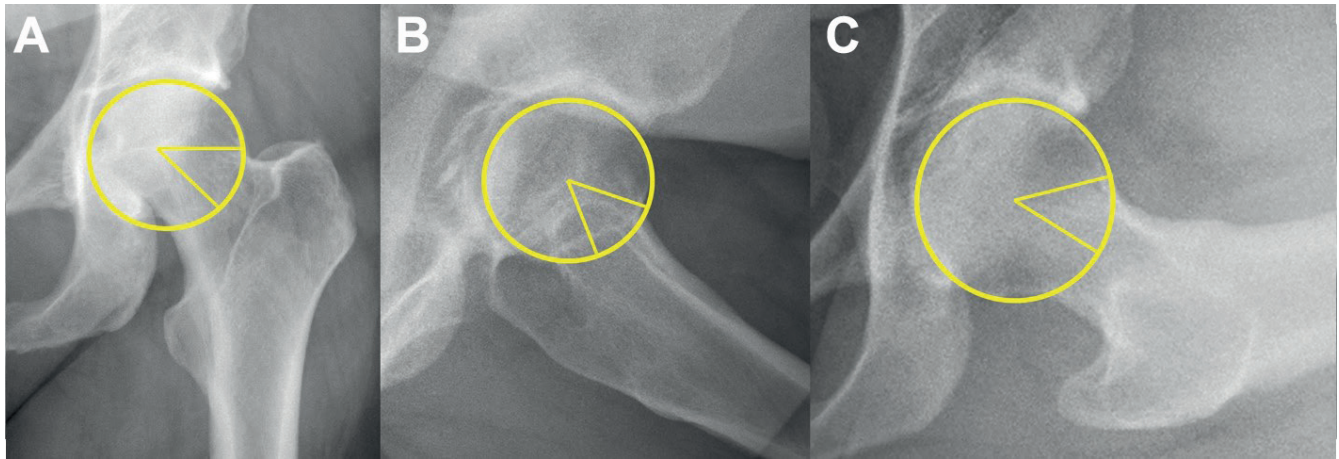


Fig. 4. Alpha angle measurement in hip anteroposterior (AP) (A), lateral (B), and Dunn (C) projections

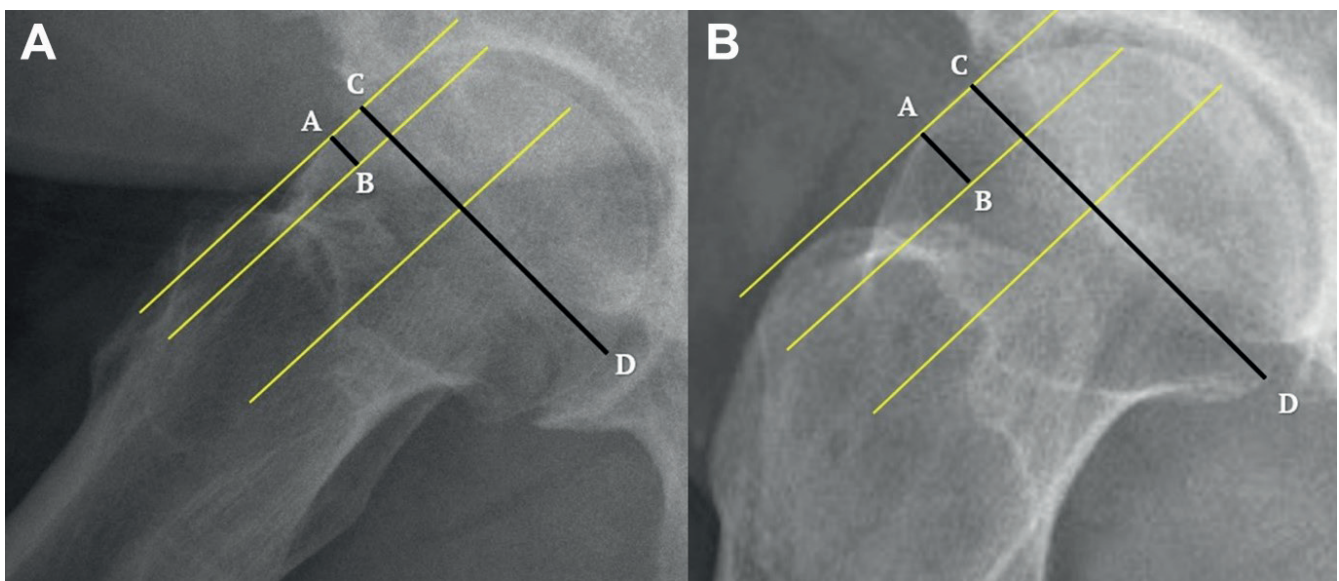


Fig. 5. Head–neck offset ratio measurement in Dunn (A) and lateral (B) projections

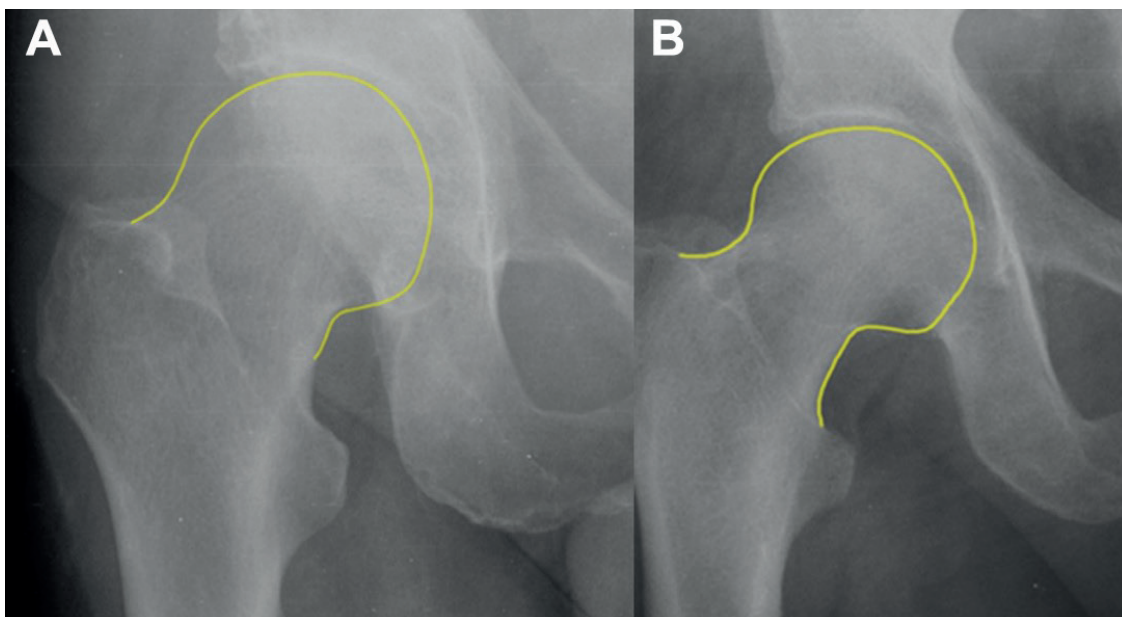


Fig. 6. Positive (A) and negative (B) pistol-grip deformity on anteroposterior hip radiographs

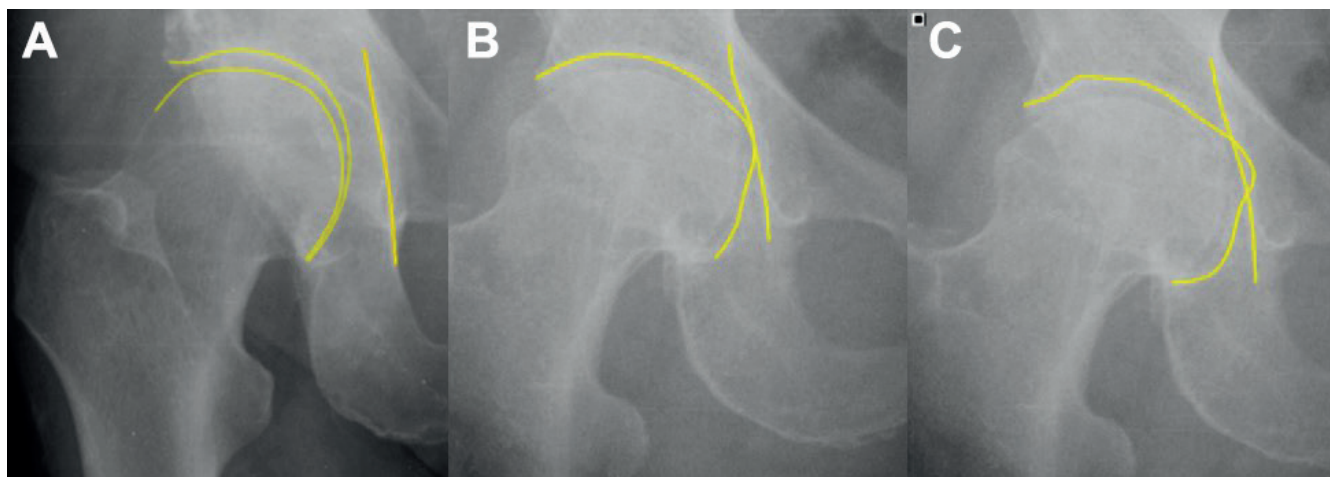


Fig. 7. Acetabular morphology variants. A. Normal variant; B. Coxa profunda; C. Acetabular protrusion

and acetabular protrusion was defined when the acetabular fossa extended medial to the ilioischial line (Fig. 7). Although the severity of acetabular protrusion can be graded using the Sotelo–Garza and Charnley classification, this assessment was beyond the scope of the present study.

Acetabular depth and acetabular index

To evaluate acetabular morphology and detect subtle features of hip dysplasia, acetabular depth and the AI were measured on AP pelvic radiographs (Fig. 8). First, a reference line (AB) was drawn between the superior margin of the pubic symphysis and the lateral edge of the acetabulum. Acetabular depth was defined as the perpendicular distance from this reference line to the deepest point of the acetabular fossa (CD). The AI was calculated as the angle between a line extending from the lateral edge of the acetabulum to the inferior margin of the teardrop and a horizontal reference line.²¹ Values greater than 42° were considered indicative of hip dysplasia.

Center-edge angle

The center-edge angle (CEA) quantifies acetabular coverage of the femoral head on AP hip radiographs. It is measured using 2 lines originating from the center of the femoral head: one extending vertically along the longitudinal axis of the pelvis and the other extending to the lateral edge of the acetabular rim (Fig. 9). A normal CEA ranges from 25° to 39° . Hip dysplasia is indicated by a CEA of less than 25° , whereas acetabular overcoverage is indicated by a CEA greater than 39° (Fig. 9A).

Neck–shaft angle

The NSA was defined as the angle between the longitudinal axes of the femoral shaft and femoral neck on AP radiographs. The axes were drawn through the midpoints of the femoral shaft and the femoral head–neck axis¹³ (Fig. 9B). The normal femoral NSA ranges from 120° to 140° . Values greater than 140° indicate coxa valga, whereas values less than 120° indicate coxa vara.

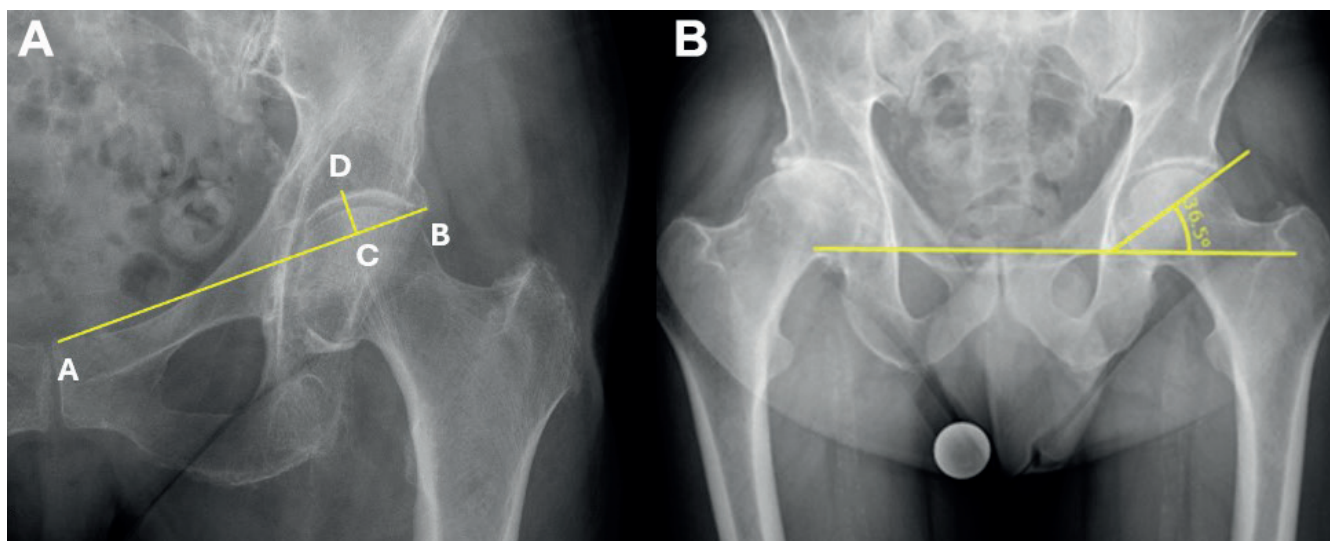


Fig. 8. Acetabular depth (A) and acetabular index (B)

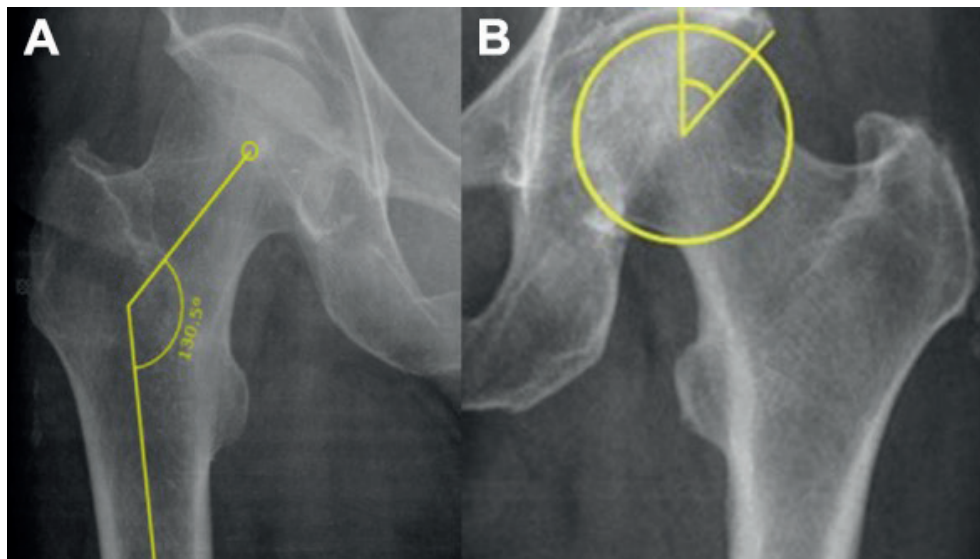


Fig. 9. Center-edge angle (A) and neck-shaft angle (B)

Crossover sign

The crossover sign was identified on AP radiographs when the outlines of the anterior and posterior acetabular walls intersected, creating a figure-of-eight appearance indicative of acetabular retroversion and pincer-type femoroacetabular impingement (FAI).¹³ (Fig. 10)

Bone quality and cortical indices

Proximal femoral bone quality was evaluated on AP pelvic radiographs using the Singh Index, the CTI, and measurements of femoral shaft width. The Singh Index is a simple radiographic classification system for assessing osteoporosis on AP pelvic radiographs.²² It evaluates the visibility of trabecular patterns in the femoral neck and proximal femur to estimate bone quality. The index ranges from grade I (loss of nearly all trabecular patterns,

indicating severe osteoporosis) to grade VI (all trabecular groups are visible, indicating normal bone structure).

Femoral shaft width was measured 10 cm below the midpoint of the lesser trochanter on AP pelvic radiographs. The CTI was calculated at the same level using the following formula: (femoral shaft width – intramedullary canal width)/femoral shaft width. The CTI reflects the relative thickness of the cortical bone of the femoral shaft.

Statistical analyses

Data distribution was assessed by visual inspection of normal Q–Q plots and the Shapiro–Wilk test. Homogeneity of variances was assessed using Levene’s test. Depending on data distribution and variance homogeneity, the independent-samples t test, Mann–Whitney U test, Welch’s t test, χ^2 test of homogeneity, or Fisher–Freeman–Halton

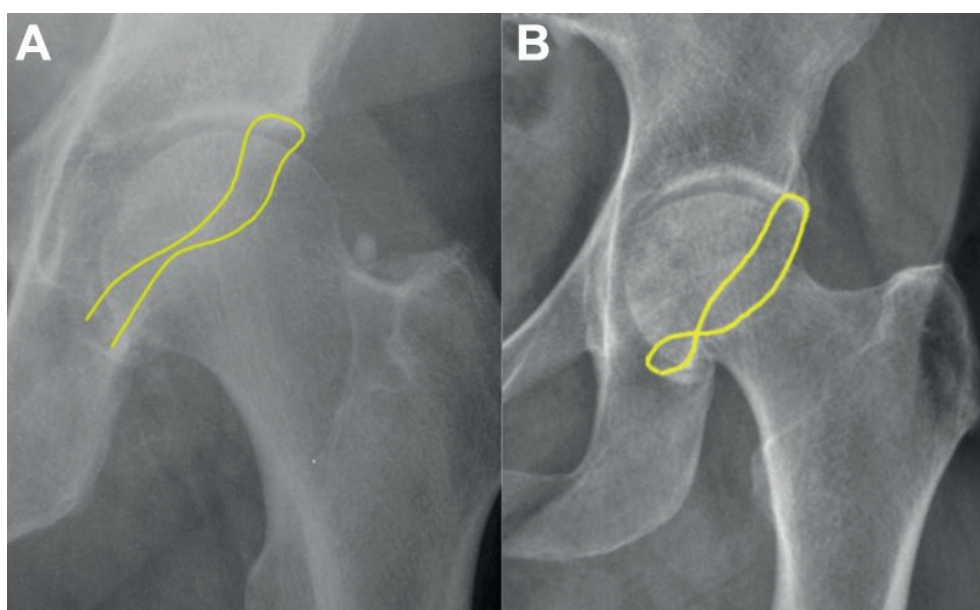


Fig. 10. Crossover sign: negative (A) and positive (B) findings

test was used to compare demographic, clinical, and radiographic characteristics between the control and OA groups. Binary logistic regression was performed to identify demographic and clinical factors (Table 1) associated with the presence or absence of OA. A best-subset selection approach was used to construct the regression model. All possible combinations of predictor variables were generated and compared using the Akaike Information Criterion (AIC) to identify the model that best balanced explanatory power and parsimony. The model with the lowest AIC value was selected as the final model for interpretation.

The final regression model was validated by assessing the key assumptions of logistic regression. First, the Box–Tidwell procedure was used to test the linearity of the relationship between continuous independent variables and the logit of the dependent variable. No significant violations were detected (all $p > 0.05$), confirming that this assumption was met. Second, variance inflation factors (VIFs) were calculated for all independent variables to assess multicollinearity; all VIF values were <5 . Additionally, receiver operating characteristic (ROC) curve analysis was performed to evaluate the discriminative performance of the model, with the area under the curve (AUC) used as a measure of classification accuracy. AUC values greater than 0.70 were interpreted as indicating good discrimination between patients with and without OA. Youden's index was used to identify the optimal cutoff point. All statistical analyses were conducted using Jamovi software (v. 2.5) (<https://www.jamovi.org>). Statistical significance was set at $p < 0.05$.

Results

Demographic and clinical characteristics of the study population are summarized in Table 1. A total of 217 participants were included, comprising 61 individuals in the control group and 156 in the OA group. The proportion of male participants was higher in the OA group (35.9%) than in the control group (21.3%), whereas women predominated in both groups (64.1% and 78.7%, respectively). Mean age did not differ significantly between the groups (OA group: 65.8 years; control group: 65.3 years; $p = 0.708$). The prevalence of comorbidities (type 2 diabetes, thyroid disorders, and gout) was similar between the groups ($p = 0.802$), as was mean body mass index (BMI) (30.6 vs 30.4 kg/m²; $p = 0.915$). Smoking prevalence was higher in the OA group (28.9%) than in the control group (20.0%), although this difference was not statistically significant ($p = 0.215$). Among smokers, the median duration of smoking did not differ significantly between the groups. Clinically, the OA group reported significantly greater pain and functional impairment. Median pain scores were higher under all conditions – at rest, during movement, and after walking – for both hips ($p < 0.001$). The mean AAOS Hip Questionnaire score was significantly higher

in the OA group (34.9) than in the control group (20.1; $p < 0.001$), indicating greater functional impairment.

The distribution of the evaluated hip side was similar between the groups. In the OA group, the mean duration of hip pain before arthroplasty was 4.2 ± 2.9 years. The median Tönnis grade was 3 for the hip scheduled for arthroplasty and 1 for the contralateral study hip. No significant difference between the groups was found in the family history of OA ($p = 0.443$). In the OA group, asymptomatic contralateral hips demonstrated a significantly higher prevalence of cam-type deformities than those in the control group. Detailed findings are presented in Table 2. An alpha angle $>50^\circ$, used as the diagnostic threshold for cam-type FAI, was observed more frequently in the OA group across all 3 radiographic projections: AP, LL, and Dunn 45° views. The mean alpha angle in the AP view was significantly greater in the OA group compared to controls (60.1° vs 52.8° , $p < 0.001$), with a moderately strong effect size (Cohen's $d = 0.77$). In the Dunn and LL projections, the effect sizes were even stronger (Dunn: 53.5° vs 46.6° , Cohen's $d = 0.95$; LL: 58.4° vs 47.9° , Cohen's $d = 1.27$; both $p < 0.001$), indicating that these views most effectively captured cam-type abnormalities. Across all projections, the presence of an alpha angle $>50^\circ$ was strongly associated with the OA group (all $p < 0.001$; Cramér's $V > 0.35$).

Head–neck offset ratios were significantly lower in the OA group than in the control group on both Dunn ($p < 0.001$) and LL ($p = 0.044$) radiographs, further supporting the higher prevalence of cam-type morphology in the OA group. In addition, pistol-grip deformity was observed more frequently in the OA group (26.3%) than in the control group (3.3%), with a moderate association (Cramér's $V = 0.26$, $p < 0.001$).

Femoral head and neck diameters were similar between the groups in the LL and Dunn projections. However, the AP view revealed a slightly larger mean femoral head diameter in the control group than in the OA group (46.3 mm vs 44.9 mm; $p = 0.034$). Femoral neck axis length was significantly greater in the control group than in the OA group (97.9 mm vs 93.2 mm; $p < 0.001$).

A binary logistic regression model evaluating proximal femoral morphology was statistically significant ($\chi^2(4) = 48.9$, $p < 0.001$) and explained 60% of the variance in OA status (Nagelkerke's $R^2 = 0.60$). The model correctly classified 82% of cases (AUC = 0.90). A larger alpha angle on the LL view ($B = 0.19$, $p < 0.001$, odds ratio (OR) = 1.21), greater femoral neck diameter on the AP view ($B = 0.26$, $p = 0.046$, OR = 1.29), and a head–neck offset ratio <0.17 on the Dunn view ($B = 1.33$, $p = 0.037$, OR = 3.78) were independently associated with an increased likelihood of OA, whereas a larger femoral head diameter on the LL view ($B = -0.33$, $p = 0.005$, OR = 0.72) was independently associated with a lower likelihood of OA (Supplementary Table 1).

These results indicate that a 1° increase in the alpha angle on the LL view was associated with a 21% increase in the odds of OA (95% confidence interval (95% CI):

Table 1. Demographic and clinical characteristics of the control and osteoarthritis (OA) groups

Parameter		Control group	OA group	p-value
Number of patients		61	156	N/A
Sex, n (%)***	male	13 (21.3)	56 (35.9)	0.038
	female	48 (78.7)	100 (64.1)	
Age [years], mean (SD)**		65.3 (8.9)	65.8 (8.3)	0.708
BMI [kg/m ²], mean (SD)**		30.4 (6.7)	30.6 (6.4)	0.915
Comorbidities, n/N (%)****	no	39/50 (78.0)	108/151 (71.5)	0.802
	diabetes	5/50 (10.0)	22/151 (14.6)	
	thyroid	3/50 (6.0)	12/151 (7.9)	
	gout	2/50 (4.0)	6/151 (4.0)	
	hepatitis	1/50 (2.0)	1/151 (0.7)	
	Lyme disease	0/50 (0.0)	2/151 (1.3)	
Sports activities, n/N (%)****	no	37/50 (74.0)	115/152 (75.7)	0.954
	athletics	2/50 (4.0)	7/152 (4.6)	
	football	1/50 (2.0)	4/152 (2.6)	
	basketball	1/50 (2.0)	5/152 (3.3)	
	dance	0/50 (0.0)	1/152 (0.7)	
	wrestling	4/50 (8.0)	11/152 (7.2)	
	other	5/50 (10.0)	9/152 (5.9)	
Smoking, n/N (%)***	no	40/50 (80.0)	108/152 (71.1)	0.215
	yes	10/50 (20.0)	44/152 (28.9)	
Duration of smoking [years], median (IQR)*		17.5 (10–30.5)	15 (10–30)	0.966
Who had OA of the hip joint, n/N (%)****	no one	45/50 (80.0)	133/152 (87.5)	0.443
	parents	2/50 (4.0)	11/152 (7.2)	
	brother/sister	1/50 (2.0)	3/152 (2.0)	
	children	0/50 (0.0)	2/152 (1.3)	
	grandparents	2/50 (4.0)	1/152 (0.7)	
	other	0/50 (0.0)	2/152 (1.3)	
Side of the hip joint to be replaced, n/N (%)	right	–	82/152 (53.9)	N/A
	left	–	70/152 (46.1)	
Duration of hip pain [years], median (IQR)		0	4 (2–5)	NA
Right hip pain at rest, median (IQR)*		0 (0–0)	1 (0–3)	<0.001
Left hip pain at rest, median (IQR)*		0 (0–0)	0 (0–3)	<0.001
Pain in the right hip during movement, median (IQR)*		0 (0–0)	4 (0–6)	<0.001
Pain in the left hip during movement, median (IQR)*		0 (0–0)	4 (0–6)	<0.001
Pain in right hip after movement, median (IQR)*		0 (0–0)	6 (0–8)	<0.001
Pain after walking 500 m, median (IQR)*		0 (0–0)	4 (0–7.25)	<0.001
AAOS, mean (SD)**		20.1 (4.8)	34.9 (6.5)	<0.001
Side of the hip joint being studied, n (%)***	right	30 (49.2)	78 (50.0)	0.914
	left	31 (50.8)	78 (50.0)	
The degree of OA of the hip joint to be replaced, median (IQR)*		–	3 (2–3)	N/A
The degree of OA of the hip joint being studied, median (IQR)*		–	1 (1–1)	N/A

AAOS – American Academy of Orthopedic Surgeons; BMI – body mass index; OA – osteoarthritis; n – number; SD – standard deviation; N/A – not applicable; Q1–Q3 – interquartile range (IQR; the 1st and 3rd quartile); *Mann–Whitney U test; **independent samples t test; *** χ^2 test of homogeneity, ****Fisher–Freeman–Halton test. For variables with missing data, categorical data are presented as n/N (%), where N represents the number of participants with available data for the respective variable.

Table 2. Radiographic measurements and findings in the control and osteoarthritis (OA) groups

Parameter		Control group, n = 61	OA group, n = 156	p-value	d
		mean (SD)	mean (SD)		
Neck–shaft angle, °*		126.7 (6.5)	130.1 (6.7)	<0.001	0.51
CTI*		0.59 (0.07)	0.61 (0.06)	0.032	0.32
Acetabular depth*		10.4 (2.6)	9.1 (2.5)	<0.001	0.54
Acetabular index*		37.2 (3.9)	35.6 (4.8)	0.029	0.33
Center-edge angle*		36.9 (6.4)	38.1 (9.6)	0.419	N/A
Alpha angle in AP projection*		52.8 (8.3)	60.1 (9.9)	<0.001	0.77
Alpha angle in Dunn projection*		46.6 (6.2)	53.5 (7.7)	<0.001	0.95
Alpha angle in LL projection*		47.9 (6.7)	58.4 (9.2)	<0.001	1.27
Head-neck offset ratio in Dunn projection*		0.18 (0.03)	0.15 (0.04)	<0.001	0.83
Head-neck offset ratio in LL projection*		0.19 (0.04)	0.16 (0.05)	0.044	0.48
Femoral head diameter in LL projection*		48.9 (5.6)	47.4 (4.3)	0.145	N/A
Femoral neck diameter in LL projection*		29.8 (4.5)	29.5 (4.3)	0.781	N/A
Femoral head diameter in Dunn projection*		46.9 (4.5)	45.7 (4.1)	0.102	N/A
Femoral neck diameter in Dunn projection*		29.3 (4.1)	28.9 (4.4)	0.595	N/A
Femoral head diameter in AP projection*		46.3 (5.1)	44.9 (4.2)	0.034	0.32
Femoral neck diameter in AP projection*		33.0 (4.5)	33.0 (3.7)	1.000	N/A
Femoral neck axis length*		97.9 (10.5)	93.2 (7.8)	<0.001	0.54
Shaft width*		31.9 (3.7)	29.9 (2.8)	<0.001	0.69
Singh index*		4 (1)	3 (1)	<0.001	0.96
Hip axis length*		114.3 (12.8)	112.1 (10.5)	0.178	N/A
Categorical radiographic morphological parameters		n (%)	n (%)	p-value	V
Pistol-grip deformity**	no	59 (96.7)	115 (73.7)	<0.001	0.26
	yes	2 (3.3)	41 (26.3)		
Crossover sign**	no	42 (68.9)	90 (57.7)	0.130	N/A
	yes	19 (31.1)	66 (42.3)		
Neck–shaft angle variant**	normal variant	46 (75.4)	114 (73.1)	0.066	N/A
	coxa vara	9 (14.8)	11 (7.1)		
	coxa valga	6 (9.8)	31 (19.9)		
CTI < 0.50**	no	56 (91.8)	149 (95.5)	0.351	N/A
	yes	5 (8.2)	6 (3.8)		
Acetabular index type***	normal variant	61 (100.0)	154 (98.7)	1.000	N/A
	acetabular dysplasia	0	2 (1.3)		
Center-edge angle categories**	normal variant	39 (63.9)	75 (48.1)	0.055	N/A
	dysplasia	1 (1.6)	12 (7.7)		
	acetabular overcoverage	21 (34.4)	69 (44.2)		
Acetabular morphology variant**	normal	19 (31.1)	105 (67.3)	<0.001	N/A
	coxa profunda	31 (50.8)	42 (26.9)		
	acetabular protrusion	11 (18.0)	9 (5.8)		
Alpha angle >50° in AP projection**	no	28 (45.9)	18 (11.5)	<0.001	0.38
	yes	33 (54.1)	138 (88.5)		
Alpha angle >50° in Dunn projection**	no	42 (75.0)	46 (37.7)	<0.001	0.35
	yes	14 (25.0)	76 (62.3)		
Alpha angle >50° in LL projection**	no	25 (69.4)	9 (17.3)	<0.001	0.53
	yes	11 (30.6)	43 (82.7)		

SD – standard deviation; d – Cohen's d (effect size); V – Cramér's V; N/A – not applicable; *independent samples T test; ** χ^2 test of homogeneity; ***Fisher–Freeman–Halton test; CTI – Cortical Thickness Index; AP – anteroposterior; LL – frog-leg lateral. Bold p-values indicate statistically significant differences between groups, defined as $p < 0.05$.

10–34%). Similarly, a 1-mm increase in femoral neck diameter on the AP view was associated with a 30% increase in the odds of OA (95% CI: 0.5–68%). Patients with a head–neck offset ratio <0.17 on the Dunn view had 3.78-fold higher odds of OA than those with a head–neck offset ratio $\geq$ 0.17 (95% CI: 1.09–13.18). In contrast, a 1-mm increase in femoral head diameter on the LL view was associated with a 28% reduction in the odds of OA (95% CI: 9–43%).

Youden's index identified optimal cutoff values of 52.6 for the alpha angle on the LL view and 29.5 mm for femoral neck diameter on the AP view, above which the likelihood of OA was increased. In contrast, a femoral head diameter of less than 43.5 mm on the LL view was associated with an increased likelihood of OA.

The ROC analysis showed that each 1° increase in the alpha angle on the LL view was associated with a 21% increase in the odds of OA. Furthermore, individuals with a head–neck offset ratio <0.17 on the Dunn view had 3.78-fold higher odds of OA (95% CI: 1.09–13.18) than those with a head–neck offset ratio $\geq$ 0.17.

Evaluation of acetabular morphology showed that acetabular protrusion and coxa profunda were more common in the control group on AP radiographs ($p < 0.001$), both representing features of pincer-type FAI. Acetabular depth and acetabular index, indicative of pincer morphology, were also significantly greater in the control group ($p < 0.001$ and $p = 0.029$, respectively). Although the crossover sign, a marker of acetabular retroversion, was more prevalent in the OA group, this difference did not reach statistical significance ($p = 0.130$).

The mean CEA did not differ significantly between the groups (38.1° in the OA group vs 36.9° in the control group, $p = 0.419$), nor did the distribution of CEA categories (normal, dysplasia, and overcoverage) ($p = 0.055$).

The NSA was significantly greater in the OA group than in the control group (130.1° vs 126.7°, $p < 0.001$), although the overall distribution of NSA categories (normal, coxa vara, and coxa valga) did not differ significantly ($p = 0.066$).

Bone quality assessment showed a significantly higher Singh Index in the control group (median: 4 vs 3; $p < 0.001$), indicating better trabecular bone structure. Femoral shaft width was also greater in the control group (31.9 mm vs 29.9 mm; $p < 0.001$). Despite this, the CTI was slightly but significantly higher in the OA group (0.61 vs 0.59; $p = 0.032$).

Discussion

Patients undergoing THA have been reported to be at an increased risk of developing HOA in the contralateral hip compared with the general population.²³ A prevailing hypothesis proposes that primary HOA arises from structural and/or morphological abnormalities of the proximal femur and acetabulum.⁶ Based on this hypothesis, we evaluated patients at increased risk of developing HOA to determine the prevalence of such abnormalities.

In our analysis, although female sex has historically been considered a potential risk factor for OA and was evaluated during model development, it was not retained in the final logistic regression model. This finding indicates that sex did not significantly differentiate the OA and control groups, whereas morphological parameters emerged as the primary predictors of OA. In the present study, radiographic features of cam-type FAI were more prevalent in the OA group. Characteristic features of cam-type FAI, including an alpha angle $>50^\circ$, pistol-grip deformity, a reduced head–neck offset ratio, and a greater femoral neck diameter on AP radiographs, were observed. These findings suggest that radiographic markers of cam-type FAI can be identified in patients at increased risk of developing HOA, offering the potential for early detection before the onset of osteoarthritic changes. In particular, the Dunn and LL views proved to be the most informative for detecting these morphological abnormalities.

Our results are consistent with those of Şahin et al.²⁴ and Tang et al.,²⁵ who also reported that both the alpha angle and the head–neck offset ratio identified a higher prevalence of proximal femoral abnormalities in a comparable population. The larger mean alpha angles observed on the Dunn and LL views, compared with the AP view, further support the concept that cam-type FAI typically involves the anterosuperior region of the femoral head–neck junction, which is best visualized on the Dunn view.²⁶

Previous studies have suggested that repetitive high-magnitude mechanical loading during skeletal development, particularly in athletes, may contribute to the development of cam-type deformities.^{27,28} However, in our cohort, the proportion of participants who engaged in organized sports for more than 1 year before the age of 18 did not differ significantly between the OA and control groups (25.3% vs 26.0%, $p = 0.954$). Likewise, although a positive family history of HOA was more common in the OA group, the difference was not statistically significant ($p = 0.443$). These findings suggest that additional or interacting risk factors may contribute to the pathogenesis of FAI and HOA.

Radiographic features of pincer-type FAI were less definitive. Indicators such as acetabular protrusion, coxa profunda, and greater acetabular depth were more common in the control group on AP radiographs, suggesting pincer morphology. Although the mean CEA remained within the normal range in both groups, a higher proportion of patients with a CEA $> 39^\circ$ and a positive crossover sign – features indicative of acetabular retroversion and pincer-type FAI – was observed in the OA group; however, these differences were not statistically significant.

The literature presents conflicting evidence regarding the relationship between pincer-type FAI and HOA. Although some population-based studies have suggested an association,⁷ prospective cohort studies have failed to confirm a causal relationship²⁹ and have reported a high prevalence of pincer-type features in asymptomatic individuals. These findings are consistent with our results,

in which several radiographic features of pincer-type FAI were more common in the control group. This may indicate either a potential protective role of pincer morphology against HOA or that additional contributing factors are required to trigger osteoarthritic changes.

Patients in the OA group demonstrated a thicker femoral cortex, reflected by higher CTI values despite a narrower femoral shaft. The increased CTI likely represents an adaptive response to elevated mechanical loading associated with early-stage OA, reflecting compensatory strengthening of the cortical bone. This interpretation is consistent with previous findings suggesting that increased cortical thickness may result from mechanical adaptation to abnormal loading conditions.³⁰

Despite excluding patients with overt femoral head subluxation due to hip dysplasia, 7.7% of patients in the OA group exhibited a CEA < 25°, and 1.3% had an AI > 42°, indicating the presence of subtle hip dysplasia. These findings reinforce the concept that even mild dysplasia, characterized by insufficient femoral head coverage, contributes to abnormal joint loading and accelerates cartilage degeneration, thereby promoting the development of HOA.³¹

Contrary to previous studies linking femoral shaft widening with advanced HOA, our findings demonstrated narrower femoral shafts in the OA group. This may reflect disease-specific bone remodeling or individual biomechanical adaptations, such as localized cortical thickening and proximal femoral shaft narrowing. Furthermore, femurs in the OA group exhibited a more valgus alignment, as evidenced by a greater NSA and reduced apparent bone density, further supporting the role of altered biomechanics in the development of radiographic changes associated with HOA.

Results of this study show that an increased femoral neck diameter in AP projection ($p = 0.046$, OR = 1.29) was positively associated with OA. Thicker femoral necks may contribute to the development of cam-type FAI, subsequently leading to the onset of HOA. Conversely, femoral head diameter in LL projection ($p = 0.005$, OR = 0.72) was negatively associated with OA, and control group patients had larger femoral heads in the AP view ($p = 0.034$), supporting previous findings by Heppenstall et al.,³² who identified a larger femoral head as a protective factor against HOA, potentially due to an increased articular surface area that reduces contact stress.

Youden's index analysis identified an optimal cut-off of 52.6° for the alpha angle in the LL projection and 29.5 mm for the femoral neck diameter in the AP projection, both indicating a higher risk of OA in patients exceeding these values. Conversely, a femoral head diameter below 43.5 mm in the LL projection was associated with an increased risk of OA. These 3 radiographic parameters may be useful in future studies to validate their effectiveness in identifying patients at high risk for developing HOA. Although CT and MRI are the gold standards for assessing such deformities as cam and pincer FAI, their use

as routine screening tools is limited by factors such as cost, accessibility, and radiation exposure (in the case of CT).

Anteroposterior and LL hip radiographs are standard imaging studies for patients presenting with hip pain. This study demonstrates that morphological abnormalities associated with an increased risk of HOA can be reliably identified using these standard radiographic views. In addition, the Dunn view proved particularly effective for measuring the alpha angle and head–neck offset ratio, providing additional diagnostic value for the detection of cam-type FAI. From a clinical perspective, these findings support the inclusion of the Dunn view when evaluating patients with hip pain, as it facilitates earlier detection of cam-type deformities that may predispose patients to HOA. Although routine population screening with radiographs is not currently justified, targeted imaging of individuals at increased risk (e.g., those with a family history of HOA or previous hip injury) may facilitate earlier identification of structural risk factors. Such an approach may support timely interventions before irreversible osteoarthritic changes develop.

Limitations of the study

This study has several limitations. Its observational case-control design prevents causal inference, and recruitment from a single center may limit generalizability. Although standardized protocols were used, radiographic measurements may still involve variability. Although all radiographs were jointly reviewed by a radiologist and an orthopedic surgeon to ensure consistency, inter-rater reliability was not formally assessed. Lastly, the cross-sectional design limits insight into disease progression. Future multicenter, longitudinal studies are needed.

Conclusions

This study demonstrated that morphological abnormalities associated with cam-type FAI are more frequently observed in patients at risk of developing HOA. Increased femoral neck diameter, a reduced head–neck offset ratio, and increased alpha angles, particularly when assessed on Dunn and LL radiographic views, were significantly associated with osteoarthritic changes. Radiographic features of pincer-type femoroacetabular impingement showed a less consistent association with OA, with some features being more prevalent in the control group, suggesting that pincer morphology alone may not be sufficient to initiate degenerative changes without additional contributing factors. Subtle indicators of hip dysplasia, greater valgus alignment, and increased cortical bone thickness were also identified in patients with early-stage OA, supporting the role of altered joint biomechanics in disease development. A larger femoral head diameter, as measured on both AP and LL radiographic views, appears

to be a protective factor against the development of HOA. A larger femoral head provides a greater articular surface area, which helps distribute mechanical loads more evenly across the joint, thereby reducing peak contact stresses on the articular cartilage. This mechanical advantage may delay the onset of cartilage degeneration and subsequent joint space narrowing.

Our findings emphasize the value of standard AP and LL hip radiographs, supplemented by the Dunn view, for identifying early structural abnormalities associated with an increased risk of HOA. Recognition of these morphological patterns may facilitate earlier risk stratification and timely intervention before substantial cartilage degeneration occurs.

Prevention of HOA involves reducing risk factors and slowing disease progression once risk factors or early pathological changes are present. Preventive strategies include early detection of structural abnormalities, identification of additional risk factors, prevention of recurrent joint injury, and preservation of long-term joint health. Risk factors may be systemic (e.g., sex, adiposity, and genetic predisposition) or joint-specific (e.g., hip morphology and previous injury). Although nonmodifiable risk factors help identify individuals at increased risk, modifiable factors may be targeted at appropriate stages of life – e.g., preventing knee injuries during adolescence or childhood obesity to reduce the risk of OA in adulthood. Prevention of post-traumatic OA focuses on minimizing the initial injury and optimizing recovery.

Our findings indicate that hip deformities, including cam morphology and acetabular dysplasia, are strongly associated with the development of HOA. Early detection and treatment of developmental dysplasia of the hip through neonatal screening and appropriate orthotic management may promote normal hip development. Clinicians should consider incorporating the Dunn view in addition to standard AP and LL radiographs when evaluating patients with hip pain or suspected FAI. In selected patients with symptomatic FAI, hip arthroscopy – including osteoplasty of the femoral head–neck junction to restore head–neck offset, debridement of damaged cartilage, and repair of labral tears – may provide greater short-term symptomatic improvement than physiotherapy, although its long-term effect on preventing OA remains uncertain. Occupational loading (e.g., farming and construction work) and participation in high-impact sports (e.g., soccer, hockey, and weightlifting) are also associated with an increased risk of HOA, partly through the development of cam deformities during adolescence.³³ Modifying training loads in high-risk activities may therefore represent an important preventive strategy. Because multiple risk factors may act synergistically, early identification of co-existing risk factors should be incorporated into preventive strategies. Further studies are needed to clarify how anatomical variations, mechanical loading, and genetic factors interact in the development of HOA.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.17297795>. The package contains the following files:

Supplementary Table 1. Top 5 candidate models ranked by their AIC values.

Data Availability Statement

The datasets generated and analysed during the current study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17979427>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

During the preparation of this work, the authors used ChatGPT to assist with language editing and phrasing; after using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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HOTAIR lncRNA variants are associated with psoriasis susceptibility: A case-control study

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Conflict of interest

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Abstract

Background. Psoriasis is a chronic, immune-mediated skin disease characterized by keratinocyte hyperproliferation and persistent inflammation. Recent studies suggest that long non-coding RNAs (lncRNAs), such as *HOX* transcript antisense RNA (*HOTAIR*), may play critical roles in regulating inflammatory pathways involved in psoriasis pathogenesis.

Objectives. This study aimed to investigate the association between *HOTAIR* gene polymorphisms (rs12826786 and rs4759314) and psoriasis susceptibility, and to assess their potential regulatory effects on gene expression using expression quantitative trait loci (eQTL) data.

Materials and methods. A case–control study including 158 patients with psoriasis and 153 controls was conducted. Genotyping of rs12826786 and rs4759314 was performed using the tetra-primer amplification refractory mutation system (T-ARMS) polymerase chain reaction (PCR). Primary associations were tested using multivariable logistic regression in an additive (per-allele) model, adjusting for sex, age in tertiles (T1 reference), smoking, and comorbidity; dominant and genotypic (3-level) models were secondary. Expression quantitative trait loci data from publicly available databases were analyzed to explore the impact of these polymorphisms on *HOTAIR* expression in skin tissue.

Results. Under a multivariable additive model (adjusted for sex, age tertiles, smoking, and comorbidity), rs12826786 was associated with higher odds of psoriasis (odds ratio (OR) = 1.52, 95% confidence interval (95% CI): 1.11–2.08; $q = 0.027$) and rs4759314 likewise (OR = 1.73, 95% CI: 1.18–2.53; $q = 0.020$). Dominant contrasts were also significant: rs12826786 (CT + TT vs CC: OR = 1.84, 95% CI: 1.12–3.05; $q = 0.027$) and rs4759314 (AG + GG vs AA: OR = 2.03, 95% CI: 1.18–3.48; $q = 0.020$). Expression quantitative trait loci analysis revealed that the rs12826786 T allele significantly upregulates *HOTAIR* expression in both sun-exposed and non-sun-exposed skin (both $p < 0.001$).

Conclusions. *HOTAIR* polymorphisms, particularly rs12826786 and rs4759314, are associated with psoriasis risk and may contribute through regulation of *HOTAIR* expression. These findings support *HOTAIR* as a potential biomarker and therapeutic target in psoriasis.

Key words: psoriasis, long noncoding RNA, *HOTAIR* RNA, single nucleotide polymorphism (SNP), gene expression regulation

Highlights

- *HOTAIR* lncRNA polymorphisms rs12826786 and rs4759314 were significantly associated with increased susceptibility to psoriasis in a case–control study.
- Multivariable logistic regression indicated a higher risk of psoriasis among carriers of the rs12826786 T allele and the rs4759314 G allele, supporting a genetic contribution to psoriasis susceptibility.
- Expression quantitative trait loci (eQTL) analysis demonstrated that the rs12826786 T allele significantly upregulates *HOTAIR* expression in human skin tissue.
- *HOTAIR* genetic variants may contribute to psoriasis pathogenesis by regulating gene expression, highlighting their potential as biomarkers and therapeutic targets in immune-mediated skin disease.

Background

Psoriasis is a chronic dermatological condition characterized by abnormal keratinocyte proliferation and immune dysregulation, resulting in persistent, incurable, scaly skin lesions. Beyond its dermatological manifestations, psoriasis is now widely recognized as a systemic disease and is associated with several comorbidities, including psychological disorders, metabolic conditions, arthritis, and cardiovascular disease (CVD).¹ The pathogenesis of psoriasis is characterized by a multifaceted interplay between genetic susceptibility, immune system dysfunction, and environmental triggers, resulting in dysregulated keratinocyte proliferation and inflammatory responses.

Substantial evidence supports a strong genetic component in psoriasis. Genome-wide association studies (GWAS) conducted in diverse populations have identified more than 100 susceptibility loci associated with the disease.^{2,3} The fact that a significant proportion of these loci are located in non-coding DNA suggests that epigenetic regulators, such as long non-coding RNAs (lncRNAs), may play a role in the development of psoriasis.⁴ In recent years, the number of studies investigating the role of non-coding RNAs in psoriasis has steadily increased, highlighting their potential importance in disease pathophysiology.^{5–8} Beyond psoriasis, immune-related lncRNAs have also shown clinical and prognostic relevance in other conditions; e.g., an immune-related lncRNA-based prognostic index was recently developed for glioblastoma and was associated with both patient survival and features of the tumor immune microenvironment (TME).⁹

lncRNAs are non-coding RNA molecules longer than 200 nt that play important roles in regulating gene expression through interactions with DNA, other RNAs, and proteins.⁹ Aberrant expression of lncRNAs, which modulate gene expression through epigenetic pathways, can impair essential biological processes, including cell differentiation, migration, apoptosis, and cell-cycle regulation.^{10,11}

The *HOTAIR* gene, located on chromosome 12 within the *HOXC* gene cluster, produces a non-coding RNA that interacts with chromatin-modifying proteins, including polycomb repressive complex 2 (PRC2) and lysine-specific

demethylase 1 (LSD1), leading to epigenetic silencing of target genes.¹² The ability of *HOTAIR* to regulate gene expression is not limited to chromatin remodeling but can also indirectly alter the expression of specific genes by sponging different miRNAs.¹³ Importantly, *HOTAIR* has been shown to regulate several key proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and macrophage inflammatory protein-1B (MIP-1B).^{14–18} These cytokines play pivotal roles in the immunopathology of psoriasis by contributing to the recruitment and activation of inflammatory cells, promoting keratinocyte proliferation, and sustaining the chronic inflammatory environment of psoriatic lesions.^{19–22} Given these findings, *HOTAIR* is increasingly recognized not only for its role in cancer biology but also as a potential regulator of inflammatory skin diseases, including psoriasis.

We considered *HOTAIR* a biologically plausible candidate in psoriasis, building on prior evidence that *HOTAIR* scaffolds chromatin-modifying complexes to direct epigenetic regulation at target loci.^{12,13,23} Beyond chromatin regulation, *HOTAIR* has been implicated in nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-related inflammatory signaling and keratinocyte responses, including an IL-22-stimulated HaCaT model involving a *HOTAIR/miR-126* axis.^{8,13,18,24} These pathways intersect with the cytokine milieu central to psoriasis pathogenesis.^{21,22} We therefore focused on 2 regulatory variants within *HOTAIR* (rs12826786 and rs4759314) which have been associated with altered *HOTAIR* transcription and susceptibility in various diseases.^{24–28} Based on this rationale, we hypothesized that these polymorphisms might modulate psoriasis susceptibility by influencing *HOTAIR* expression and downstream inflammatory programs.

Objectives

The *HOTAIR* gene contains numerous functional single-nucleotide polymorphisms (SNPs) that may influence its expression and function. Variants such as rs12826786 and

rs4759314 have been linked to altered *HOTAIR* transcription and susceptibility to several conditions, including gastric cardia adenocarcinoma, pancreatic cancer, breast cancer, and autism spectrum disorder (ASD).^{25–28} However, their role in psoriasis remains to be fully elucidated. In this context, the present study aimed to investigate the association between *HOTAIR* polymorphisms and psoriasis risk and to explore potential genotype-dependent regulatory effects that may contribute to disease pathogenesis through the modulation of inflammatory responses.

Materials and methods

Study population

The study comprised 158 patients diagnosed with psoriasis as the patient group and 153 healthy individuals without a history of psoriasis as the control group. All participants were unrelated individuals of self-reported Turkish ethnicity residing in the northeastern Black Sea region of Turkey. This region is known to be ethnically homogeneous, and no evidence of recent migration or admixture was present in the recruitment history.

The diagnosis of psoriasis was made by physicians at the Dermatology Clinic of Giresun University Faculty of Medicine Research and Application Hospital (Giresun, Turkey). It was confirmed that the participants included in the patient group did not have any malignant, autoimmune, or systemic inflammatory disease. The control group consisted of individuals with no history of malignant, autoimmune, or systemic inflammatory disease. They also had no family history of psoriasis and had presented to Giresun University Faculty of Medicine Research and Application Hospital for routine health check-ups.

This study was approved by the Clinical Research Ethics Committee of Giresun University on December 23, 2021 (approval No. 09) and was conducted in accordance with the ethical principles of the Declaration of Helsinki. All participants or their legal guardians provided written informed consent before inclusion in the study. We used the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) case–control checklist when reporting our study.

Genotyping of rs12826786 and rs4759314 polymorphisms

Genomic DNA was extracted from venous blood using a commercial polymerase chain reaction (PCR) template kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manufacturer’s instructions. DNA quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and samples were stored at –20°C.

Genotyping was performed using tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) with the FastStart™ High Fidelity PCR System (Roche Diagnostics GmbH). Primers were designed using the National Center for Biotechnology Information (NCBI) and Ensembl genome databases and verified using NCBI Primer-BLAST (National Institutes of Health (NIH), Bethesda, USA). Polymerase chain reaction was performed on a Bio-Rad T100 thermal cycler (Bio-Rad, Hercules, USA).

Each 25-μL reaction included buffer, deoxynucleoside triphosphates (dNTPs), dimethyl sulfoxide (DMSO), enzyme, primers, DNA, and water. The thermocycling conditions were as follows: 94°C for 1 min, followed by 35 cycles of 94°C for 2 min, 57°C for 30 s, and 72°C for 45 s. Primer sequences are listed in Table 1.

Bioinformatics analysis

To explore the potential regulatory effects of the studied variants, expression quantitative trait loci (eQTL) data were retrieved from and inspected in the Genotype-Tissue Expression (GTEx) Portal (v8; <https://www.gtexportal.org>; accessed August 29, 2025). Specifically, we queried publicly available single-tissue cis-eQTL summary results for *HOTAIR* in sun-exposed and non-sun-exposed skin. Genotype–expression associations and p-values were generated within the GTEx consortium pipeline using additive genotype coding (0/1/2) and linear regression with standard GTEx covariate adjustment, as described in the GTEx Portal documentation and the GTEx v8 primary publication.^{29,30} We did not download or analyze individual-level GTEx expression data or rerun any regression models; GTEx results were used solely as external functional annotation.

Table 1. Specifications of the designed tetra-ARMS PCR primers

SNP ID	Primer	Primer sequence (5' to 3')		Fragment length (bp)
		inner	outer	
rs12826786	F	CGCTGGCCGGGCTTCGTGAATTATAC	ATCTGTCCAGTCGCTCGTCCCTGAGC	203 (C allele) 145 (T allele) 293 (2 outer primers)
	R	CGGCAGAGGGAAGGAGCTTAGGATAACGA	TGTGTTTGGTCTTGTCGAGGCCAGTTT	
rs4759314	F	GCTTGGAAGGGATATAACAGGCAAA	CTTTGAGATTTAGGGACTTGCAGAGA	181 (A allele) 247 (G allele) 373 (2 outer primers)
	R	TTTTCAGGTTTATTAACTTGCATCCTCC	CCAAGGTAGGGAAGTCTCTTTTCTCTG	

SNP – single-nucleotide polymorphism; F – forward primer; R – reverse primer; bp – base pairs; PCR – polymerase chain reaction; tetra-ARMS-PCR – tetra-primer amplification refractory mutation system PCR.

Statistical analyses

All tests were two-sided ($\alpha = 0.05$). Continuous variables were summarized as means $\pm$ standard deviations (SDs) when parametric assumptions were met and as medians [Q1–Q3] otherwise; categorical variables were summarized as n (%). The p -values are reported to 3 decimal places, with $p < 0.001$ reported when appropriate. Normality of continuous variables was assessed within each group (cases and controls) using the Shapiro–Wilk test and normal Q–Q plots, and homogeneity of variances between groups was assessed using Levene’s test (Supplementary Table 1 and Supplementary Fig. 1). Hardy–Weinberg equilibrium (HWE) in controls was evaluated for each SNP using Pearson’s goodness-of-fit χ^2 test (degrees of freedom (df) = 1) when all expected genotype counts were ≥ 5 and a two-sided exact conditional test otherwise; mid- p -values were additionally obtained using the HardyWeinberg package (<https://cran.r-project.org/web/packages/HardyWeinberg/index.html>) in R (R Foundation for Statistical Computing, Vienna, Austria) (Supplementary Table 2).

For the primary case–control analysis, psoriasis susceptibility (case vs control) was modeled using 3 prespecified multivariable logistic regression models: Model 1 (additive, per risk allele), Model 2 (dominant, heterozygotes plus risk-allele homozygotes vs common-allele homozygotes), and Model 3 (3-level genotypic model, 2 df). All models were adjusted for sex, age (tertiles; T1 as reference), smoking status (any vs none), and the presence of any comorbidity.

Linearity of the logit for age was examined using the Box–Tidwell procedure by adding an age $\times$ $\ln(\text{age})$ interaction term to preliminary additive logistic models for each SNP, with age entered as a continuous covariate. The statistical significance of this term ($p < 0.05$) indicated violation of the linearity assumption. Therefore, age was categorized into tertiles and entered as 2 dummy variables (T2 and T3; T1 as reference) in all reported logistic regressions; full Box–Tidwell results are presented in Supplementary Table 3.

For each genetic model, we report the logistic regression coefficient (B, log odds) with its standard error (SE), the corresponding adjusted odds ratio ($\text{OR} = e^B$) with 95% confidence intervals (95% CIs), and Wald p -values for individual coefficients. We also report likelihood-ratio χ^2 statistics, with degrees of freedom and p -values, comparing covariate-only models with models additionally including the SNP term(s), so that likelihood-ratio test (LRT) p -values represent the incremental fit of the genetic term(s) over the covariates.

Model fit and calibration are summarized using the Hosmer–Lemeshow goodness-of-fit p -value and Nagelkerke R^2 , and discrimination is summarized using the area under the receiver operating characteristic curve (ROC AUC) with 95% CIs (Supplementary Table 4). Variance inflation factors (VIFs) were used to assess multicollinearity (Supplementary Table 5); outlier and influence diagnostics, including standardized residuals, leverage, Cook’s

distance, and difference-in-betas statistics (DFBETAs), and robustness refits excluding high-leverage observations are shown in Supplementary Tables 6 and 7. All multivariable logistic regression models were fitted on the complete-case dataset ($n = 300$) after excluding participants with missing data on genotype or covariates.

Multiplicity in the primary case–control comparisons was controlled using the Benjamini–Hochberg false discovery rate (FDR) procedure (target $q = 0.05$) within each SNP across the 5 prespecified logistic tests: additive, dominant, genotypic omnibus, and the 2 pairwise genotypic contrasts ($m = 5$). Within-case genotype–clinical analyses were considered exploratory. No multiplicity correction was applied for these secondary endpoints; therefore, the p -values are interpreted descriptively and with caution regarding potential inflation of the type I error rate.

For binary clinical endpoints within psoriasis cases, we constructed 2×3 contingency tables (endpoint $\times$ 3-level genotype) and used Pearson’s χ^2 test of independence (df = 2) when expected cell counts were adequate. When expected counts were small (minimum expected count < 5), we report the exact two-sided p -value from the Fisher–Freeman–Halton (FFH) exact test (Supplementary Table 8). The linear-by-linear association test (df = 1) is additionally reported to assess an ordinal genotype dose trend ($0 \rightarrow 1 \rightarrow 2$), where appropriate. We also performed prespecified dominant-model 2×2 sensitivity analyses (major-allele homozygotes vs minor-allele carriers) using two-sided Fisher’s exact test (Supplementary Table 9). For continuous clinical endpoints, Spearman’s rank correlation coefficient (ρ) was used to test monotonic allele-dose trends using the additive genotype code 0/1/2 (Supplementary Table 10).

All descriptive and regression analyses were performed using IBM SPSS v. 25.0 (IBM Corp., Armonk, USA). Hardy–Weinberg equilibrium exact and mid- p -values were calculated in R using the HardyWeinberg package.

Results

The study comprised 311 participants, including 158 cases and 153 controls. An a priori power analysis indicated that the sample size was adequate to detect a moderate effect size ($\omega = 0.3$) with 95% power at $\alpha = 0.05$. The demographic and clinical characteristics of the study population are summarized in Table 2.

Median age was higher in cases than in controls (48.0 [35.0–58.3] years vs 31.5 [23.0–49.0] years, $n = 158$ vs $n = 153$, $p < 0.001$, Mann–Whitney U test), consistent with the non-normal distribution of age in both groups (Shapiro–Wilk $p = 0.029$ in cases and $p < 0.001$ in controls; Supplementary Table 1). Sex distribution was similar between groups (female: 51.3% vs 62.1%; Pearson’s χ^2 test, df = 1, $p = 0.054$), with expected cell counts satisfying the χ^2 assumptions. Smoking status also did not differ between groups (32.7% vs 29.1%; Pearson’s χ^2 test, df = 1, $p = 0.508$).

Table 2. Demographic and clinical characteristics of the case and control groups

Parameter		Cases (n = 158)	Controls (n = 153)	p-value
Age [years], median [Q1–Q3]		48.0 [35.0–58.3]	31.5 [23.0–49.0]	<0.001 ^a
Sex, n (%)	female	81 (51.3)	95 (62.1)	0.054 ^b
	male	77 (48.7)	58 (37.9)	
BMI [kg/m ²], median [Q1–Q3]		26.4 [23.9–29.8]	–	–
Smoking status, n (%)	yes	49 (32.7)	44 (29.1)	0.508 ^c
	no	101 (67.3)	107 (70.9)	
Age at onset [years], median [Q1–Q3]		28.0 [18.0–41.0]	–	–
Disease duration [years], median [Q1–Q3]		14.0 [6.0–23.0]	–	–
Positive family history, n (%)		72 (45.6)	–	–
Psoriatic arthritis, n (%)		33 (20.9)	–	–
PASI, median [Q1–Q3]		6.0 [2.0–12.0]	–	–

Q1–Q3 – first-to-third quartile; df – degrees of freedom; BMI – body mass index; PASI – Psoriasis Area and Severity Index. Superscripts denote tests for between-group comparisons (cases vs controls): ^aMann–Whitney U test (n = 158 vs n = 153); ^{b,c}Pearson's χ^2 test (df = 1, two-sided). Variables without values in controls (BMI and case-only clinical characteristics) were not compared between groups; therefore, no statistical test or p-value is reported ("–"). All p-values are 2-sided and reported to 3 decimal places (with p < 0.001 reported when appropriate).

Case-only summaries were as follows: body mass index (BMI), 26.4 [23.9–29.8] kg/m²; age at onset, 28.0 [18.0–41.0] years; disease duration, 14.0 [6.0–23.0] years; and Psoriasis Area and Severity Index (PASI), 6.0 [2.0–12.0]. A positive family history was reported in 45.6% of patients, and 20.9% had psoriatic arthritis.

Genotype and allele distributions by case–control status are shown in Table 3. For example, for rs12826786, TT was observed in 22.8% of cases and 14.4% of controls; for

rs4759314, GG was observed in 15.1% and 6.5%, respectively. Minor allele frequencies were 33.2% vs 23.5% for T and 26.3% vs 14.7% for G.

We fitted 3 prespecified multivariable logistic models per SNP: Model 1 (additive), Model 2 (dominant), and Model 3 (genotypic, 3-level; 2 df). In adjusted logistic models including sex, age tertiles, smoking, and comorbidity, both loci were associated with case status across the primary genetic models (Table 4). For rs12826786, the additive model showed OR = 1.515 (95% CI: 1.105–2.076, p = 0.010; LRT p = 0.009), the dominant model showed OR = 1.843 (95% CI: 1.115–3.046, p = 0.017; LRT p = 0.016), and the genotypic 3-level omnibus test was significant (2 df; LRT p = 0.032; TT vs CC OR = 2.299, p = 0.013). For rs4759314, the additive model showed OR = 1.729 (95% CI: 1.180–2.534, p = 0.005; LRT p = 0.004), the dominant model showed OR = 2.028 (95% CI: 1.183–3.475, p = 0.010; LRT p = 0.009), and the genotypic omnibus test was significant (LRT p = 0.016; GG vs AA OR = 3.104, p = 0.012).

Across these models, overall calibration was acceptable (Hosmer–Lemeshow p > 0.70), and explanatory power was modest (Nagelkerke R² = 0.17), indicating that the genetic terms improved prediction beyond the covariates alone. BH–FDR q-values within each SNP (m = 5) are reported alongside p-values in Table 4 and did not change the statistical conclusions. For completeness, the corresponding log-odds coefficients (B) and SEs are also provided in Table 4.

Sensitivity analyses excluding high-leverage observations (Supplementary Table 7) did not change the statistical conclusions for the primary additive and dominant contrasts. For rs4759314, however, the genotypic omnibus test (2 df) and the GG vs AA pairwise comparison were attenuated and were no longer significant, whereas the additive and dominant associations remained significant.

Across binary clinical endpoints, including psoriatic arthritis, family history, joint pain, joint involvement,

Table 3. Genotype and allele distributions of SNPs in patients with psoriasis and controls

SNP/genotype or allele	Cases (n = 158)	Controls (n = 153)
rs12826786		
CC	89 (56.3%)	103 (67.3%)
CT	33 (20.9%)	28 (18.3%)
TT	36 (22.8%)	22 (14.4%)
CT+TT	69 (43.7%)	50 (32.7%)
Alleles		
C	211 (66.8%)	234 (76.5%)
T	105 (33.2%)	72 (23.5%)
rs4759314		
AA	99 (62.7%)	118 (77.1%)
AG	35 (22.2%)	25 (16.3%)
GG	24 (15.1%)	10 (6.5%)
AG+GG	59 (37.3%)	35 (22.8%)
Alleles		
A	233 (73.7%)	261 (85.3%)
G	83 (26.3%)	45 (14.7%)

SNP – single-nucleotide polymorphism. Values are presented as counts (column percentages). No hypothesis tests are presented in this table; multivariable associations between these SNPs and psoriasis status are shown in Table 4.

Table 4. Multivariable logistic regression analysis using prespecified genetic models for each SNP, including pairwise genotypic contrasts

SNP	Model/contrast	B (SE)	OR (95% CI)	Wald p	Omnibus χ^2 (df)	Omnibus p (LRT)	Hosmer–Lemeshow p-value	Nagelkerke R ²	BH–FDR q
rs12826786	Model 1 – Additive (per T allele)	0.415 (0.161)	1.515 (1.105–2.076)	0.010	6.874 (1)	0.009	0.723	0.166	0.027
	Model 2 – Dominant (CT+TT vs CC)	0.612 (0.256)	1.843 (1.115–3.046)	0.017	5.795 (1)	0.016	0.764	–	0.027
	Model 3 – Genotypic three-level omnibus	–	–	–	6.874 (2)	0.032	0.722	0.166	0.040
	CT vs CC	0.410 (0.321)	1.506 (0.804–2.824)	0.201	–	–	–	–	0.201
	TT vs CC	0.832 (0.336)	2.299 (1.190–4.441)	0.013	–	–	–	–	0.027
rs4759314	Model 1 – Additive (per G allele)	0.548 (0.195)	1.729 (1.180–2.534)	0.005	8.293 (1)	0.004	0.890	0.172	0.020
	Model 2 – Dominant (AG+GG vs AA)	0.707 (0.275)	2.028 (1.183–3.475)	0.010	6.760 (1)	0.009	0.916	–	0.020
	Model 3 – Genotypic three-level omnibus	–	–	–	8.322 (2)	0.016	0.927	0.172	0.020
	AG vs AA	0.505 (0.317)	1.656 (0.889–3.085)	0.112	–	–	–	–	0.112
	GG vs AA	1.133 (0.449)	3.104 (1.287–7.487)	0.012	–	–	–	–	0.020

SNP – single-nucleotide polymorphism; B – regression coefficient; SE – standard error; OR – odds ratio; 95% CI – 95% confidence interval; df – degrees of freedom; LRT – likelihood-ratio test; BH–FDR – Benjamini–Hochberg false discovery rate; q – FDR-adjusted p-value; Nagelkerke R² – Nagelkerke's pseudo-R². Models were adjusted for sex, age tertiles (T1 as reference), smoking status (any), and any comorbidity. B represents the logistic regression coefficient (log odds) with its SE; OR = e^B. Wald p-values represent coefficient-level tests, whereas omnibus p-values from the LRT evaluate the incremental fit of the genetic term over the covariate-only model. Nagelkerke R² and the Hosmer–Lemeshow p-value summarize overall model fit and calibration. Multiplicity within each SNP across 5 prespecified tests, additive, dominant, genotypic omnibus, and the 2 pairwise genotypic contrasts, was controlled using the BH–FDR procedure (target q = 0.05; m = 5); the corresponding q-values are shown. All p-values are two-sided and reported to 3 decimal places.

nail, scalp, and genital involvement, and the PASI-10 category, no between-genotype differences were observed using Pearson's χ^2 test (2×3 ; df = 2), with expected cell counts satisfying the χ^2 assumptions. Where examined, linear-by-linear association (LBL; df = 1) trend tests were also non-significant. Dominant 2×2 sensitivity analyses using two-sided Fisher's exact test were likewise null. Full statistics are provided in Supplementary Tables 8 and 9. In exploratory case-only analyses of continuous variables, Spearman's rank correlations (n = 153) showed no monotonic allele-dose trends using the genotype dose 0/1/2. Consistent with this, for rs12826786, the correlations with disease duration ($\rho = -0.077$, $p = 0.340$), age at onset ($\rho = 0.049$, $p = 0.544$), and BMI ($\rho = -0.003$, $p = 0.968$) were all nonsignificant. Likewise, for rs4759314, the correlations with disease duration ($\rho = 0.053$, $p = 0.514$), age at onset ($\rho = -0.012$, $p = 0.883$), and BMI ($\rho = -0.101$, $p = 0.211$) were nonsignificant (Supplementary Table 10).

Interrogation of GTEx v8 single-tissue cis-eQTL data indicated that rs12826786 was strongly associated with *HOTAIR* expression in human skin (Fig. 1). In both sun-exposed (n = 751) and non-sun-exposed (n = 649) skin tissues, individuals carrying the T allele showed progressively

increased *HOTAIR* expression in a genotype-dependent manner. Specifically, TT homozygotes exhibited the highest expression levels, followed by heterozygous CT individuals, whereas CC homozygotes had the lowest expression levels. These differences were highly statistically significant in both sun-exposed and non-sun-exposed skin (two-sided $p < 0.001$ for both tissues in the GTEx cis-eQTL linear regression, using default GTEx covariates), indicating a strong association between the T allele and increased *HOTAIR* expression. This finding supports the hypothesis that the T allele of rs12826786 may upregulate *HOTAIR* transcription in skin, providing a potential mechanistic explanation for its observed association with increased psoriasis risk in our study.

Hardy–Weinberg equilibrium was evaluated within each group under p^2 , $2pq$, and q^2 expectations. For rs12826786, all expected counts were ≥ 5 , and Pearson's χ^2 goodness-of-fit test (df = 1; n = 153 controls, n = 158 cases) indicated departures from HWE in both controls ($\chi^2 = 36.953$, $p < 0.001$) and cases ($\chi^2 = 44.268$, $p < 0.001$). For rs4759314, expected counts were ≥ 5 in cases, which also deviated from HWE ($\chi^2 = 28.956$, $p < 0.001$). In controls, however, the expected count for minor-allele homozygotes was < 5 ($E = 3.3$), rendering

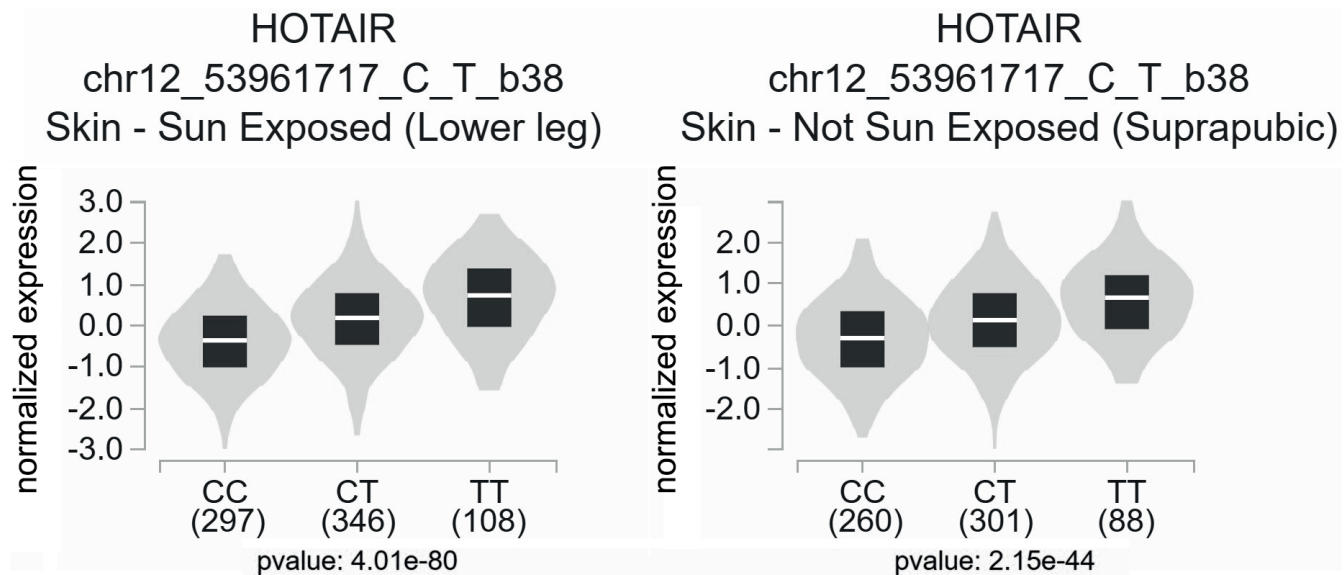


Fig. 1. Normalized expression levels from GTEx v8 are shown for sun-exposed skin (lower leg, $n = 751$) and non-sun-exposed skin (suprapubic, $n = 649$), stratified by rs12826786 genotype (CC, CT, and TT). Carriers of the T allele (CT and TT) exhibited higher *HOTAIR* expression than CC homozygotes in both tissues. The p-values shown for each tissue (both $p < 0.001$) correspond to the GTEx v8 single-tissue cis-eQTL linear regression test (two-sided), in which normalized *HOTAIR* expression was regressed on rs12826786 genotype, additively coded as 0, 1, or 2 T alleles. Data and p-values were obtained from the GTEx Portal; no additional statistical analyses of these data were performed by the authors

eQTL – expression quantitative trait loci.

the χ^2 test asymptotically unreliable; therefore, we report the corresponding exact two-sided HWE p-value in Supplementary Table 2, with Pearson's χ^2 shown for comparison.

These discrepancies are unlikely to stem from technical genotyping errors, as randomly selected samples were re-genotyped to validate the initial findings, thereby increasing confidence in data accuracy. Importantly, although genotyping errors are a potential source of deviation from HWE, they are not the only possible explanation. Various biological and demographic factors, including random genetic drift, heterozygote advantage, non-random mating, population stratification or admixture, inbreeding, and structural genetic variation, such as copy number variants, may also lead to deviations from equilibrium assumptions.³¹

To assess robustness, we repeated allelic case–control tests after excluding the control genotypes that contributed most to the HWE departure: TT for rs12826786 and GG for rs4759314. Effect sizes remained stable and significant, e.g., for rs4759314: OR = 2.705 [1.587–4.610] before exclusion vs OR = 2.852 [1.691–4.810] after exclusion; both Pearson's χ^2 tests, $df = 1$; $n = 153$ controls; $p < 0.001$. These findings suggest that the observed deviations did not materially bias the associations.

Discussion

In this study, we investigated the association between 2 specific SNPs, rs12826786 and rs4759314, and susceptibility to psoriasis. To our knowledge, this is the first study to investigate the association between psoriasis and

the *HOTAIR* rs12826786 and rs4759314 polymorphisms in the Turkish population. Our findings indicate that both SNPs are significantly associated with increased susceptibility to psoriasis.

Our genotypic analysis demonstrated that rs12826786 was significantly associated with psoriasis susceptibility under both the codominant, or genotypic 3-level, model (2 df) and the dominant model (CT + TT vs CC). At the allelic level, the presence of the T allele was linked to an increased risk of developing psoriasis. These findings are consistent with those of a previous study conducted in an Iranian population, in which Rakhshan et al. reported the T allele of rs12826786 as a risk factor for psoriasis (OR = 1.35; $p = 0.040$). Consistently, they also identified a significant association between the CT + TT genotypes and psoriasis in the dominant model (OR = 1.59; $p = 0.020$).¹⁴

In contrast, a study conducted in the Han Chinese population reported a protective role of the T allele of rs12826786.⁷ According to that study, individuals with CT + TT genotypes had a lower risk of psoriasis (OR = 0.70; $p = 0.049$). This discrepancy suggests potential ethnic variation in the functional impact of this SNP. Such population-specific effects are common in genetic association studies and may reflect underlying differences in environmental interactions or sample characteristics.

In our study, the rs4759314 polymorphism showed a significant association with psoriasis under both the genotypic 3-level model (2 df) and the dominant genetic model. Allelic analysis further supported these findings, with the G allele being more common in patients than in healthy controls. These results are consistent with

those of a previous study conducted in the Han Chinese population, which also reported a significant association between rs4759314 and psoriasis under similar genetic models: the dominant model (OR = 3.78; $p = 0.002$) and the overdominant model (OR = 3.79; $p < 0.001$).⁷ By contrast, the study by Rakhshan et al. in the Iranian population did not detect any significant association between this polymorphism and psoriasis under any genetic model.¹⁴

This inconsistency may reflect ethnic differences in allele frequencies, population-specific linkage disequilibrium patterns around the *HOTAIR* locus, or variations in environmental exposures and clinical characteristics. In sensitivity analyses excluding high-leverage observations (Supplementary Table 6), our primary models for rs4759314, additive and dominant, remained significant, whereas the genotypic omnibus test (2 df) and the GG vs AA pairwise comparison were attenuated and were no longer significant. These patterns are consistent with G allele-related susceptibility rather than a stable homozygote-specific effect and highlight sensitivity to influential observations and small cell counts.

In exploratory case-only analyses, we found no robust genotype–phenotype associations for PASI, age at onset, or psoriatic arthritis (Supplementary Tables 8–10). Psoriasis Area and Severity Index measured at a single visit, and influenced by ongoing treatment, may be insensitive to modest genetic effects; therefore, larger longitudinal datasets will be needed to assess severity endpoints.

The biological plausibility of these associations is supported by the known functions of *HOTAIR* in epigenetic and inflammatory regulation. *HOTAIR* acts as a scaffold for chromatin-modifying complexes (PRC2/LSD1) to shape transcriptional programs^{12,13,23} and has been linked to NF- κ B-driven responses and the regulation of cytokines and chemokines implicated in the maintenance of psoriatic lesions^{13,24}; this is consistent with the established importance of TNF- α , IL-6, and related mediators in psoriasis pathogenesis.^{19–22} Within this framework, genetic variation at rs12826786, located in the promoter region, and rs4759314, with functional effects on *HOTAIR* expression in reporter and cell assays,^{25–28,32,33} may contribute to disease risk by modulating *HOTAIR* levels and downstream inflammatory programs that intersect with the IL-23/IL-17 (Th17) axis.^{34,35}

A recent expression-based study conducted in Turkey evaluated *HOTAIR* lncRNA expression levels in psoriatic lesions and perilesional healthy skin.³⁶ Although that study, which evaluated *HOTAIR* expression in psoriatic vs non-lesional skin, did not report a statistically significant difference (fold change: 0.92; $p = 0.218$), it is important to note that its sample size was limited to 15 patients, which may have reduced the statistical power to detect meaningful expression differences.

Moreover, functional evidence from eQTL analyses suggests that the T allele of rs12826786 is associated with increased transcriptional activity of *HOTAIR*. This regulatory effect may explain the observed association between

the T allele and increased psoriasis risk, as higher *HOTAIR* expression has been linked to enhanced activation of inflammatory pathways, including NF- κ B signaling and up-regulation of cytokines such as IL-6 and iNOS.^{17,31}

Limitations of the study

This study has several limitations that should be acknowledged. First, although an a priori power analysis confirmed sufficient statistical power, the sample size was relatively modest and limited to a single geographic region, which may affect the generalizability of the findings to broader populations. Second, although public eQTL resources support a transcriptional effect of rs12826786 on *HOTAIR*, these data are indirect. We did not perform in vitro or in vivo experiments to assess allele-specific effects. Future work should include promoter–reporter assays and CRISPRi/CRISPRa perturbations in psoriasis-relevant cells, such as primary keratinocytes and macrophages, to define the regulatory roles of rs12826786 and rs4759314 in psoriasis. Third, genotype-specific contrasts, such as rs4759314 GG vs AA, were sensitive to leverage and small cell counts, as shown by the robustness refits (Supplementary Table 6), and should therefore be interpreted with caution. Finally, cytokine levels, such as IL-6 and TNF- α , which could further elucidate the inflammatory consequences of *HOTAIR* variation, were not measured. Future studies incorporating cytokine profiling may help clarify the downstream immunological consequences of *HOTAIR* dysregulation in psoriasis.

Conclusions

Our findings provide further evidence supporting the involvement of *HOTAIR* gene polymorphisms in genetic susceptibility to psoriasis. Both rs12826786 and rs4759314 were significantly associated with the disease, particularly under the additive and dominant models, with supportive evidence from a genotypic 3-level omnibus test (2 df). These associations are strengthened by eQTL data demonstrating that the rs12826786 T allele upregulates *HOTAIR* expression in skin tissue, potentially influencing inflammatory pathways implicated in psoriasis pathogenesis.

Overall, our study highlights the relevance of lncRNAs, especially *HOTAIR*, as potential biomarkers and therapeutic targets in psoriasis and emphasizes the need for further functional and longitudinal studies to elucidate the precise mechanisms by which *HOTAIR* contributes to disease development.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.18031314>. The package contains the following files:

Supplementary Fig. 1. Q–Q plots for continuous variables used in group comparisons (assumption check for normality).

Supplementary Table 1. Assumption checks for continuous variables (Shapiro–Wilk, Levene’s; summary-choice rules and test decisions).

Supplementary Table 2. HWE in cases and controls (Pearson’s χ^2 , two-sided exact and mid-p-values).

Supplementary Table 3. Box–Tidwell tests for linearity of the logit with respect to age in additive logistic models.

Supplementary Table 4. Model discrimination (ROC–AUC with 95% CIs) for prespecified logistic models.

Supplementary Table 5. Multicollinearity diagnostics (VIF/tolerance; condition indices) for logistic regression covariates and SNP terms.

Supplementary Table 6. Outlier and influence diagnostics (standardized residuals, leverage/hat, Cook’s distance, DFBETAs) for multivariable logistic models.

Supplementary Table 7. Robustness refits excluding high-leverage observations (adjusted ORs with 95% CIs; Wald and LRT p; Hosmer–Lemeshow p; Nagelkerke R^2).

Supplementary Table 8. Case-only genotype $\times$ binary clinical endpoints (2×3 Pearson’s χ^2 with LLT; minimum expected counts reported).

Supplementary Table 9. Dominant-model sensitivity analyses (2×2 ; unadjusted ORs with 95% CIs; Fisher’s exact, two-sided p-values).

Supplementary Table 10. Case-only continuous endpoints vs genotype dose (Spearman’s ρ and p for, age at onset, disease duration, BMI).

Data Availability Statement

The de-identified subject-level dataset underlying the analyses is openly available in Zenodo at <https://doi.org/10.5281/zenodo.17598034>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Cross-cultural adaptation and assessment of psychometric properties of the Polish version of the Hairdex Life Quality Questionnaire (Hairdex-PL)

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Abstract

Background. Hair and scalp disorders substantially impair health-related quality of life (HRQoL), yet no validated Polish-language disease-specific instrument is currently available to assess this burden.

Objectives. This study aimed to translate, cross-culturally adapt, and assess the structural validity and internal consistency of the Polish version of the Hairdex questionnaire (Hairdex-PL).

Materials and methods. The original 48-item German Hairdex questionnaire was translated and culturally adapted according to the Beaton guidelines. Psychometric evaluation was performed in 245 adults with hair loss or scalp disorders recruited through an online survey. Structural validity was assessed using confirmatory factor analysis (CFA), followed by exploratory factor analysis (EFA) when the predefined model demonstrated inadequate fit. Internal consistency was assessed using Cronbach's α and McDonald's ω .

Results. A total of 245 participants (55.9% women; 44.1% men; mean age 35.8 ± 9.7 years) were included in the final analysis. Confirmatory factor analysis did not confirm the original 5-factor structure (root mean square error of approximation (RMSEA) = 0.234; comparative fit index (CFI) = 0.744; Tucker–Lewis index (TLI) = 0.749; standardized root mean square residual (SRMR) = 0.192). Exploratory factor analysis retained a single factor explaining 38.2% of the total variance. The greatest impairment in HRQoL was observed in the Self-confidence and Emotions domains, corresponding to the greatest impairment in the same items in the original Hairdex, whereas Functioning and Symptoms were the least affected. Stigmatization had an intermediate mean score. Internal consistency was high (Cronbach's α = 0.960; McDonald's ω = 0.963).

Conclusions. Hairdex-PL showed high internal consistency and provided preliminary psychometric evidence supporting its use to assess disease-specific HRQoL in Polish-speaking individuals with hair and scalp disorders. Although confirmatory factor analysis did not support the original 5-factor structure, exploratory factor analysis identified a single factor in the present sample; this exploratory finding requires confirmation in an independent population. This study provides new evidence regarding its underlying measurement structure and supports further evaluation of the instrument in clinical and research settings.

Key words: quality of life, psychometric properties, cross-cultural adaptation, alopecia, patient-reported outcome measures

Highlights

- Hairdex-PL provides a disease-specific tool for assessing HRQoL in patients with hair and scalp disorders.
- Hairdex-PL demonstrated high internal consistency, with Cronbach's $\alpha = 0.960$ and McDonald's $\omega = 0.963$.
- Exploratory factor analysis identified a single-factor structure, differing from the original 5-factor Hairdex model.
- Hair loss and scalp disorders most strongly affected self-confidence and emotions, highlighting their psychosocial impact.

Background

Hair loss and scalp disorders are among the most common conditions encountered in dermatological practice, and their impact on affected patients' quality of life can be immense.^{1–4} Androgenetic alopecia, the most prevalent form of hair loss, affects up to 70–80% of men and 40–50% of women of European ancestry by the age of 70 years, with prevalence rising progressively with age.^{2,5,6} Alopecia areata, the 2nd most common form, carries an estimated lifetime risk of approx. 2.1%, typically presenting between the ages of 25 and 35 years.⁷ Although these conditions are rarely life-threatening, their high prevalence and substantial psychosocial burden make them an important public health issue.^{3–5}

Despite the availability of effective pharmacological and supportive treatments, the psychosocial burden of alopecia areata is increasingly recognized.^{8,9} Because of their visible and aesthetic nature, these conditions markedly impair health-related quality of life (HRQoL), frequently leading to reduced self-esteem, social anxiety, social withdrawal, and self-criticism.⁹ Among patients with alopecia areata, the lifetime prevalence of psychiatric disorders reaches 66–74%, with depression and generalized anxiety disorder affecting approx. 38–39% and 39–62% of patients, respectively.⁷ Consequently, these disorders often impair occupational and daily functioning.⁹

Clinicians have traditionally assessed the severity of hair and scalp disorders using scales such as the Norwood–Hamilton and Ludwig classifications and the Psoriasis Area and Severity Index (PASI).^{10–12} However, these instruments assess primarily the objective extent of disease and do not adequately reflect the patient's subjective experience, as clinical severity correlates poorly with patient-perceived HRQoL.¹³ Similarly, widely used dermatology-specific questionnaires, such as the Dermatology Life Quality Index (DLQI) and Skindex-29, provide standardized measures of HRQoL in skin disease but do not address concerns specific to hair and scalp disorders.^{14,15} Consequently, there is growing emphasis on patient-reported outcome measures (PROMs), which provide a standardized assessment of HRQoL from the patient's perspective and better capture the lived experience of these conditions.^{16,17}

The Hairdex is a German disease-specific 48-item questionnaire derived from and validated against

the Skindex^{18,19} and developed to assess the impact of hair and scalp disorders on patients' HRQoL.³ However, no Polish-language version of this instrument is currently available, limiting the use of a standardized disease-specific measure in both clinical practice and research in Poland. Therefore, the aim of this study was to perform the cross-cultural adaptation and assess selected psychometric properties, including structural validity and internal consistency, of the Polish version of the Hairdex (Hairdex-PL) questionnaire in patients with hair and scalp disorders.

Materials and methods

Study population

Data were collected between August 2025 and January 2026 using an anonymous online survey distributed via social media platforms, internet forums, and Facebook groups dedicated to individuals experiencing hair loss and scalp disorders. Participants were recruited using a non-probability convenience sampling strategy. As recommendations for psychometric validation studies suggest recruiting 5–10 participants per questionnaire item,²⁰ and as the Hairdex³ questionnaire consists of 48 items, we aimed to reach a sample size range of 240–480 participants. Eligibility criteria included age ≥ 18 years, self-reported hair loss or scalp-related problems, and provision of informed consent. Before accessing the questionnaire, all participants confirmed that they were at least 18 years of age, experienced hair loss, and voluntarily agreed to participate in the study. Information regarding physician-confirmed diagnosis and the type of hair loss was collected separately as part of the demographic questionnaire. A total of 294 individuals participated in the study. Forty-nine respondents completed the sociodemographic section but did not proceed to the Hairdex-PL questionnaire and were therefore excluded from further psychometric analyses. Consequently, the final analytical sample comprised 245 participants. The survey consisted of 2 sections. The 1st section collected demographic and clinical information, including age, sex, place of residence, education, socioeconomic status, duration of hair loss, concomitant diseases, physician-confirmed

diagnosis, and the type of hair loss. The 2nd section comprised the Hairdex-PL questionnaire. To ensure data quality, the Webankieta platform automatically prevented multiple questionnaire submissions using IP-based filtering. Researchers had no access to participants' IP addresses, preserving anonymity and protecting personal data throughout the study.

According to the opinion of the Bioethics Committee at Wrocław Medical University (opinion No. 296/2025, 03.07.2025), this study was not classified as a medical experiment under Polish regulations and, therefore, did not require approval by the Bioethics Committee.

Hairdex questionnaire

The Hairdex questionnaire is a 48-item disease-specific PROM developed by Fischer et al. to assess HRQoL in patients with hair and scalp disorders.³ The instrument was originally derived from the Skindex questionnaire and subsequently modified to address concerns specific to individuals affected by hair loss and scalp diseases.^{18,19} The final version of Hairdex comprises 48 items organized into 5 domains: Symptoms, Functioning, Emotions, Self-confidence, and Stigmatization. Each item is rated on a 5-point Likert scale ranging from 0 ("never") to 4 ("always"). Responses are treated as ordinal variables in psychometric analyses.³ In the original validation study involving female patients with diffuse alopecia and androgenetic alopecia across 3 hair-loss visibility levels, exploratory factor analysis supported the proposed 5-domain structure, explaining approx. 69% of the total variance. Internal consistency ranged from 0.55 to 0.82 across domains, with the highest reliability in the Self-confidence domain and the lowest in the Symptoms domain.³

Instrument translation and cultural and language adaptation

Permission to translate and culturally adapt the Hairdex questionnaire into Polish was obtained from the copyright holder of the original instrument before commencing the study. The translation and cross-cultural adaptation process followed the internationally accepted recommendations proposed by Beaton et al.²¹ First, 2 bilingual translators whose native language was Polish independently translated the original German version of the Hairdex questionnaire into Polish (forward translation). The 2 forward translations were subsequently compared and synthesized into a single preliminary Polish version through discussion and consensus. Two bilingual translators who were native German speakers then independently back-translated the synthesized Polish version into German and were blinded to the original questionnaire (back-translation). The purpose of the back-translation was to verify conceptual equivalence and identify potential discrepancies between the translated and original versions.

A 10-member expert committee subsequently reviewed all translated versions, including the original questionnaire, the forward translations, the synthesized Polish version, and the back-translations. The committee consisted of a health promotion specialist, 3 nurses, a paramedic, a dermatologist, a methodologist, a cosmetologist, a trichologist, and a patient with physician-confirmed androgenetic alopecia. The expert panel evaluated the semantic, idiomatic, experiential, and conceptual equivalence of all questionnaire items and reached consensus on the final pre-final Polish version. The pre-final version was subsequently pilot-tested among 35 adults experiencing hair loss to evaluate the clarity, comprehensibility, and cultural relevance of the translated questionnaire. Participants reported no difficulties in understanding the Hairdex-PL items. Minor linguistic refinements concerned only the wording of selected sociodemographic questions, primarily to improve clarity and inclusiveness, and did not affect the content or conceptual meaning of the Hairdex-PL items. The Hairdex-PL is provided in the Supplementary Material (<https://doi.org/10.5281/zenodo.22108376>).

Statistical analyses

Statistical analyses were performed using R v. 4.5.2 (R Foundation for Statistical Computing, Vienna, Austria) together with RStudio and the lavaan, psych, psy, and diagram packages.^{22–25} No data were missing, as participants were required to complete all questionnaire items before submitting the survey. Descriptive statistics are presented as means with standard deviations (SDs), medians with interquartile ranges (IQRs), ranges, and frequencies with percentages, as appropriate. Structural validity was initially assessed using confirmatory factor analysis (CFA) to evaluate the fit of the original 5-factor structure of the Hairdex questionnaire. Because the questionnaire items were measured using ordinal Likert-scale responses, the robust weighted least squares estimator with mean and variance adjustment (WLSMV) was applied. Model fit was evaluated using the standardized root mean square residual (SRMR), root mean square error of approximation (RMSEA), comparative fit index (CFI), and Tucker–Lewis index (TLI). Following the recommendation of Hu and Bentler, acceptable model fit was defined as SRMR < 0.09 together with at least one of the following criteria: CFI > 0.96, TLI > 0.96, or RMSEA < 0.06.²⁶ As the predefined 5-factor model demonstrated inadequate fit, exploratory factor analysis (EFA) was subsequently performed to identify the underlying factor structure. The suitability of the data for factor analysis was assessed using the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity. We performed EFA using a Pearson correlation matrix, Principal Axis Factoring (PAF) as the extraction method, and oblimin rotation. Principal Axis Factoring was selected to identify latent factors based

on the common variance shared among items, while oblimin rotation was used because potential HRQoL dimensions were expected to be correlated. The number of retained factors was determined using the prespecified Kaiser criterion (eigenvalue >1) as the factor-retention rule for this exploratory analysis. Factor loadings of ≥ 0.30 were considered meaningful. Internal consistency was assessed using Cronbach's alpha (α) and McDonald's omega (ω) coefficients. Corrected item-total correlations and Cronbach's α if item deleted were additionally calculated to evaluate the contribution of individual items to the internal consistency of the questionnaire. Cronbach's α values were interpreted as follows: $\alpha \geq 0.90$ – excellent; 0.80 – 0.89 – good; 0.70 – 0.79 – acceptable; 0.60 – 0.69 – questionable; 0.50 – 0.59 – poor; and $\alpha < 0.50$ – unacceptable. Floor and ceiling effects were evaluated for each questionnaire item. A floor effect was defined as the percentage of participants selecting the lowest possible response category, whereas a ceiling effect was defined as the percentage selecting the highest possible response category. Proportions exceeding 15% were considered indicative of substantial floor or ceiling effects. Statistical significance was set at $p < 0.05$.

Results

Participant characteristics

A total of 294 individuals entered the survey. Of these, 49 did not complete the Hairdex-PL questionnaire and were excluded from the psychometric analyses, resulting in a final analytical sample of 245 participants. The mean age was 35.84 ± 9.71 years; 55.92% of participants were women and 44.08% were men. Most respondents had higher education (73.06%), lived in cities with more than 100,000 inhabitants (56.73%), and reported good socioeconomic status (64.90%). Table 1 presents the detailed demographic and clinical characteristics of the study population.

Hairdex-PL scores

No normative values have been established for the Hairdex-PL questionnaire; therefore, the obtained scores should be interpreted descriptively. Hairdex-PL total and subscale scores range from 0 to 100, with higher scores indicating poorer HRQoL. The mean total Hairdex-PL score was 28.71 ± 14.85 . Among the 5 subscales, Self-confidence (43.04 ± 15.60) had the highest mean score, followed by Emotions (35.91 ± 21.79) and Stigmatization (25.29 ± 16.21), indicating the greatest impairment in the first 2 domains. In contrast, Functioning (17.83 ± 18.39) and Symptoms (22.40 ± 15.75) had the lowest mean scores, suggesting a lower impact on HRQoL in these areas. Table 2 presents descriptive statistics for the total Hairdex-PL score and individual subscales.

Table 1. Demographic and clinical characteristics of the study population

Parameter	Total (n = 245)
Age [years]	mean (SD)
	35.84 (9.71)
	median (quartiles)
	35 (29–40)
Sex	range
	18–70
	n
	245
BMI [kg/m ²]	female
	137 (55.92%)
	male
	108 (44.08%)
Place of residence	mean (SD)
	25.25 (4.37)
	median (quartiles)
	24.8 (22.3–27.5)
Education level	range
	16.4–39.5
	n
	245
Socio-economic status	city with up to 100,000 inhabitants
	55 (22.45%)
	city with more than 100,000 inhabitants
	139 (56.73%)
Chronic diseases	rural area
	51 (20.82%)
	vocational
	10 (4.08%)
Type of chronic diseases*	secondary
	56 (22.86%)
	higher
	179 (73.06%)
Scalp or skin diseases	good
	159 (64.90%)
	medium
	78 (31.84%)
Type of scalp or skin diseases*	unsatisfactory
	8 (3.27%)
	no
	123 (50.20%)
Duration of the hair loss problem since it was noticed	yes
	122 (49.80%)
	cardiovascular diseases
	29 (11.84%)
Type of hair loss	metabolic diseases
	14 (5.71%)
	autoimmune diseases
	27 (11.02%)
not diagnosed by a doctor	hormonal disorders
	37 (15.10%)
	mental illnesses
	38 (15.51%)
Type of hair loss	allergic diseases
	33 (13.47%)
	other
	18 (7.35%)
Type of hair loss	I prefer not to answer
	6 (2.45%)
	no
	173 (70.61%)
Type of hair loss	yes
	72 (29.39%)
	acne
	23 (9.39%)
Type of hair loss	psoriasis
	10 (4.08%)
	atopic dermatitis
	15 (6.12%)
Type of hair loss	seborrheic dermatitis
	28 (11.43%)
	other
	8 (3.27%)
Type of hair loss	up to 1 year
	38 (15.51%)
	1–2 years
	44 (17.96%)
Type of hair loss	2–5 years
	57 (23.27%)
	over 5 years
	106 (43.27%)
Type of hair loss	androgenetic alopecia
	78 (31.84%)
	telogen effluvium
	16 (6.53%)
Type of hair loss	alopecia in the course of immune diseases
	13 (5.31%)
	alopecia in the course of infectious diseases
	5 (2.04%)
Type of hair loss	not diagnosed by a doctor
	133 (54.29%)

* multiple choice question – percentages do not sum up to 100.

Table 2. Descriptive statistics for the Hairdex total score and subscale scores

Subscale	n	Missing	Mean	SD	Median	Min	Max	Q1	Q3
Hairdex total	245	0	28.71	14.85	26.56	4.17	67.71	16.67	37.5
Hairdex subscales									
Symptoms	245	0	22.4	15.75	18.75	0	62.5	9.38	34.38
Functioning	245	0	17.83	18.39	10.42	0	72.92	4.17	27.08
Emotions	245	0	35.91	21.79	34.09	0	75	18.18	54.55
Self-confidence	245	0	43.04	15.6	41.67	16.67	83.33	30.56	52.78
Stigmatization	245	0	25.29	16.21	21.88	3.12	71.88	12.5	34.38

SD – standard deviation, Q1 – lower quartile, Q3 – upper quartile.

Table 3. Analysis of individual questionnaire items

Item	Floor effect [%]	Ceiling effect [%]	Missing [%]	Item	Floor effect [%]	Ceiling effect [%]	Missing [%]
1	53.9	0.0	0.0	25	73.9	0.0	0.0
2	64.5	0.0	0.0	26	56.7	0.0	0.0
3	18.4	0.0	0.0	27	87.8	0.0	0.0
4	41.6	0.0	0.0	28	32.2	0.0	0.0
5	32.2	0.0	0.0	29	67.3	0.0	0.0
6	42.0	0.0	0.0	30	15.1	0.0	0.0
7	65.7	0.0	0.0	31	0.0	19.6	0.0
8	70.2	0.0	0.0	32	41.6	0.0	0.0
9	60.0	0.0	0.0	33	0.0	5.7	0.0
10	33.5	0.0	0.0	34	65.3	0.0	0.0
11	60.4	0.0	0.0	35	0.0	4.1	0.0
12	31.0	0.0	0.0	36	29.8	0.0	0.0
13	8.2	0.0	0.0	37	59.2	0.0	0.0
14	64.1	0.0	0.0	38	71.0	0.0	0.0
15	24.9	0.0	0.0	39	0.0	6.9	0.0
16	46.5	0.0	0.0	40	0.0	9.8	0.0
17	71.4	0.0	0.0	41	0.0	11.4	0.0
18	38.0	0.0	0.0	42	65.3	0.0	0.0
19	40.4	0.0	0.0	43	0.0	23.3	0.0
20	60.0	0.0	0.0	44	37.6	0.0	0.0
21	31.4	0.0	0.0	45	33.1	0.0	0.0
22	63.3	0.0	0.0	46	46.9	0.0	0.0
23	16.7	0.0	0.0	47	24.9	0.0	0.0
24	22.0	0.0	0.0	48	43.7	0.0	0.0

Analysis of individual questionnaire items

Analysis of floor and ceiling effects is presented in Table 3. Substantial floor effects were identified for several items, particularly those assessing physical symptoms, with several items exceeding the predefined 15% threshold. The pronounced floor effects observed for several symptom-related items likely reflect the clinical characteristics of the study sample, which predominantly comprised patients with hair disorders not typically associated with scalp symptoms such as pain, burning, or pruritus. In contrast, ceiling effects were generally negligible; however, moderate ceiling effects were observed for items 31 (19.6%) and 43 (23.3%).

Confirmatory and exploratory factor analysis

The CFA of the predefined 5-factor model demonstrated inadequate fit to the data (RMSEA = 0.234, CFI = 0.744, TLI = 0.749, SRMR = 0.192). Therefore, EFA was performed to further investigate the latent structure of the Hairdex-PL questionnaire. The data were suitable for factor analysis, as indicated by a KMO measure of sampling adequacy of 0.948 and a statistically significant Bartlett's test of sphericity ($p < 0.001$), suggesting the correlation matrix was appropriate for factor analysis. Only 1 factor had an eigenvalue greater than 1 and was therefore retained according

Table 4. Factor loadings of the Hairdex questionnaire items derived from exploratory factor analysis

Item	Loading	Item	Loading	Item	Loading
1	0.095	17	0.773	33	0.602
2	0.448	18	0.335	34	0.756
3	0.543	19	0.212	35	0.341
4	0.724	20	0.841	36	0.605
5	0.805	21	0.775	37	0.792
6	0.841	22	0.478	38	0.597
7	0.181	23	0.748	39	0.575
8	0.789	24	0.521	40	0.223
9	0.586	25	0.756	41	0.305
10	0.140	26	0.848	42	0.595
11	0.813	27	0.213	43	0.364
12	0.825	28	0.807	44	0.764
13	0.583	29	0.702	45	0.631
14	0.813	30	0.690	46	0.691
15	0.757	31	0.159	47	0.542
16	0.343	32	0.774	48	0.291

to the prespecified Kaiser criterion, explaining 38.19% of the total variance. Forty of the 48 items demonstrated factor loadings of ≥ 0.30 on the retained factor. The 8 items with loadings below 0.30 were items 1, 7, 10, 19, 27, 31, 40, and 48, most of which also demonstrated pronounced floor effects. Because only 1 factor met the retention criterion, and most questionnaire items had meaningful loadings, the 1-factor solution was the most parsimonious exploratory representation of the data in this sample. Table 4 presents factor loadings for all Hairdex-PL items.

The Hairdex-PL questionnaire showed excellent internal consistency, with a Cronbach's α of 0.960 and a McDonald's ω of 0.963. Corrected item-total correlations were positive for all items, ranging from 0.148 to 0.813, indicating that each item contributed positively to the overall scale. Cronbach's α values after deleting individual items ranged from 0.958 to 0.961, suggesting that removing any single item would not meaningfully improve the questionnaire's internal consistency. Table 5 presents detailed item-level reliability statistics.

Table 5. Internal consistency of the Polish version of the Hairdex questionnaire: Corrected item-total correlations, Cronbach's α if item deleted, and McDonald's omega

Item	α if item deleted	Item-total correlation	Item	α if item deleted	Item-total correlation	Overall Cronbach's α	Overall McDonald's ω
1	0.961	0.148	25	0.959	0.724	0.960	0.963
2	0.96	0.472	26	0.958	0.804		
3	0.96	0.562	27	0.961	0.248		
4	0.959	0.705	28	0.958	0.791		
5	0.959	0.783	29	0.959	0.659		
6	0.958	0.813	30	0.959	0.684		
7	0.961	0.233	31	0.961	0.166		
8	0.959	0.751	32	0.959	0.751		
9	0.959	0.593	33	0.959	0.605		
10	0.961	0.203	34	0.959	0.724		
11	0.959	0.776	35	0.96	0.343		
12	0.958	0.802	36	0.96	0.574		
13	0.959	0.581	37	0.959	0.751		
14	0.959	0.776	38	0.96	0.561		
15	0.959	0.744	39	0.96	0.557		
16	0.96	0.371	40	0.961	0.223		
17	0.959	0.732	41	0.961	0.302		
18	0.96	0.392	42	0.96	0.562		
19	0.961	0.278	43	0.961	0.353		
20	0.959	0.791	44	0.959	0.73		
21	0.959	0.752	45	0.959	0.641		
22	0.96	0.468	46	0.959	0.659		
23	0.959	0.738	47	0.96	0.535		
24	0.96	0.543	48	0.961	0.294		

Discussion

This study presents the first cross-cultural adaptation and psychometric evaluation of the Hairdex-PL questionnaire for use in the Polish population. The Polish version demonstrated high internal consistency and overall satisfactory psychometric performance. Although confirmatory factor analysis did not support the originally proposed 5-factor structure, exploratory factor analysis identified a dominant general factor in the present sample. Taken together, these findings indicate that the pattern of domain scores was broadly consistent with previous studies, while providing new evidence on the underlying measurement structure of the Polish adaptation.

The domain-level pattern observed here closely mirrors that of the original German instrument and the Swedish adaptation, in which Self-confidence and Emotions consistently showed the greatest impairment and Symptoms the least.^{3,27} A more detailed comparison with the original German Hairdex showed that the Self-confidence and Emotions scores were 29.98 and 37.01, respectively, whereas the Symptoms and Functioning scores were higher than in the Polish version.³ In the Swedish validation, Self-confidence (45.0) and Emotions (36.5) produced the highest scores, almost identical to our values, supporting the cross-cultural stability of the construct that Hairdex captures.²⁷ The instrument has also been applied, without formal psychometric validation, in Turkish and Chinese populations, where it likewise detected the greatest burden in the emotional and self-perception domains and discriminated HRQoL across clinical and sociodemographic subgroups.^{16,28} This consistency across linguistically and culturally diverse settings reinforces the view that hair and scalp disorders affect patients principally through emotional and self-image pathways rather than physical symptoms.^{29–32}

Regarding factor structure, our confirmatory analysis indicated inadequate fit for the 5-factor model (RMSEA = 0.234; CFI = 0.744; TLI = 0.749; SRMR = 0.192), and exploratory analysis retained a single factor explaining 38.19% of the variance. At first glance, this appears to diverge from the original study, in which a 5-factor solution accounted for approx. 69% of the variance.³ However, these variance estimates are not directly comparable across studies because different analytical approaches were used; notably, the Swedish principal component analysis (PCA) was performed on domain-level scores rather than on all 48 individual items.^{3,27} This apparent discrepancy likely reflects both methodological differences between PCA and exploratory factor analysis and differences in study populations; PCA tends to yield higher explained variance, and neither previous study subjected the 5-domain model to a confirmatory test. To our knowledge, this study is the first to evaluate the structural validity of the Hairdex using CFA, which is a principal methodological contribution of this work. The CFA findings indicate that the original 5-factor model was not supported in the present Polish sample. The subsequent EFA

identified a dominant general factor. The 1-factor solution was considered the most parsimonious exploratory interpretation because only 1 factor met the prespecified Kaiser criterion and 40 of the 48 items demonstrated meaningful loadings on the retained factor. However, this finding should be interpreted as exploratory and sample-specific rather than as definitive evidence of unidimensionality. This caution is particularly warranted because the retained factor explained 38.19% of the total variance and several items demonstrated relatively low factor loadings. Future studies should assess the reproducibility of this structure in independent samples, ideally by testing a prespecified 1-factor model using CFA and comparing it with theoretically plausible multidimensional models.

The strong internal consistency of the Hairdex-PL aligns with both previous validations and reinforces this interpretation. The Swedish Hairdex-S reported a total Cronbach's α of 0.92,²⁷ and the original German instrument reported domain-level values of 0.55–0.82, with the lowest reliability for the Symptoms subscale.³ Our total α of 0.960 and ω of 0.963 exceed these figures and indicate excellent reliability. Corrected item–total correlations were positive but heterogeneous (0.148–0.813), indicating that some items were only weakly associated with the total score. The high overall α and ω can coexist with low item–total correlations for individual items because these coefficients summarize reliability across all 48 items and reflect scale length and the covariance pattern of the full item set. Removal of any single item changed α only marginally (0.958–0.961); therefore, item deletion was not considered justified on the basis of internal consistency alone.

The items with the lowest factor loadings and item–total correlations were predominantly symptom-related (e.g., items 1, 7, 10, and 27) and corresponded to the items showing the most pronounced floor effects, several of which exceeded the conventional 15% threshold for an acceptable floor effect.³³ One possible explanation for this pattern is the clinical composition of our sample; however, the present data cannot determine whether the low loadings reflect sample-specific response distributions, limitations in item performance, or both. Our participants were recruited online and included a substantial proportion of individuals with androgenetic alopecia or hair loss without physician-confirmed diagnosis. This sample composition may have contributed to the low prevalence of scalp symptoms such as pain, burning, or pruritus and, consequently, to the pronounced floor effects. By contrast, the Swedish cohort was recruited from a hospital dermatology register and included a substantial proportion of patients with alopecia totalis or universalis,²⁷ representing more severe disease in which a greater symptom burden would attenuate such floor effects. This interpretation is consistent with the original German work, in which the Symptoms subscale was the weakest performer yet was deliberately retained to preserve comparability with other dermatological instruments,³ and with broader

evidence that floor and ceiling effects frequently arise from a mismatch between the severity range of an instrument and the characteristics of the population in which it is applied.³³ It also accords with the well-documented observation that clinical severity and patient-perceived HRQoL are only weakly correlated in hair loss.^{3,14,34,35}

In the Polish clinical and research context, the availability of a cross-culturally adapted, hair- and scalp-specific HRQoL instrument addresses a clear gap. Dermatology-specific instruments such as the DLQI and Skindex-29, including their Polish adaptations, do not capture concerns specific to hair loss, and clinical severity scales correlate poorly with the patient's subjective experience.^{13–15} The psychosocial burden of hair loss is increasingly recognized as a substantial component of disease impact, affecting self-esteem, social functioning, and mental health,^{8,29} and disease-specific instruments are considered essential for capturing this dimension in both clinical and research settings.³⁶ Our findings, showing that the greatest burden lies in Self-confidence and Emotions, suggest that psychosocial aspects may represent an important component of the HRQoL burden associated with hair and scalp disorders.³ After confirming its factorial structure, temporal reliability, and responsiveness, the Hairdex-PL may support routine monitoring of patient-reported burden and assessment of treatment-related changes. Taken together, these exploratory findings indicate that the total score may provide a useful summary measure of hair- and scalp-related HRQoL. However, the relative validity of the total and domain-level scores requires further evaluation in independent samples before making definitive scoring recommendations.

Limitations of the study

This study has several limitations that should be considered when interpreting the findings. First, although the sample size met current recommendations for studies evaluating psychometric properties, the study population included patients with various hair and scalp disorders rather than a single diagnostic entity. This clinical heterogeneity may have influenced participants' responses and contributed to the observed factor structure. Future studies involving diagnostically homogeneous patient groups are warranted to further evaluate the structural properties of the Hairdex-PL questionnaire. Second, participants were recruited using a convenience sampling strategy through an anonymous online survey distributed via social media platforms and internet forums. Consequently, individuals without internet access or those not engaged in online communities may have been underrepresented, which may limit the generalizability of the findings to the broader population of patients with hair and scalp disorders. Furthermore, 133 participants (54.29%) reported that a physician had not diagnosed their hair loss. This category indicates a lack of diagnostic confirmation rather than a confirmed absence of disease; nevertheless, it may have

increased sample heterogeneity and limited the generalizability of the findings to populations with physician-confirmed diagnoses. Finally, the present study did not evaluate test–retest reliability. Therefore, future studies should examine the temporal stability of the Hairdex-PL questionnaire by administering the instrument at 2 time points in clinically stable patients.

Conclusions

The Hairdex-PL demonstrated high internal consistency and provided preliminary psychometric evidence supporting its use to assess disease-specific health-related quality of life in Polish-speaking individuals with hair and scalp disorders. Although the original 5-factor structure was not supported in the present sample, the domain score pattern was broadly consistent with that reported in previous international studies. Future studies should evaluate additional psychometric properties, particularly test–retest reliability, and examine the reproducibility of the exploratory factor structure and determine the most appropriate scoring model in independent samples.

Supplementary data

Supplementary materials are available at <https://doi.org/10.5281/zenodo.22108376>. The package contains the following files:

The Polish version of the Hairdex Life Quality Questionnaire (Hairdex-PL): the final Polish-language version of the 48-item Hairdex questionnaire developed through translation and cross-cultural adaptation.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available in Zenodo at <https://doi.org/10.5281/zenodo.22108332>.

Consent for publication of personal information

Not applicable.

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Investigation of the effect of berberine on memory and learning in aged rats undergoing sevoflurane anesthesia

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Conflict of interest

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Abstract

Background. Sevoflurane is a widely used inhalation anesthetic during surgical procedures. Experimental animal models have demonstrated that exposure to sevoflurane leads to cognitive dysfunction. The molecular mechanisms underlying sevoflurane-induced neurotoxicity, as well as the potential neuroprotective mechanisms of berberine, have not yet been fully elucidated.

Objectives. The current investigation sought to evaluate the effects of berberine on memory and learning in aged rats undergoing sevoflurane anesthesia.

Materials and methods. Fifty-six male Sprague Dawley rats aged 20–24 weeks were assigned to 4 groups (n = 14/group): control, berberine, sevoflurane + berberine, and sevoflurane. The control group was exposed to 50% O₂ and 50% air at a flow rate of 2 L/min for 6 h. The sevoflurane group underwent inhalation anesthesia with 3% sevoflurane mixed with 50% O₂ and 50% air at 2 L/min for 6 h. In the sevoflurane + berberine group, berberine was administered intraperitoneally at a dose of 50 mg/kg/day, starting 1 week before sevoflurane anesthesia. Following these procedures, the Morris water maze (MWM), elevated plus maze (EPM), and novel object recognition tests were conducted. Brain tissues obtained from the remaining 7 rats were preserved for TUNEL staining. The expression levels of the investigated microRNAs and BDNF (brain-derived neurotrophic factor) in the frontal lobe, hippocampus, and plasma were determined using real-time quantitative polymerase chain reaction (qPCR), while BDNF levels were measured using enzyme-linked immunosorbent assay (ELISA).

Results. In the berberine group, compared with the sevoflurane group, plasma, frontal lobe, and hippocampal levels of microRNAs 9, 124, and 132 and BDNF were significantly increased, and TUNEL staining revealed a statistically significant reduction in apoptotic bodies.

Conclusions. This study indicates that berberine exerts beneficial effects on memory and learning at the molecular level in the geriatric population.

Key words: microRNA, berberine, sevoflurane, geriatric anesthesia

Highlights

- Berberine mitigates sevoflurane-induced cognitive impairment in aged rats, significantly improving memory and learning performance in behavioral tests.
- Neuroprotective effects of berberine are mediated by microRNA regulation, with increased expression of miR-9, miR-124, and miR-132 in the hippocampus and frontal cortex.
- Berberine restores brain-derived neurotrophic factor (BDNF) levels, counteracting sevoflurane-associated neurotoxicity at both molecular and tissue levels.
- Reduced neuronal apoptosis following berberine treatment, as evidenced by decreased TUNEL-positive cells, highlights its potential role in preventing anesthesia-related neurodegeneration in the elderly.

Background

Postoperative cognitive dysfunction (POCD) is a form of cognitive impairment that may develop following anesthesia administration and surgical procedures. It is characterized by deficits in multiple cognitive domains, including information processing, visuospatial ability, executive function, attention, concentration, memory, and psychomotor speed.¹ The prevalence of cognitive dysfunction depends on several factors, including age, education level, sex, comorbidities, type of surgery, and assessment methods.² Postoperative cognitive dysfunction represents a common postoperative complication that affects the central nervous system in older adult patients after surgery and is associated with increased mortality, greater pain and distress, prolonged hospital stays, and higher healthcare costs.³ Investigating the pathophysiology of POCD may help identify new therapeutic targets, improve postoperative quality of life in older adults, and reduce clinical and social burdens. However, the pathophysiological mechanisms underpinning POCD remain unclear, effective preventive or therapeutic interventions are currently lacking, and no effective therapies are currently available.

Sevoflurane is a widely used volatile anesthetic agent owing to its rapid onset, fast induction, and precise controllability. Nevertheless, accumulating evidence indicates that exposure to sevoflurane-based anesthesia, particularly when repeated, may induce neuropathological alterations and contribute to long-term cognitive impairment in humans and animals.⁴ Experimental studies have demonstrated that sevoflurane increases hippocampal neuronal inflammation and apoptosis in aged rats.⁵ Proposed mechanisms underlying sevoflurane-induced POCD include neuroinflammation, alterations in neurotransmitter systems, reductions in brain-derived neurotrophic factor (BDNF) levels, mitochondrial oxidative stress, and dysregulation of amyloid- β metabolism.⁶

Berberine is a naturally occurring benzylisoquinoline alkaloid present in several medicinal plants and exhibits a broad spectrum of pharmacological activities.⁷ In addition to its well-documented anti-inflammatory, antioxidant, antitumor, and cholesterol-lowering properties,^{8–13} berberine has been shown to exert neuroprotective effects

and to ameliorate diabetes-associated cognitive decline in mice.^{14,15} Moreover, its protective effects in neurodegenerative disorders and aging-related cognitive impairment have attracted increasing attention.¹⁶

MicroRNAs (miRNAs) are small endogenous non-coding RNA molecules, approx. 21 nucleotides in length, that act as key epigenetic regulators of gene expression.¹⁷ Among them, microRNA-9 (miR-9) is highly expressed in the mammalian brain and plays a critical role in neuronal development and function by modulating the expression of ion channels, enzymes, transcription factors, and other essential molecular targets.¹⁸ MicroRNA-124 (miR-124) is essential for neuronal differentiation and maturation and facilitates neural reprogramming and functional integration of newly generated neurons.¹⁹ MicroRNAs miR-132 and miR-212 are co-transcribed from a shared genomic locus and are closely linked to BDNF signaling through the cyclic AMP response element-binding protein (CREB), a key transcriptional regulator of genes involved in synaptic plasticity and memory formation.²⁰

MicroRNA-134 (miR-134), a brain-specific miRNA, has been implicated in the regulation of synaptic activity and dendritic spine morphology.²¹ Together with miR-132 and miR-138, miR-134 contributes to the fine-tuning of dendritic spine structure, which represents the primary site of excitatory synaptic transmission. In hippocampal circuits – widely used as experimental models for learning and memory – miR-138 is abundantly expressed and serves as a critical regulator of excitatory synaptic activity in pyramidal neurons.²² We hypothesize that berberine administration prior to sevoflurane exposure would attenuate anesthesia-induced cognitive dysfunction by modulating key molecular pathways, particularly through the upregulation of BDNF, alteration of microRNA expression profiles, and reduction of neuronal apoptosis.

Objectives

In this study, we aimed to investigate the roles of miR-9, miR-124, miR-132, miR-134, miR-138, and BDNF and examine the neuroprotective effects of berberine in sevoflurane-

induced neurotoxicity in aged rats. Specifically, we sought to evaluate the impact of berberine on memory and learning in aged rats exposed to sevoflurane-based anesthesia.

Materials and methods

Fifty-six male Sprague Dawley rats (20–24 weeks old, weighing 350–500 g) were obtained from the Eskişehir Osmangazi University Saki Yenilli Experimental Animal Production and Application Laboratory (Eskişehir, Turkey). All experimental procedures were approved by the Animal Experiments Local Ethics Committee of Eskişehir Osmangazi University, Turkey (decision No. 893-1, issued on June 16, 2022). Animals were housed under controlled conditions (19–21°C, 12-h light/dark cycle) with ad libitum access to food and water. Rats were randomly assigned to 4 groups ($n = 14$ per group): control, sevoflurane, berberine, and sevoflurane + berberine. Berberine (50 mg/kg, intraperitoneally) was administered once daily for 7 days in the berberine and sevoflurane + berberine groups. The selected dose was below the reported LD₅₀ of berberine (57.61 mg/kg).²³ On the final day, rats in the sevoflurane and sevoflurane + berberine groups received inhalation anesthesia with 3% sevoflurane in a 50% O₂/air mixture for 6 h (2 L/min). Control animals were exposed to the same gas mixture without sevoflurane. Inclusion of a berberine-only group allowed evaluation of the independent effects of berberine in the absence of anesthesia. Twenty-four hours after anesthesia was designated as day 0. Behavioral assessments were conducted between days 1 and 5, including the elevated plus maze (EPM), novel object recognition, and Morris water maze (MWM), using standard protocols. After completion of behavioral testing, blood samples were collected, and the rats were euthanized. The hippocampus and frontal lobes were dissected for molecular and biochemical analyses. Tissues from 7 rats per group were used for histological evaluation, and the remaining samples were allocated to real-time quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) analyses.

Anxiety-like behavior was assessed using the EPM by recording the time spent in the open and closed arms and the number of arm entries. Spatial learning and memory were evaluated using the MWM by measuring escape latency, time spent in the target quadrant, swimming speed, and total distance traveled. Recognition memory was assessed using the novel object recognition test by calculating the relative exploration index and novelty preference ratio. Total RNA was isolated from hippocampal, frontal lobe, and serum samples, followed by cDNA synthesis. Expression levels of miR-9, miR-124, miR-132, miR-134, miR-138, and BDNF were quantified using SYBR Green-based quantitative PCR, and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are provided in Table 1. Brain-derived neurotrophic factor protein concentrations were measured using a commercially available ELISA kit

Table 1. Primer sequences used for qPCR analysis

Gene	Reverse/ forward primer	Primer sequence
miR-132	reverse	5'-CCTCGGACCAGCTGCTTT-3'
miR-132	forward	5'-AGCCTATATCCATTCTTGG-3'
miR-134	reverse	5'-CGGCTAACGTGGCAAGCT-3'
miR-134	forward	5'-AAGACAGACCATGGAGCAGAAATC-3'
miR-138	reverse	5'-AAGGGACAGACAGATACCCAC-3'
miR-138	forward	5'-TGAGCCTCAGACCCCTTCT-3'
BDNF	reverse	5'-AATCGCCAGCCAATTCTCTTT-3'
BDNF	forward	5'-CTACGAGACCAAGTGCAATCC-3'

miR – microRNA; BDNF – brain-derived neurotrophic factor; qPCR – real-time quantitative polymerase chain reaction.

according to the manufacturer's instructions (cat. No. 201-11-0477-96T; Shanghai Sunred Biological Technology Co., Ltd., Shanghai, China). Brain tissues were fixed, paraffin-embedded, and sectioned for histopathological evaluation. Apoptosis in the hippocampus was assessed using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and the apoptotic index was calculated as the percentage of TUNEL-positive cells. Immunohistochemical staining was performed, and staining intensity was quantified using the H-score method by blinded observers. Morphometric damage in the cortical and hippocampal regions was graded using a standardized semi-quantitative scale.

Statistical analyses

All analyses were conducted on 4 independent groups using IBM SPSS Statistics v. 28 (IBM Corp., Armonk, USA). Continuous outcomes were summarized using mean $\pm$ standard deviation (SD) and median (interquartile range (IQR)). Before inferential testing, distributional assumptions were assessed with the Shapiro–Wilk test and visual inspection of Q–Q plots; homogeneity of variances was examined using the Levene or Brown–Forsythe test. When assumptions were met, between-group differences were evaluated using one-way analysis of variance (ANOVA); otherwise, the Kruskal–Wallis test was applied. For outcomes with a significant omnibus result, pairwise post hoc comparisons employed Tukey's honestly significant difference (HSD) test (after ANOVA) or Dunn's test (after Kruskal–Wallis), with family-wise error controlled using the Holm or Bonferroni adjustment. All tests were two-tailed with $\alpha = 0.05$, and exact p-values are reported (very small values denoted as $p < 0.001$); significance levels are indicated as $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results

Results of the object recognition test are summarized in Table 2, and assumption checks are provided in Supplementary Table 1. As the assumptions of normality

Table 2. Object recognition test results

Variable	Control (n = 14) median [Q1, Q3]	Berberine (n = 12) median [Q1, Q3]	Sevoflurane + berberine (n = 14) median [Q1, Q3]	Sevoflurane (n = 13) median [Q1, Q3]	p-value*	Post hoc comparisons (adjusted p-value**)
Familiarization I	19.000 [10.000, 41.000]	28.000 [20.000, 44.500]	26.000 [15.000, 34.000]	25.000 [15.000, 30.000]	0.716	not significant
Familiarization II	13.000 [7.000, 24.000]	20.500 [10.000, 25.500]	16.000 [7.000, 27.000]	20.000 [14.000, 28.000]	0.670	not significant
Familiar object	8.500 [3.000, 20.000]	5.500 [1.000, 9.500]	13.500 [10.000, 17.000]	11.000 [5.000, 20.000]	0.019	1–2 (0.703) 1–3 (0.735) 1–4 (1.000) 2–3 (0.014) 2–4 (0.150) 3–4 (1.000)
Novel object	16.000 [12.000, 21.000]	14.500 [9.500, 21.000]	10.500 [8.000, 18.000]	6.000 [3.000, 9.000]	0.001	1–2 (1.000) 1–3 (0.580) 1–4 (0.001) 2–3 (0.019) 2–4 (0.001) 3–4 (0.143)

*Kruskal–Wallis Test; **Dunn’s test with Bonferroni correction. Q1 – 1st quartile; Q3 – 3rd quartile. Values in bold are statistically significant

Table 3. Object exploration and preference ratios across experimental groups

Variable	Control (n = 14) mean ±SD	Berberine (n = 12) mean ±SD	Sevoflurane + berberine (n = 14) mean ±SD	Sevoflurane (n = 13) mean ±SD	p-value	Post hoc comparisons (adjusted p-value)
Relative exploration time	0.309 ±0.332	0.405 ±0.319	−0.118 ±0.230	−0.375 ±0.378	<0.001*	1–2 (0.869) ^a 1–3 (0.005)^a 1–4 (<0.001)^a 2–3 (0.001)^a 2–4 (<0.001)^a 3–4 (0.166) ^a
Data presentation	median [Q1, Q3]	median [Q1, Q3]	median [Q1, Q3]	median [Q1, Q3]	–	–
Preference ratio	0.707 [0.481, 0.812]	0.688 [0.553, 0.821]	0.427 [0.333, 0.533]	0.211 [0.161, 0.429]	<0.001**	1–2 (1.000) ^b 1–3 (0.069) ^b 1–4 (<0.001)^b 2–3 (0.186) ^b 2–4 (0.002)^b 3–4 (0.768) ^b

*one-way analysis of variance (ANOVA); **Kruskal–Wallis test; ^aTukey’s honestly significant difference (HSD) test; ^b Dunn’s test with Bonferroni correction; SD – standard deviation; Q1 – 1st quartile; Q3 – 3rd quartile. Values in bold are statistically significant.

and/or homogeneity of variance were violated, between-group comparisons were performed using the Kruskal–Wallis test followed by Dunn–Bonferroni post hoc test.

Exploration times during the familiarization phases (phases I and II) did not differ among groups ($p = 0.716$ and $p = 0.670$), indicating comparable baseline exploratory behavior. For the familiar object, a significant group effect was observed ($p = 0.019$), with reduced exploration time in the berberine group compared with the sevoflurane + berberine group (adjusted $p = 0.014$). No other pairwise differences were significant.

Exploration of the novel object differed significantly among groups ($p = 0.001$). The sevoflurane group showed markedly reduced exploration compared with the control and berberine groups (both adjusted $p = 0.001$). In addition, the berberine group exhibited greater novel object exploration than the sevoflurane + berberine group (adjusted

$p = 0.019$), suggesting preservation of recognition performance with berberine treatment.

Novel object preference results are presented in Table 3, with assumption checks shown in Supplementary Table 2. Relative exploration time differed significantly among groups ($p < 0.001$); the control and berberine groups demonstrated higher values than both sevoflurane-exposed groups (all adjusted $p \leq 0.005$), while no difference was observed between the control and berberine groups. Preference ratio also varied significantly ($p < 0.001$), with lower values in the sevoflurane group compared with the control and berberine groups (adjusted $p < 0.01$).

The ELISA results for hippocampal tissue, frontal lobe tissue, and blood samples are summarized in Table 4. Hippocampal biomarker levels differed significantly among groups ($p = 0.02$), with higher concentrations in the sevoflurane + berberine group than in the control group (adjusted $p = 0.014$). Frontal lobe concentrations also showed

Table 4. Comparison of ELISA measurements of tissue and blood samples among groups

Variable	Control mean $\pm$ SD (n = 7)	Berberine mean $\pm$ SD (n = 7)	Sevo + berberine mean $\pm$ SD (n = 7)	Sevoflurane mean $\pm$ SD (n = 7)	p-value	Post hoc comparisons (adjusted p-value)
Hippocampus	0.678 $\pm$ 0.101	0.761 $\pm$ 0.077	0.858 $\pm$ 0.119	0.804 $\pm$ 0.103	0.020*	1–2 (0.436) ^a 1–3 (0.014)^a 1–4 (0.122) ^a 2–3 (0.300) ^a 2–4 (0.859) ^a 3–4 (0.747) ^a
Data presentation	median [Q1, Q3] (n = 7)	median [Q1, Q3] (n = 7)	median [Q1, Q3] (n = 7)	median [Q1, Q3] (n = 7)	–	–
Frontal lobe	0.490 [0.463, 0.590]	0.551 [0.511, 0.597]	0.307 [0.230, 0.339]	0.254 [0.237, 0.328]	0.003**	1–2 (1.000) ^b 1–3 (0.089) ^b 1–4 (0.163) ^b 2–3 (0.017)^b 2–4 (0.035)^b 3–4 (1.000) ^b
Data presentation	median [Q1, Q3] (n = 12)	median [Q1, Q3] (n = 11)	median [Q1, Q3] (n = 12)	median [Q1, Q3] (n = 11)	–	–
Blood ^c	1.028 [0.951, 1.161]	1.220 [1.196, 1.313]	1.064 [0.805, 1.388]	1.300 [1.083, 1.448]	0.027**	1–2 (0.093) ^b 1–3 (1.000) ^b 1–4 (0.053) ^b 2–3 (0.977) ^b 2–4 (1.000) ^b 3–4 (0.671) ^b

*one-way analysis of variance (ANOVA); **Kruskal–Wallis test; ^aTukey’s honestly significant difference (HSD) test; ^bDunn’s test with Bonferroni correction; ^cfor the blood variable, the Kruskal–Wallis test was significant (p = 0.027); however, no pairwise comparisons were significant after Bonferroni correction. ELISA – enzyme-linked immunosorbent assay. Values in bold are statistically significant.

significant group differences (p = 0.003), with higher levels in the control and berberine groups compared with the sevoflurane and sevoflurane + berberine groups. Blood biomarker levels differed at the group level (p = 0.027); however, no pairwise comparisons remained significant after correction.

Hippocampal expression levels of all examined miRNAs and BDNF differed significantly among groups (all p < 0.001; Table 5). Sevoflurane exposure was associated with pronounced downregulation of miRNAs and BDNF, whereas berberine treatment significantly attenuated these molecular alterations.

Significant group-dependent differences were observed for all frontal lobe miRNAs and BDNF (all p < 0.001; Table 6). Sevoflurane produced the lowest expression levels, while berberine administration resulted in higher expression of miR-9, miR-124, miR-132, miR-134, miR-138, and BDNF.

Serum miRNA and BDNF results are shown in Table 7. Significant group differences were detected for all markers except miR-9 (p = 0.086). Sevoflurane exposure significantly reduced serum miR-124, miR-132, miR-134, miR-138, and BDNF levels, whereas berberine treatment reversed these reductions and yielded the highest overall expression levels. Representative TUNEL-stained histopathological images of the hippocampus and cortex are presented in Fig. 1 and Fig. 2, respectively.

Across behavioral, biochemical, and molecular analyses, sevoflurane consistently impaired recognition-related performance and suppressed miRNA and BDNF expression in brain tissue and serum. Berberine treatment mitigated

these effects, preserving cognitive performance and restoring molecular profiles.

Discussion

This study measured hippocampal, frontal lobe, and serum levels of BDNF and miR-9, miR-124, miR-132, miR-134, and miR-138, which are regulators of dendritic outgrowth, synaptic plasticity, long-term potentiation, memory, and learning, and demonstrated that berberine attenuated the adverse effects of sevoflurane. TUNEL staining also demonstrated a statistically significant reduction in the apoptotic index and damage scores in the hippocampus and cortex of the berberine-treated groups.

Behavioral assessments showed that sevoflurane exposure impaired cognitive performance, as reflected by reduced exploration of novel objects in the object recognition test and decreased time spent in the open arms of the EPM. The MWM further confirmed spatial memory deficits, with prolonged escape latency and reduced time spent in the target quadrant. In contrast, berberine treatment ameliorated these impairments, improving exploration indices, memory retention, and anxiety-related behaviors. At the molecular level, hippocampal analyses revealed that berberine markedly upregulated miR-9, miR-132, miR-134, and BDNF expression, while sevoflurane significantly suppressed miR-9, miR-124, miR-132, and BDNF levels. Frontal lobe qPCR data showed a similar pattern, with miR-9 and miR-124 downregulated and miR-138 markedly

Table 5. Comparison of qPCR measurements of miRNAs and BDNF in hippocampus samples among groups

Variable	Control (n = 7) median [Q1, Q3]	Berberine (n = 7) median [Q1, Q3]	Sevoflurane + berberine (n = 7) median [Q1, Q3]	Sevoflurane n = 7) median [Q1, Q3]	p-value*	Post hoc comparisons (adjusted p-value**)
miR-9	0.510 [0.302, 1.062]	3.189 [2.896, 3.452]	0.232 [0.298, 0.361]	0.031 [0.028, 0.040]	<0.001	1–2 (0.285) 1–3 (1.000) 1–4 (0.031) 2–3 (0.031) 2–4 (<0.001) 3–4 (0.285)
miR-124	1.168 [0.896, 1.760]	1.770 [1.418, 2.116]	0.727 [0.551, 0.973]	0.315 [0.239, 0.354]	<0.001	1–2 (1.000) 1–3 (0.713) 1–4 (0.008) 2–3 (0.051) 2–4 (<0.001) 3–4 (0.585)
miR-132	1.557 [1.289, 1.746]	4.460 [2.998, 5.363]	1.929 [1.315, 2.392]	0.318 [0.289, 0.366]	<0.001	1–2 (0.150) 1–3 (1.000) 1–4 (0.126) 2–3 (0.384) 2–4 (<0.001) 3–4 (0.042)
miR-134	3.319 [2.693, 3.797]	6.590 [5.262, 7.239]	1.606 [1.326, 2.144]	1.553 [1.392, 1.666]	<0.001	1–2 (0.585) 1–3 (0.264) 1–4 (0.126) 2–3 (0.001) 2–4 (<0.001) 3–4 (1.000)
miR-138	1.707 [1.066, 1.825]	0.601 [0.459, 0.858]	0.859 [0.502, 1.105]	1.486 [1.476, 2.316]	<0.001	1–2 (0.010) 1–3 (1.000) 1–4 (1.000) 2–3 (0.081) 2–4 (0.001) 3–4 (0.862)
BDNF	1.756 [1.381, 2.289]	1.324 [1.577, 1.882]	1.294 [0.915, 1.514]	0.409 [0.297, 0.452]	<0.001	1–2 (0.244) 1–3 (1.000) 1–4 (0.038) 2–3 (0.038) 2–4 (<0.001) 3–4 (0.244)

*Kruskal–Wallis Test; **Dunn's test with Bonferroni correction. miR – microRNA; BDNF – brain-derived neurotrophic factor; qPCR – real-time quantitative polymerase chain reaction. Values in bold are statistically significant.

reduced in the sevoflurane group, whereas berberine preserved expression levels closer to those of the control group. Serum analyses supported these findings, demonstrating significantly reduced miR-124 and BDNF levels in the sevoflurane group and a marked elevation of BDNF in the berberine group. Collectively, these results indicate that berberine mitigates sevoflurane-induced cognitive and molecular impairments by preserving memory performance and normalizing miRNA and BDNF expression.

Previous studies have examined the effects of sevoflurane on memory. In one study, 60 male Wistar rats (200–250 g) were divided into 6 groups: control, diabetic control, sevoflurane anesthesia, isoflurane anesthesia, diabetic sevoflurane anesthesia, and diabetic isoflurane anesthesia. Animals received 2.5% sevoflurane or 1.5% isoflurane for 2 h, and the MWM test was conducted 1 week later. While the diabetic control and sevoflurane anesthesia groups showed no significant changes, the isoflurane group had a longer escape latency than the controls, suggesting impaired

learning and memory. Diabetic rats exposed to either anesthetic also spent less time in the target quadrant than the controls.²⁴ Unlike that study, which initiated MWM testing 1 week after exposure, we began testing 1 day after sevoflurane exposure and found a statistically significant increase in escape latency in the sevoflurane group.

Brain-derived neurotrophic factor, a member of the neurotrophin family, activates intracellular signaling pathways such as mitogen-activated protein kinase/extracellular signal-regulated kinase and phosphoinositide 3-kinase through stimulation of tropomyosin receptor kinase B. It has been shown to upregulate miR-132 expression in cortical neuron cultures.²⁵ MicroRNA-132 promotes primary neuron growth. Consistent with these findings, our study demonstrated significantly higher BDNF and miR-132 levels in the frontal cortex and hippocampus of the berberine groups compared with the sevoflurane group. The relationship between BDNF and miR-132 underscores the significance of our results.

Table 6. Frontal lobe miRNA and BDNF expression levels among groups

Variable	Control (n = 7) median [Q1, Q3]	Berberine (n = 7) median [Q1, Q3]	Sevoflurane + berberine (n = 7) median [Q1, Q3]	Sevoflurane (n = 7) median [Q1, Q3]	p-value	Post hoc comparisons (adjusted p-value)
miR-9	1.5958 [1.3006, 1.7962]	4.4164 [2.7036, 5.5331]	0.2470 [0.1738, 0.3451]	0.0302 [0.0192, 0.0392]	<0.001**	1–2 (1.000) ^b 1–3 (0.476) ^b 1–4 (0.005)^b 2–3 (0.015)^b 2–4 (<0.001)^b 3–4 (0.668) ^b
miR-124	1.1810 [0.7873, 1.2620]	1.9725 [1.5083, 2.5917]	0.7321 [0.6433, 0.8332]	0.0576 [0.0524, 0.0830]	<0.001**	1–2 (0.476) ^b 1–3 (1.000) ^b 1–4 (0.022)^b 2–3 (0.044)^b 2–4 (<0.001)^b 3–4 (0.285) ^b
miR-132	1.3078 [1.0057, 1.4851]	1.8392 [1.7357, 2.9694]	0.8649 [0.7227, 1.3353]	0.5772 [0.4571, 0.7413]	<0.001**	1–2 (0.547) ^b 1–3 (1.000) ^b 1–4 (0.089) ^b 2–3 (0.028)^b 2–4 (<0.001)^b 3–4 (1.000) ^b
miR-138	1.2220 [0.9394, 1.4128]	2.4260 [2.0820, 2.8448]	0.3786 [0.2720, 0.4576]	0.2635 [0.2402, 0.3199]	<0.001**	1–2 (1.000) ^b 1–3 (0.163) ^b 1–4 (0.028)^b 2–3 (0.002)^b 2–4 (<0.001)^b 3–4 (1.000) ^b
BDNF	1.7205 [1.3541, 2.0645]	2.2304 [1.7699, 4.0179]	1.1083 [0.9288, 1.6123]	0.4772 [0.3298, 0.4890]	<0.001**	1–2 (1.000) ^b 1–3 (1.000) ^b 1–4 (0.007)^b 2–3 (0.307) ^b 2–4 (<0.001)^b 3–4 (0.177) ^b
Data presentation	mean ±SD	mean ±SD	mean ±SD	mean ±SD	–	–
miR-134	3.21 ±0.88	6.36 ±1.76	1.79 ±0.65	1.59 ±0.35	<0.001*	1–2 (<0.001)^a 1–3 (0.081) ^a 1–4 (0.037)^a 2–3 (<0.001)^a 2–4 (<0.001)^a 3–4 (0.981) ^a

*one-way analysis of variance (ANOVA); **Kruskal–Wallis test; ^aTukey’s honestly significant difference (HSD) test; ^bDunn’s test with Bonferroni correction. miR – microRNA; BDNF – brain-derived neurotrophic factor; qPCR – quantitative polymerase chain reaction. Values in bold are statistically significant.

Our study also revealed higher miR-9 expression, increased active microglia, and reduced neuronal cell counts in rats, similar to findings from a study in mice with late-stage Alzheimer’s disease.²⁶ In the present study, hippocampal miR-9 expression was markedly suppressed following sevoflurane exposure, while berberine administration significantly increased miR-9 levels compared with the sevoflurane group, suggesting a potential protective role against anesthesia-induced neurodegeneration.

MicroRNA-124 plays a central role in neuronal cell proliferation, differentiation, migration, memory formation, apoptosis, and neurodegenerative diseases. Experimental studies have shown that reduced miR-124 expression is associated with increased neuronal apoptosis and impaired axonal growth.^{27,28} In line with these observations, the present study demonstrated significantly reduced miR-124 expression and increased hippocampal apoptosis in the sevoflurane group, as confirmed

by TUNEL staining, whereas berberine treatment attenuated these effects.

MicroRNA-134, a brain-specific microRNA primarily localized to the synpto-dendritic region, is thought to negatively regulate dendritic spine development and plasticity.²⁹ Recent findings indicate that miR-134 inhibits hippocampal synaptic plasticity by targeting plasticity-related proteins, such as LIM kinase 1, resulting in cognitive deficits. In contrast, our study found increased hippocampal miR-134 expression in the berberine group.

MicroRNA-138 has been shown to reduce dendritic spine size and suppress excitatory synaptic transmission in hippocampal neurons.³⁰ Elevated miR-138 levels inhibit SIRT1 signaling, impair axonal growth, and are associated with stress-related behavioral alterations.^{31,32} Consistent with these findings, hippocampal miR-138 expression was significantly higher in the sevoflurane group and accompanied by reduced BDNF levels, whereas

Table 7. Serum miRNA and BDNF expression levels among groups

Variable	Control (n = 10) median [Q1, Q3]	Berberine (n = 10) median [Q1, Q3]	Sevoflurane + Berberine (n = 10) median [Q1, Q3]	Sevoflurane (n = 10) median [Q1, Q3]	p*	Post-hoc comparisons (adjusted p-value**)
miR-9	1.0321 [0.9235, 1.2588]	1.2418 [0.8247, 1.6607]	0.8461 [0.6471, 1.2854]	0.7562 [0.5148, 0.9717]	0.086	not significant
miR-124	0.7471 [0.6764, 0.7916]	0.9967 [0.4750, 1.1589]	0.3488 [0.2973, 0.3753]	0.0357 [0.0161, 0.0374]	<0.001	1–2 (1.000) 1–3 (0.096) 1–4 (<0.001) 2–3 (0.112) 2–4 (<0.001) 3–4 (0.151)
miR-132	1.2839 [0.8409, 1.8089]	0.5110 [0.3978, 0.6397]	0.5359 [0.3209, 0.6792]	1.6602 [0.8766, 1.6589]	<0.001	1–2 (0.003) 1–3 (0.004) 1–4 (1.000) 2–3 (1.000) 2–4 (0.001) 3–4 (0.002)
miR-134	1.3263 [0.9337, 1.5594]	0.6035 [0.5605, 0.6666]	0.9375 [0.6579, 1.4756]	1.1480 [1.1322, 1.1704]	<0.001	1–2 (0.034) 1–3 (1.000) 1–4 (<0.001) 2–3 (0.770) 2–4 (0.193) 3–4 (0.001)
miR-138	1.5863 [1.3528, 1.9145]	2.3118 [1.8627, 3.2393]	0.8288 [0.6017, 1.2684]	0.5734 [0.4087, 0.8303]	<0.001	1–2 (1.000) 1–3 (0.077) 1–4 (0.002) 2–3 (0.002) 2–4 (<0.001) 3–4 (1.000)
BDNF	0.6699 [0.4104, 1.0830]	14.8665 [7.7008, 16.8860]	5.7023 [2.3426, 5.9230]	0.2259 [0.1272, 0.2732]	<0.001	1–2 (<0.001) 1–3 (0.198) 1–4 (0.799) 2–3 (0.406) 2–4 (<0.001) 3–4 (0.002)

*Kruskal–Wallis test; **Dunn's test with Bonferroni correction. miR – microRNA; BDNF – brain-derived neurotrophic factor; qPCR – quantitative polymerase chain reaction. Values in bold are statistically significant.

berberine treatment normalized both miR-138 and BDNF expression.

Saha et al.³³ established an epilepsy model in male Wistar rats by intraperitoneal administration of pentylenetetrazol (30 mg/kg) every other day for 6 weeks. Rats with pentylenetetrazol-induced epilepsy were treated with 50, 100, and 200 mg/kg of berberine and 25, 50, and 100 mg/kg of nano-berberine. Outcomes included seizure scores, the percentage of rats experiencing seizures, histopathological scores, oxidative stress, apoptosis, and inflammation. None of the animals in the nano-berberine group exhibited seizures. This effect was attributed to significantly increased levels of antioxidants, such as superoxide dismutase and glutathione, in the nano-berberine group, whereas standard berberine did not produce a comparable antioxidant response. These results suggest that the superior antioxidant properties of nano-berberine may be due to its higher plasma concentration compared with standard berberine. Nano-berberine exhibited greater antioxidant properties than berberine.

In the present study, berberine hydrochloride was freshly prepared in 0.9% isotonic saline immediately before administration. Histological TUNEL staining showed

significantly reduced apoptosis in the berberine-treated group compared with the sevoflurane group; however, the absence of significant differences in behavioral tests may be attributed to the rapid metabolism, slow elimination, and short half-life of berberine, which may limit plasma levels. This limitation could potentially be addressed by developing novel nano-formulations with improved bioavailability. Future studies using nano-berberine are expected to yield more pronounced effects than both sevoflurane and the control group.

Previous studies have demonstrated that miR-124, miR-132, miR-134, and miR-138 play critical roles in neuronal differentiation, neurite outgrowth, dendritic spine remodeling, synaptic plasticity, and axonal regeneration, and that dysregulation of these microRNAs is associated with stress-related, anesthesia-induced, and age-related cognitive impairment and affective behavioral changes.^{28–32} In this context, our findings suggest that berberine exerts neuroprotective effects, potentially through modulation of miRNA expression, BDNF levels, and reduced apoptosis. It is important to acknowledge that berberine has a broad pharmacological profile. In addition to its effects on neuronal signaling,

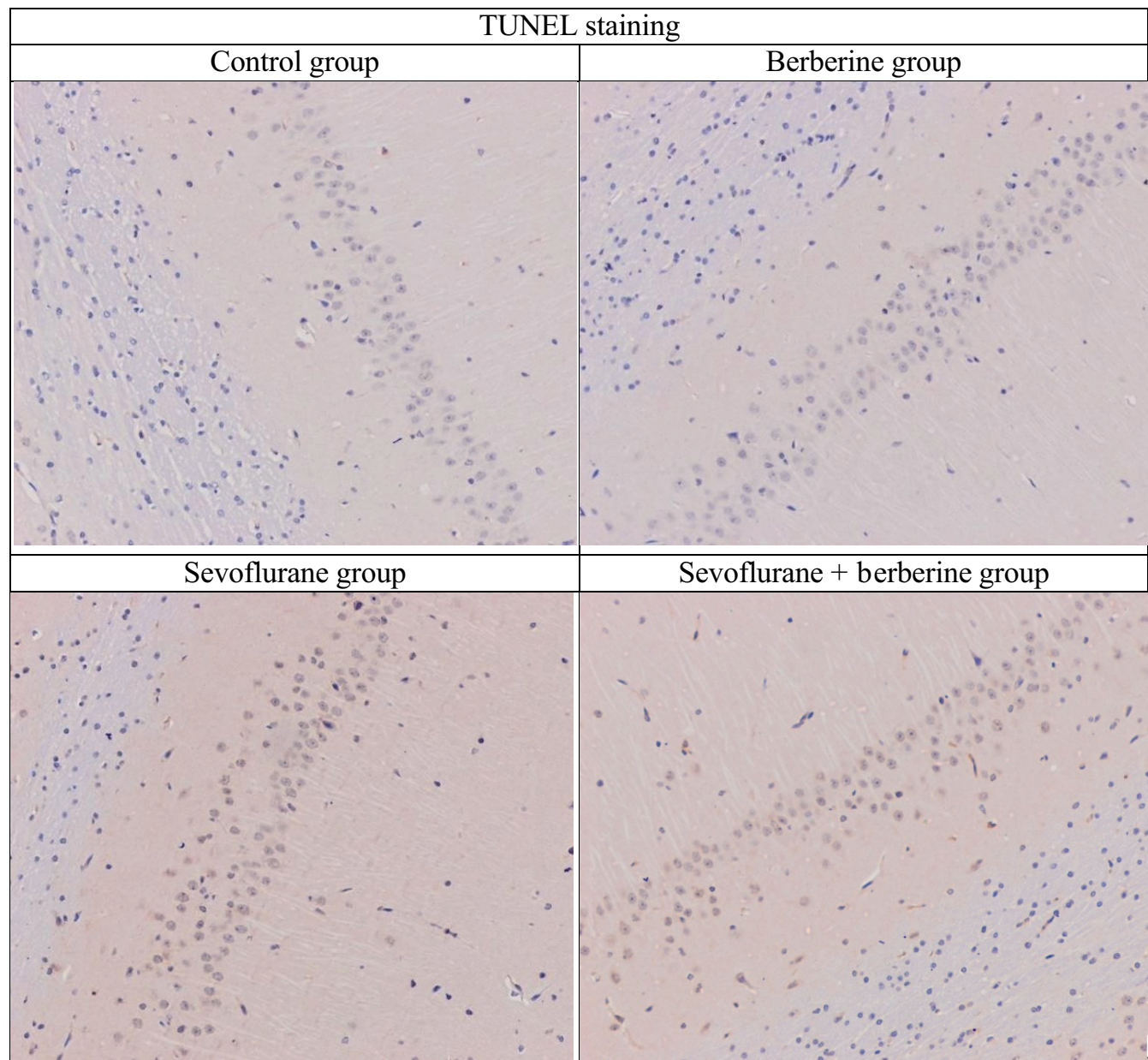


Fig. 1. Histopathological images of the hippocampus at $\times 200$ magnification with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

berberine is known to exert anti-inflammatory, antioxidant, and metabolic effects, all of which could contribute to the observed behavioral improvements.^{34,35} Furthermore, although rodent models provide valuable mechanistic insight, interspecies differences in brain structure, pharmacokinetics, and immune responses limit direct translational applicability to postoperative cognitive dysfunction in humans.³⁶ Additionally, our study was conducted exclusively in male animals, which may overlook potential sex-based differences in response to berberine, as previous studies have shown that pharmacokinetic and pharmacodynamic profiles can vary significantly by sex.³⁷

Finally, the observed miRNA alterations are consistent with emerging evidence that integrative bioinformatics and machine learning approaches can identify miRNA-driven regulatory networks and protective biomarkers in complex

diseases, including neurocognitive and oncological conditions.³⁸ Future studies should therefore incorporate both sexes, explore longer-term outcomes, and evaluate alternative dosing strategies to better inform clinical applications.

Limitations of the study

Our study had several limitations. First, isotonic fluid maintenance was not administered for 6 h during sevoflurane anesthesia, and neither the minimum alveolar concentration of sevoflurane nor mean arterial blood pressure were continuously monitored. Second, the anti-inflammatory effects of berberine were not specifically evaluated, and the 1-week pre-anesthesia administration of berberine may be insufficient to assess its potential for long-term neuroprotection. In this study, our primary objective was

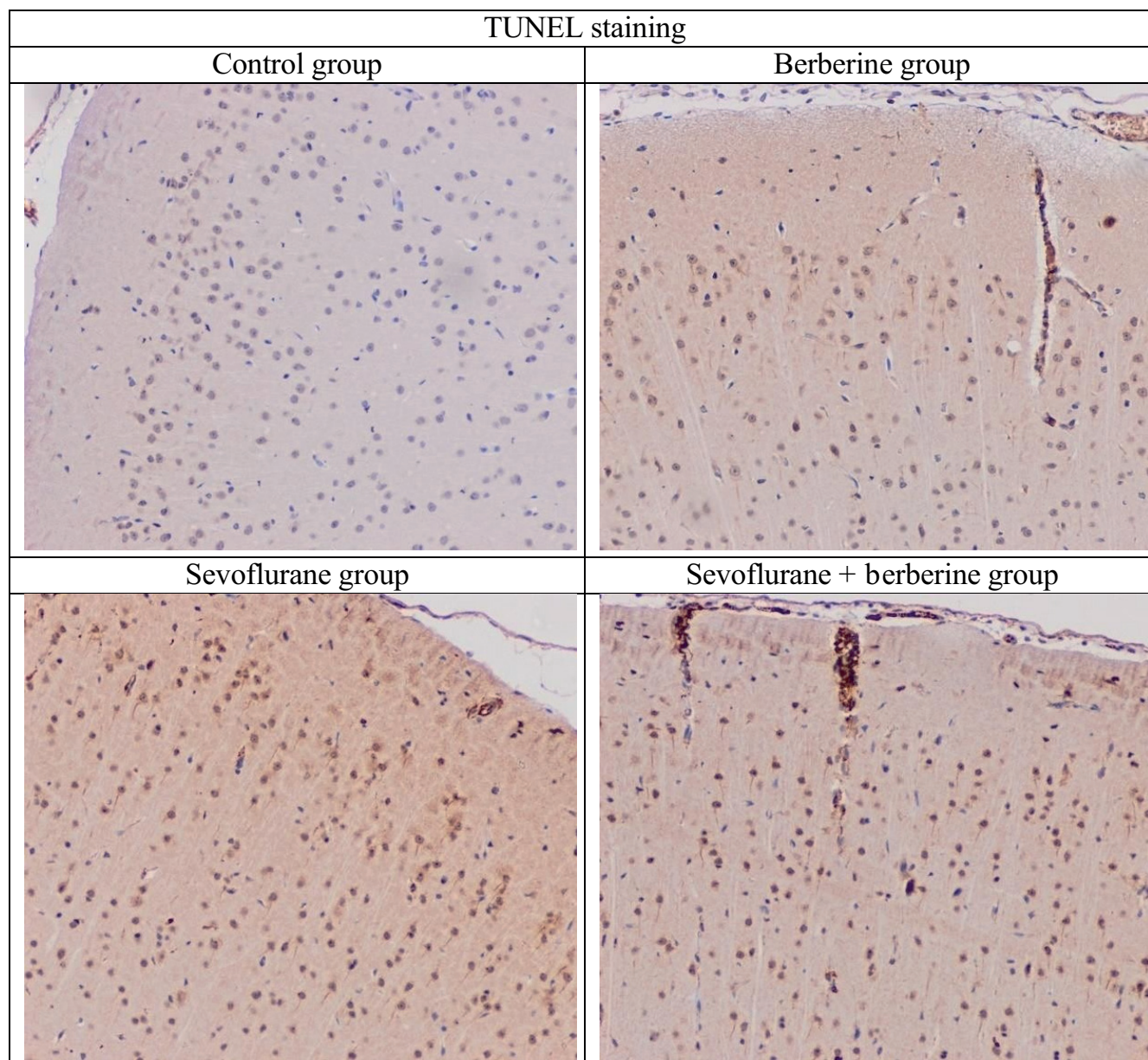


Fig. 2. Histopathological images of the cortex at $\times 200$ magnification with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

to assess the prophylactic effects of berberine in the early phase following anesthesia; therefore, berberine administration was not continued post-anesthesia, and we recognize that this may have limited our ability to assess the sustainability of its effects. In addition, we acknowledge that the short interval between anesthesia, behavioral testing, and tissue collection may not fully capture delayed or persistent cognitive deficits, which are indeed critical in the context of POCD. Finally, the lack of nano-berberine administration in our study is a limitation.

Conclusions

This study evaluated the hippocampal, frontal lobe, and serum levels of BDNF and miRNAs 9, 124, 132, 134,

and 138, which regulate dendritic outgrowth, synaptic plasticity, long-term potentiation, memory, and learning, and showed that berberine ameliorated the detrimental effects of sevoflurane. TUNEL staining also demonstrated a significant reduction in the apoptotic index and damage scores in the hippocampus and cortex of the berberine-treated groups. However, the absence of significant differences among groups in behavioral tests such as the MWM and EPM suggests that these effects may not translate into observable clinical improvement. To enhance clinical relevance, further research should examine berberine in nano-formulations, evaluate longer treatment durations, and assess continued administration after sevoflurane exposure. These findings may help optimize the neuroprotective potential and clinical relevance of berberine.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.17822503>. The package contains the following files:

Supplementary Table 1. Results of assumption checks for variables in Table 2 (with p-values).

Supplementary Table 2. Results of assumption checks for variables in Table 3 (with p-values).

Supplementary Table 3. Results of assumption checks for variables in Table 4 (with p-values).

Supplementary Table 4. Results of assumption checks for variables in Table 5 (with p-values).

Supplementary Table 5. Results of assumption checks for variables in Table 6 (with p-values).

Supplementary Table 6. Results of assumption checks for variables in Table 7 (with p-values).

Data Availability Statement

The datasets supporting the findings of the current study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17831138>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Machine learning identifies autophagy biomarkers driving microvascular injury in cardiac ischemia/reperfusion

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

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Conflict of interest

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Abstract

Background. Ischemic heart disease (IHD) remains a leading global cause of mortality. While reperfusion is essential for treating ischemic cardiomyopathy, it paradoxically induces myocardial ischemia/reperfusion (I/R) injury targeting the microvascular endothelium. The link between autophagy and microvascular damage in myocardial I/R requires clarification.

Objectives. This study aimed to identify autophagy-related biomarkers of myocardial I/R injury using integrated bioinformatics and experimental validation, characterize the associated molecular and immune features, and evaluate the role of Myc in cardiac microvascular endothelial injury.

Materials and methods. Bioinformatics analysis of Gene Expression Omnibus (GEO) multi-chip datasets intersected autophagy-related genes (HADB) with differentially expressed genes (DEGs) to identify signature biomarkers using machine-learning algorithms. Diagnostic efficacy was validated using a nomogram. Mechanistic exploration through gene set enrichment analysis (GSEA) and immune profiling revealed I/R subtypes. Biomarker expression was externally validated in the GSE168610 dataset. Myocardial I/R models were assessed using echocardiography (cardiac function), Evans blue/TTC staining (infarction area), and ink perfusion (microvascular density). Hub gene expression was quantified using quantitative polymerase chain reaction (qPCR), western blot (WB), and immunohistochemistry (IHC). In vitro, c-Myc-knockout cardiac endothelial cells underwent hypoxia/reoxygenation (H/R), followed by analysis of autophagy (WB), apoptosis (TUNEL), and autophagic vacuoles (transmission electron microscopy (TEM)).

Results. Seven autophagy-related DEGs were identified and linked to muscle proliferation, protease activation, and oncogenesis. Four signature genes (*Casp4*, *Cdkn1a*, *Myc*, and *Rgs19*) were identified by 3 machine-learning algorithms, with diagnostic validation. In the development cohort, the AUCs ranged from 0.756 to 0.876. In the external-validation cohort, the AUC was 1.000 for *Casp4*, *Myc*, and *Rgs19* and 0.875 for *Cdkn1a*. GSEA linked these genes to tricarboxylic acid cycle (TCA) dysregulation and immune infiltration. Unsupervised clustering identified 2 molecular subtypes. C1 exhibited higher expression of 6 of the 7 autophagy-related DEGs, whereas C2 showed higher proportions of mast cells and activated dendritic cells and enrichment of mitochondrial and energy-metabolism pathways. c-Myc knockout reduced endothelial apoptosis and autophagic injury.

Conclusions. c-Myc emerged as a critical driver linking excessive autophagy to microvascular damage, providing a promising diagnostic and therapeutic target for reperfusion injury management.

Key words: autophagy, biomarkers, myocardial ischemia, reperfusion injury, microcirculation

Highlights

- In the development cohort, the AUCs ranged from 0.756 to 0.876; in the external-validation cohort, the AUCs ranged from 0.875 to 1.000.
- The study demonstrates that *c-Myc* is a critical regulator connecting excessive autophagy with microvascular endothelial injury during myocardial reperfusion, positioning it as a potential therapeutic target.
- Unsupervised clustering identified 2 molecular subtypes. C1 showed higher expression of 6 of the 7 autophagy-related DEGs and greater M0 macrophage infiltration, whereas C2 showed higher proportions of mast cells and activated dendritic cells and enrichment of mitochondrial and energy-metabolism pathways.
- Biomarker expression was validated in external datasets and I/R models using qPCR, Western blot, and immunohistochemistry, confirming their association with cardiac dysfunction, infarct size, and microvascular density.

Background

Ischemic heart disease (IHD) remains one of the leading causes of mortality worldwide, with both its prevalence and mortality increasing substantially on a global scale.¹ Over the past 3 decades, the incidence and mortality of IHD have increased by 103.5% and 60.4%, respectively, and are projected to continue rising over the next 2 decades.² Although early reperfusion remains the cornerstone of treatment for ischemic cardiomyopathy, it can paradoxically induce ischemia/reperfusion (I/R) injury. Myocardial I/R injury is characterized by tissue damage resulting from insufficient blood supply during ischemia, which is exacerbated upon reperfusion.³ The pathological manifestations of myocardial I/R include myocardial stunning, the no-reflow phenomenon, reperfusion arrhythmias, and lethal reperfusion injury.⁴ The no-reflow phenomenon, driven by microvascular injury, is a fundamental mechanism of myocardial I/R injury and an independent predictor of mortality and recurrent myocardial infarction.^{5,6} Compared with cardiomyocytes, cardiac microvascular endothelial cells sustain earlier and more severe injury during myocardial I/R.⁷ This injury is closely associated with several pathogenic mechanisms, including calcium overload,⁸ endoplasmic reticulum stress,⁹ mitochondrial dysfunction, and impaired mitochondrial quality control.^{10,11} Despite extensive research aimed at elucidating the mechanisms underlying myocardial I/R-induced microvascular injury and developing novel therapeutic strategies, early diagnosis, timely intervention, and a comprehensive understanding of the underlying mechanisms remain major challenges.

With increasing insight into the pathophysiological mechanisms of microvascular injury during myocardial I/R, autophagy has emerged as a promising therapeutic target. Autophagy, a fundamental intracellular degradation and recycling process, plays an essential role in cardiovascular homeostasis by removing damaged mitochondria and protein aggregates.¹² While physiological levels of autophagy support cellular homeostasis,

excessive activation may lead to cellular dysfunction and death.¹³ Accumulating evidence has linked dysregulated autophagy to the initiation and progression of microvascular injury during myocardial I/R. Cardiac microvascular endothelial cells, which account for more than 60% of the total cardiac cell population, possess a tightly regulated autophagic system. Impaired autophagy can compromise endothelial cell integrity.¹⁴ Experimental models of myocardial I/R have demonstrated excessive autophagy in cardiac microvascular endothelial cells and have shown that inhibition of autophagy attenuates cellular injury and apoptosis.^{15,16} Conversely, autophagy activation has also been reported to exert protective effects against myocardial I/R injury, highlighting its dual role in the I/R process.¹⁷ Therefore, a comprehensive evaluation of autophagy may improve our understanding of the molecular mechanisms underlying the no-reflow phenomenon associated with microvascular I/R injury and facilitate the identification of potential diagnostic biomarkers.

Objectives

Despite advances in cardiac I/R research at the genomic level, studies specifically investigating autophagy-related genes in the context of myocardial I/R-induced microvascular injury remain limited. This gap hinders a comprehensive understanding of the underlying molecular mechanisms and the identification of potential diagnostic biomarkers. Therefore, the present study was designed to identify and characterize key autophagy-related genes associated with microvascular injury during myocardial I/R.

This study aimed to identify novel biomarkers for the diagnosis of myocardial I/R injury by integrating bioinformatics and experimental validation. It also investigated the functional role of dysregulated autophagy in microvascular injury, identified key biomarkers, and explored the mechanistic link between autophagy, microvascular injury, and the regulator *c-Myc*.

Materials and methods

Microarray data retrieval and pre-processing

Cardiac I/R datasets were retrieved from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>) using the keywords “myocardial ischemia/reperfusion injury” and “cardiac ischemia/reperfusion injury”. Seven datasets (GSE108940, GSE115568, GSE4105, GSE58486, GSE61592, GSE83472, and GSE168610) were retrieved from the GEO database on June 5, 2024. A combined multi-chip cohort comprising 25 sham samples and 28 I/R samples from GSE108940, GSE115568, GSE4105, GSE58486, GSE61592, and GSE83472 (Supplementary Table 1) underwent batch-effect correction using the SVA R package (<https://www.bioconductor.org/packages/release/bioc/html/sva.html>). The effectiveness of batch correction was assessed using principal component analysis (PCA) plots generated with the ggplot2 package (<https://cran.r-project.org/web/packages/ggplot2/index.html>). The first 6 datasets were used to identify differentially expressed genes (DEGs), whereas GSE168610 was used for external validation.

DEGs identification

Differential expression analysis between the sham and I/R groups was performed using the limma R package (<https://bioconductor.org/packages/release/bioc/html/limma.html>). Heatmaps and volcano plots were generated using the pheatmap (<https://cran.r-project.org/web/packages/pheatmap/index.html>) and ggplot2 packages. Genes with a $p < 0.05$ and an absolute $\log_2$ fold change ($|\log_2\text{FC}| > 1.0$) were considered DEGs. The top 20 DEGs were visualized in a heatmap.

Identification of DEGs related to autophagy and functional enrichment analysis

A total of 215 autophagy-related genes (ARGs) were retrieved from the Human Autophagy Database (HADb; <http://autophagy.lu>) and converted to their mouse homologs. Differentially expressed autophagy-related genes (ARDEGs) were identified using Venn diagram analysis by intersecting the DEGs with the ARGs. Functional enrichment analysis of ARDEGs was performed using Gene Ontology (GO) (<http://geneontology.org>) analysis (biological process (BP), cellular component (CC), and molecular function (MF)) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp>) pathway analysis (ClusterProfiler; <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>). Results with a $p < 0.05$ were visualized as bubble plots using ggplot2.

Recognition of hub genes based on well-established machine learning algorithms

Machine-learning algorithms – including least absolute shrinkage and selection operator (LASSO; glmnet R package (<https://cran.r-project.org/web/packages/glmnet/index.html>)), support vector machine–recursive feature elimination (SVM-RFE; e1071/MSVM-RFE), and Random Forest (RF; randomForest (<https://cran.r-project.org/web/packages/randomForest/index.html>)) – were used to identify signature genes associated with cardiac microvascular I/R injury. Using the 7 shared ARDEGs as input, LASSO with 10-fold cross-validation was applied to calculate regression coefficients and identify the optimal features based on partial likelihood deviance and λ -logarithm curves (cv.glmnet; <https://www.rdocumentation.org/packages/glmnet/versions/5.0/topics/cv.glmnet>). SVM-RFE performed sequential backward feature selection to identify key hub genes at the highest 5-fold cross-validation accuracy (highlighted by a red circle). Random Forest ranked gene importance using an ensemble of decision trees. Hub genes were defined as those identified by all 3 machine-learning algorithms, as illustrated in the Venn diagram.

Nomogram construction and ROC evaluation

To further evaluate the predictive performance of the model, a nomogram was constructed by integrating the 4 hub genes using the rms package (<https://cran.r-project.org/web/packages/rms/index.html>) in R. A score was assigned to the expression level of each gene, and the total score was used to estimate the probability of I/R injury. Model performance was evaluated using decision curve analysis (DCA) and calibration curves. In addition, the area under the receiver operating characteristic (ROC) curve (AUC) was calculated to assess the diagnostic performance of each candidate biomarker.

Single-gene gene set enrichment analysis

Single-gene gene set enrichment analysis (GSEA) of the 4 diagnostic genes was performed using the ClusterProfiler package. To investigate the association between the underlying molecular mechanisms and gene expression phenotypes, Hallmark gene sets were retrieved from the Molecular Signatures Database (MSigDB; version accessed May 18, 2024) (<https://www.gsea-msigdb.org/gsea/msigdb/mouse/genesets.jsp?collection=MH>). Enrichment plots were generated to display the top 5 positively and negatively enriched pathways for each gene in the 2 study groups.

Immune cell infiltration analysis

The CIBERSORT R package (<https://github.com/Moonerss/CIBERSORT>) was used to estimate the relative

proportions of immune cell populations in the combined multi-chip datasets from the I/R and sham groups. Based on gene expression data, the CIBERSORT algorithm was applied to estimate immune cell abundance by deconvolution, and the results were visualized as bar plots. In addition, the ggplot2 package was used to generate lollipop plots illustrating the correlations between the expression of each hub gene and immune cell abundance.

Unsupervised clustering analysis of I/R subtypes

Ischemia/reperfusion subtypes were identified using consensus clustering with the ConsensusClusterPlus R package (<https://www.rdocumentation.org/packages/ConsensusClusterPlus/versions/1.36.0/topics/ConsensusClusterPlus>) based on the expression profiles of AR-DEGs (1,000 iterations). The optimal number of clusters was determined using the consensus matrix, cumulative distribution function (CDF) index, and the relative change in the area under the CDF curve. Intercluster heterogeneity was assessed using PCA and box plots. Functional differences, including immune cell infiltration and KEGG/GO pathway enrichment, were compared between Cluster 1 and Cluster 2.

Validation of hub genes within the dataset

The GSE168610 dataset served as an external validation cohort. Raw data retrieved from the GEO database underwent expression matrix transformation using Perl and normalization with the limma R package. Expression levels of the 4 hub genes were extracted, compared between groups using the Wilcoxon rank-sum test, and visualized as violin plots. Diagnostic performance was evaluated using receiver operating characteristic (ROC) curves, and the area under the curve (AUC) was calculated for each biomarker.

Animals

A total of 60 healthy adult male C57BL/6J mice (8–10 weeks old, 18–22 g) were obtained from the Guangxi Medical University Laboratory Animal Center (Nanning, China). The mice were housed at 21–23°C with a relative humidity of 55 ± 5%. Before the experiments, they had ad libitum access to food and water and were maintained under a 12-h light/dark cycle. The animal facility was cleaned regularly to maintain standardized housing conditions. All animal experiments were conducted in accordance with the applicable Chinese regulations and were approved by the Animal Ethics Committee of Guangxi Medical University (approval No. 202401020).

Myocardial I/R model establishment and electrocardiogram and echocardiography analysis

Mice were randomly assigned to the sham (n = 30) or I/R (n = 30) group using a lottery-drawing method. Anesthesia was induced by intraperitoneal administration of avertin (1.25%, 0.2 mL/10 g). Myocardial ischemia/reperfusion was induced by 45 min of left anterior descending coronary artery (LAD) ligation using an 8-0 silk suture, followed by 120 min of reperfusion.^{18,19} Sham-operated mice underwent the same surgical procedure without LAD ligation. Cardiac function was assessed after reperfusion using a VINNO D700 VET ultrasound system under 1–2% isoflurane anesthesia. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were measured using B-mode and M-mode imaging over 3 consecutive cardiac cycles. Electrocardiography (ECG) was performed before ischemia and after reperfusion using an ECG-3306G system (Sanyue, Guangzhou, China).

Subsequently, mice designated for Evans blue/TTC staining (n = 16) and gelatin-ink perfusion (n = 16) underwent intracardiac perfusion with the respective solutions under continuous anesthesia before heart excision. Mice assigned to the tissue analysis cohort (n = 12) and the molecular analysis cohort (n = 16) were euthanized by anesthetic overdose, and the hearts were immediately harvested and snap-frozen at –80°C. All subjectively assessed outcomes (specifically histological scoring, infarct size analysis, and behavioral video analysis) were evaluated by an independent investigator blinded to the experimental group assignments.

Evans blue and TCC staining analysis

After 120 min of reperfusion, myocardial infarct size was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) and Evans blue staining. A 2% Evans blue solution was retrogradely perfused through the right ventricle until cyanosis of the extremities was observed. Hearts were excised, rinsed with saline, frozen at –20°C for 10 min, and sectioned into 1-mm-thick slices. The sections were incubated in the dark with 1% TTC (Leagene Biotechnology, Anhui, China) at 37°C for 30 min. Infarct size was quantified as the proportion of white ischemic tissue relative to the total left ventricular area using ImageJ (National Institutes of Health (NIH), Bethesda, USA).

Gelatin-ink perfusion

Following 120 min of reperfusion, hearts were perfused through the right ventricle with 4% gelatin-ink to achieve uniform cardiac staining. The tissues were harvested, fixed in 4% paraformaldehyde, and sectioned. Microvascular architecture was examined at ×20 magnification using an Olympus BX53 light microscope (Olympus Corp., Tokyo, Japan), and images were acquired for quantitative morphometric analysis.

Immunohistochemical staining

Cardiac tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 μm . After deparaffinization and antigen retrieval, endogenous peroxidase activity was blocked with 3% H_2O_2 , followed by blocking with 3% bovine serum albumin (BSA) at room temperature for 30 min. Sections were incubated overnight at 4°C with anti-c-Myc (1:200; HA721182; Huabio, Hangzhou, China) or anti-caspase-4 (1:300; ER60011; Huabio) primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. 3,3'-Diaminobenzidine (DAB) was used as the chromogenic substrate, followed by hematoxylin counterstaining. Images were acquired using an Olympus BX53 light microscope, and positively stained areas were quantified using ImageJ.

Cell culture and hypoxia/reoxygenation model

Cardiac microvascular endothelial cells (CMECs) were obtained from Procell Life Science (cat. No. CP-M129; Procell, Wuhan, China). Quality control confirmed CD31 immunofluorescence positivity $\geq 90\%$, the absence of HIV-1, HBV, HCV, and mycoplasma (polymerase chain reaction (PCR)-tested), and the absence of microbial contamination (culture-verified). Cells were cultured in mouse cardiac microvascular endothelial cell complete medium (ECM; cat. No. CM-M129; Procell) at 37°C in a humidified atmosphere containing 5% CO_2 . At 80–90% confluence, CMECs between passages 3 and 8 were subjected to hypoxia in glucose- and fetal bovine serum (FBS)-free Dulbecco's modified Eagle's medium (DMEM; cat. No. PM150122; Procell) under 1% O_2 , 5% CO_2 , and 94% N_2 for 24 h, followed by reoxygenation in complete medium under 95% O_2 and 5% CO_2 for 24 h.

siRNA transfection

Cells were seeded and cultured for 24 h to achieve 70–80% confluence before transfection with siRNA targeting *Myc* (*si-Myc*) (sequence: TGGAGATGATGACC-GAGTTAC; Hanbio Co., Ltd., Shanghai, China) using Lipo8000™ transfection reagent (C0533; Beyotime Biotechnology, Shanghai, China). The transfection complex was prepared by mixing the siRNA with Lipo8000™ in antibiotic- and serum-free DMEM, followed by incubation at room temperature for 20 min. The transfection complex was then added to the CMEC culture and incubated for 24 h. Subsequently, the transfection medium was replaced with glucose- and serum-free DMEM, and the cells were subjected to 24 h of hypoxia followed by 24 h of reoxygenation.

TUNEL staining assay

To evaluate apoptosis in CMECs in vitro, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Beyotime Biotechnology) was used. After hypoxia/reoxygenation (H/R) treatment, CMECs at an appropriate density were selected for TUNEL staining. According to the manufacturer's instructions, the cells were washed, fixed, permeabilized, and incubated with the TUNEL reaction mixture at 37°C in the dark. Images were acquired using a fluorescence microscope (200 $\times$ magnification; Zeiss Axiocam 506 Color; Carl Zeiss AG, Jena, Germany).

Transmission electron microscopy of mitochondria

Cell pellets (1×10^7 cells) were fixed in 2.5% glutaraldehyde at 4°C for 24 h, rinsed with phosphate-buffered saline (PBS), and post-fixed in 1% osmium tetroxide for 2 h. En bloc staining was performed with uranyl acetate in 70% ethanol for 3 h. After graded ethanol dehydration, samples were treated with propylene oxide, infiltrated with Eponate 12 resin (hardener: DDSA/NMA/DMP-30), and polymerized at 45°C for 12 h, followed by 72°C for 24 h. Ultrathin sections (70 nm) were prepared using a Leica UC-7 ultramicrotome (Leica Camera AG, Wetzlar, Germany), stained with lead citrate, and examined using a JEOL JEM-1400 transmission electron microscope (TEM) (80 kV; Morada G3 CCD; JEOL Ltd., Tokyo, Japan). Mitophagy was identified by the presence of double-membrane autophagosomes containing mitochondria with disrupted cristae.

RT-qPCR assay

Total RNA was isolated from myocardial tissues and cultured cells using TRIzol reagent (Solarbio, Beijing, China). RNA concentration and purity (A260/A280 ratio) were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Complementary DNA (cDNA) was synthesized from 1,000 ng of RNA using HyperScript III RT SuperMix (R202; EnzyArtisan Biotech, Shanghai, China). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed using $\times 2$ S6 Universal SYBR Mix (Q204-1; EnzyArtisan Biotech) on a 7500 Real-Time PCR System (Thermo Fisher Scientific). Primer sequences are listed in Supplementary Table 2. The thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. For RT-qPCR, 5 independent myocardial tissue samples per group and 6 independent cell samples per group were analyzed. Each RNA sample was measured in 3 technical replicates, and the technical-replicate Ct values were averaged before downstream analysis. Gene expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Western blot assay

Cardiac tissues were homogenized in radioimmuno-precipitation assay (RIPA) buffer (R0010; Solarbio) containing protease and phosphatase inhibitors (P1261; Solarbio). Protein concentration was determined using a bicinchoninic acid (BCA) assay kit (P0398S; Beyotime Biotechnology) at 562 nm. Equal amounts of protein (20 µg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (ISEQ00010; MilliporeSigma, St. Louis, USA), and blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 2 h at room temperature. Membranes were incubated overnight at 4°C with the following primary antibodies: anti-caspase-4 (1:500; TA5130; Abmart, Shanghai, China), anti-c-Myc (1:1,000; T55150; Abmart), anti-Cdkn1a (1:1,000; #382492; Zen-Bioscience, Chengdu, China), anti-Bax (1:1,000; #50599-2-Ig; Proteintech, Wuhan, China), anti-Bcl-2 (1:1,000; #26593-1-AP; Proteintech), anti-LC3 (1:1,000; #382687; Zen-Bioscience), anti-GAPDH (1:10,000; #301341; Zen-Bioscience), and anti-β-tubulin (1:5,000; #48375; Signalway Antibody, USA). After washing with TBST, HRP-conjugated secondary antibodies (1:10,000; #301341; Zen-Bioscience) were applied for 1 h at room temperature. Protein bands were visualized using an e-BLOT Touch Imager (Ebio-trade Life Science, Shanghai, China) and quantified using ImageJ.

Statistical analyses

All bioinformatics and statistical analyses were performed using R v. 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria). Statistical analyses and graphical visualizations were generated using GraphPad Prism v. 8.0 (GraphPad Software, San Diego, USA). All statistical analyses were performed in accordance with the statistical guidelines of *Advances in Clinical and Experimental Medicine*.²⁰ The results of all assumption checks for parametric tests are presented in Supplementary Table 5.

The normality of data distribution was assessed using the Shapiro–Wilk test. Data are presented as the mean ± standard deviation (SD) for normally distributed variables or as the median with the 1st and 3rd quartiles (Q1 and Q3) for non-normally distributed variables. Differences between 2 independent groups were analyzed using the Mann–Whitney U test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A $p < 0.05$ was considered statistically significant.

Results

Identification of DEGs

After batch-effect correction, PCA demonstrated a more homogeneous distribution of samples across all datasets (Fig. 1A). A total of 402 DEGs were identified across the 6 datasets, including 168 downregulated and 234 upregulated genes, using the thresholds of $p < 0.05$ and an absolute $\log_2$ fold change ($|\log_2 FC| > 1.0$) (Fig. 1B). A clustered heatmap further illustrates the expression profiles of the top 20 DEGs in the 2 study groups (Fig. 1C).

Functional enrichment analysis of ARDEGs

The Venn diagram in Fig. 2A shows that 7 genes were identified at the intersection of the DEGs and ARGs. These 7 genes (*Casp4*, *Cdkn1a*, *Edem1*, *Egfr*, *Hif1a*, *Myc*, and *Rgs19*) were significantly upregulated in the I/R group compared with the sham group (Fig. 2B). To gain further functional insight, GO enrichment analysis was performed to characterize the BP, CC, and MF associated with the ARDEGs. As shown in Fig. 2C, the ARDEGs were significantly enriched in several biological processes, including the regulation of smooth muscle cell proliferation, positive regulation of microRNA (miRNA) metabolic processes, and regulation of miRNA transcription. With respect to cellular components, the ARDEGs were significantly associated with euchromatin, RNA polymerase II transcription regulator complexes, and membrane rafts. Molecular function analysis identified significant enrichment for ubiquitin or ubiquitin-like protein ligase binding, E-box binding, and protein kinase activator activity. Furthermore, KEGG pathway analysis demonstrated significant enrichment in pathways including proteoglycans in cancer, bladder cancer, the ErbB signaling pathway, the HIF-1 signaling pathway, hepatitis C, and the JAK-STAT signaling pathway (Fig. 2D).

Identification of the hub genes via machine learning algorithms

Based on the 7 shared genes, 3 machine-learning algorithms – LASSO, SVM-RFE, and RF – were used to identify the most promising candidate biomarker genes. The LASSO coefficient path plot identified 7 genes with nonzero regression coefficients. Using 10-fold cross-validation, the optimal λ value corresponding to the minimum cross-validation error was selected, resulting in the identification of 6 key feature genes (Fig. 3A). The performance of the SVM-RFE model was evaluated using 5-fold cross-validation, leading to the identification

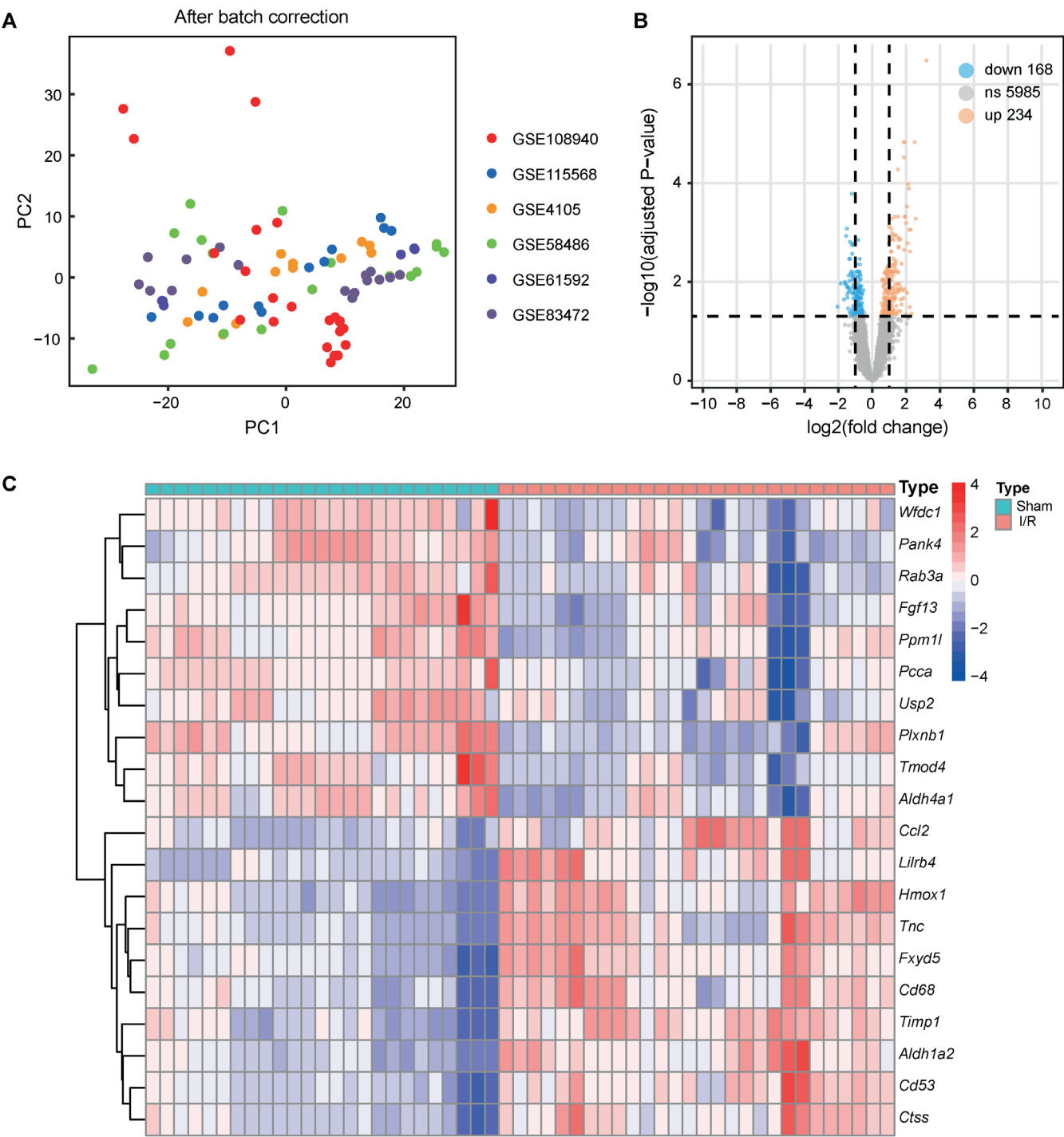
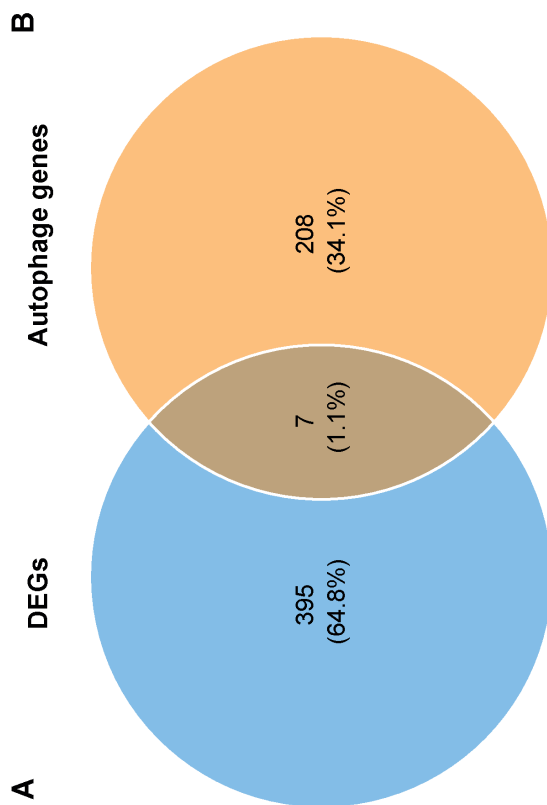


Fig. 1. Identification of differentially expressed genes (DEGs) associated with myocardial ischemia/reperfusion (I/R). A. Principal component analysis (PCA) plot showing gene expression profiles after batch-effect correction; B. Volcano plot showing DEGs between the sham and I/R groups; C. Heatmap showing the top 20 DEGs between the 2 groups

of 6 key genes with the lowest error rate and highest classification accuracy (Fig. 3B). Subsequently, the RF algorithm identified 7 genes with importance scores >2.0 as potential biomarkers for I/R injury (Fig. 3C). Ultimately, 4 hub genes (*Casp4*, *Cdkn1a*, *Myc*, and *Rgs19*) were consistently identified by all 3 algorithms, as illustrated in the Venn diagram (Fig. 3D).

Evaluation of diagnostic efficacy for hub biomarkers via nomogram

To further evaluate the predictive performance for myocardial I/R injury, a nomogram was constructed using the 4 candidate hub biomarkers (*Casp4*, *Cdkn1a*, *Myc*, and *Rgs19*), providing a simple and reliable diagnostic



Gene	Gene description	I/R vs Sham
<i>Casp4</i>	Caspase 4	Up-regulated
<i>Cdkn1a</i>	Cyclin dependent kinase inhibitor 1A	Up-regulated
<i>Edem1</i>	ER degradation enhancing alpha-mannosidase like protein 1	Up-regulated
<i>Egfr</i>	Epidermal growth factor receptor	Up-regulated
<i>Hif1a</i>	Hypoxia-inducible factors 1-alpha	Up-regulated
<i>Myc</i>	Myelocytomatosis	Up-regulated
<i>Rgs19</i>	Regulator of G protein signaling 19	Up-regulated

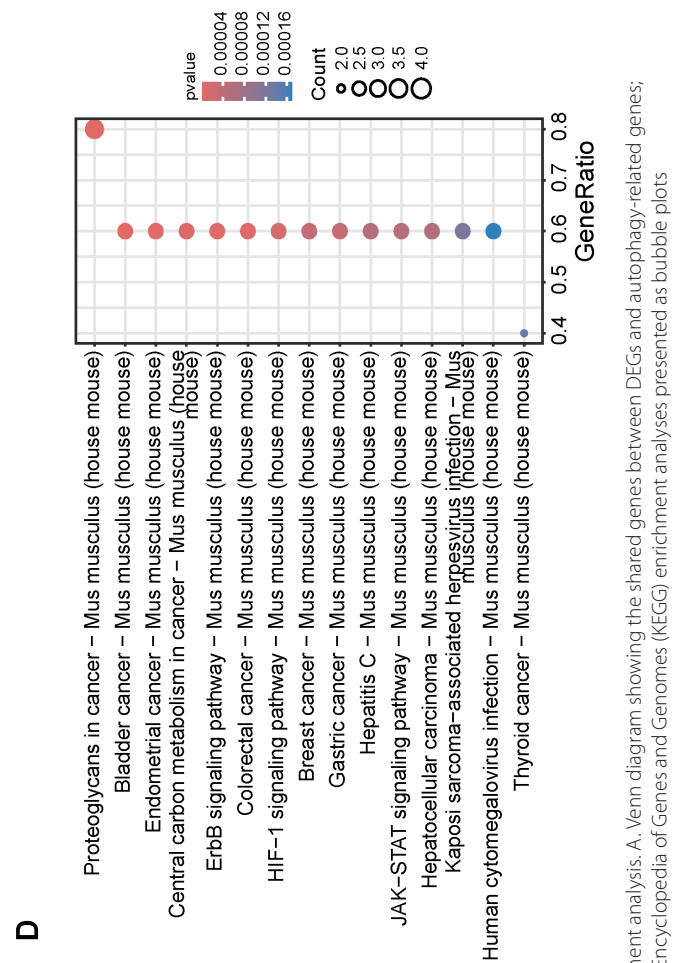
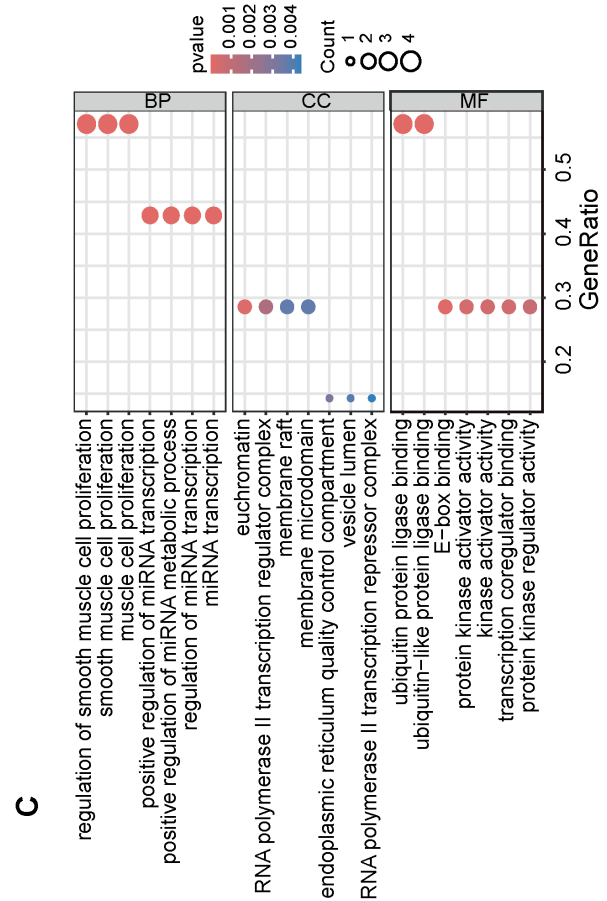


Fig. 2. Identification of autophagy-related genes (ARDEGs) and functional enrichment analysis. A. Venn diagram showing the shared genes between DEGs and autophagy-related genes; B. Table showing upregulation of all 7 shared genes in the I/R group; C,D. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses presented as bubble plots

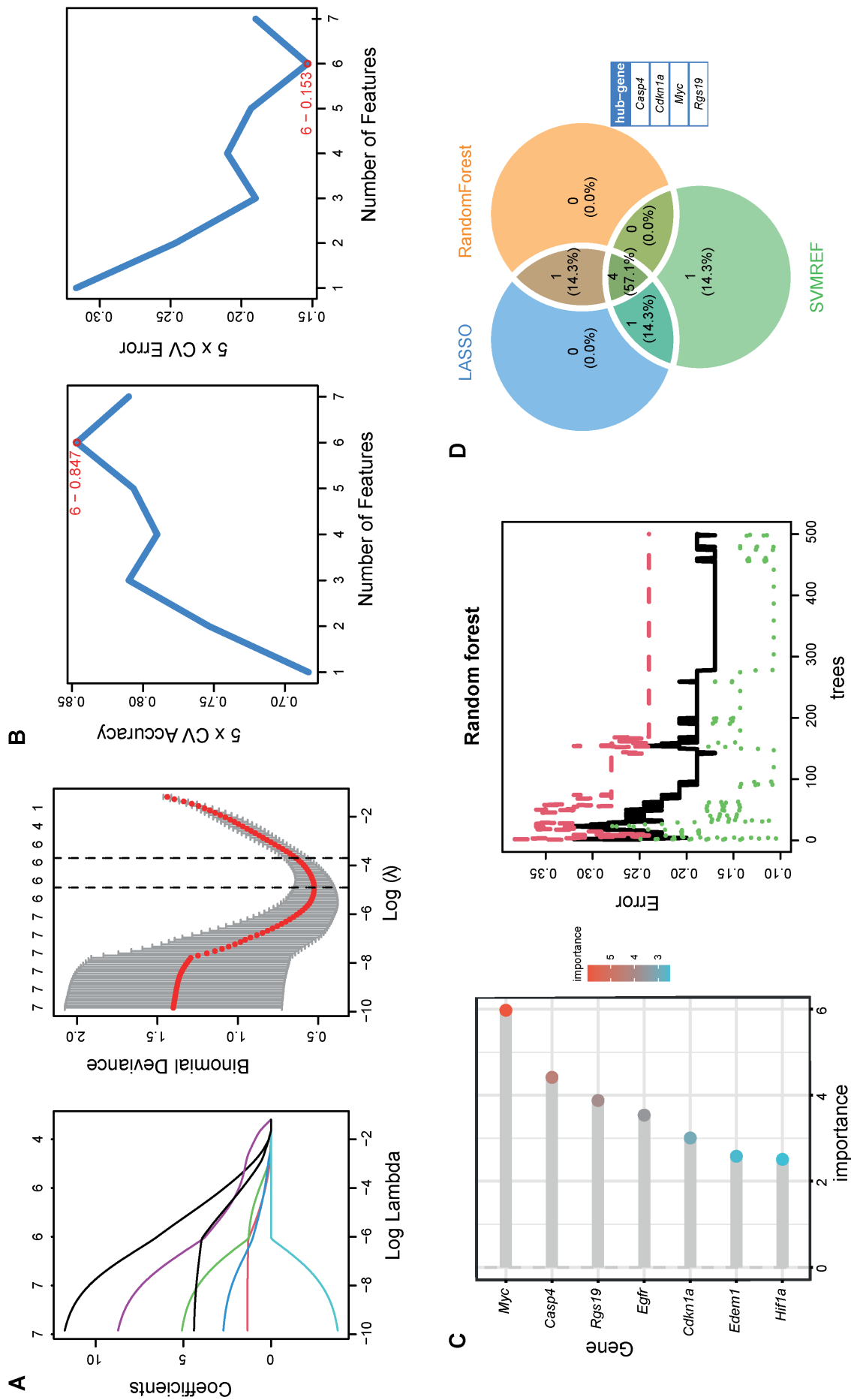


Fig. 3. Identification of candidate diagnostic biomarkers for myocardial ischemia/reperfusion (I/R) using 3 machine-learning algorithms. A. Six key genes identified using the least absolute shrinkage and selection operator (LASSO) model; B. Support vector machine–recursive feature elimination (SVM-RFE) identified 6 candidate genes associated with I/R; C. The top 7 candidate genes identified using the Random Forest model; D. Venn diagram showing the 4 genes identified by all 3 machine-learning algorithms

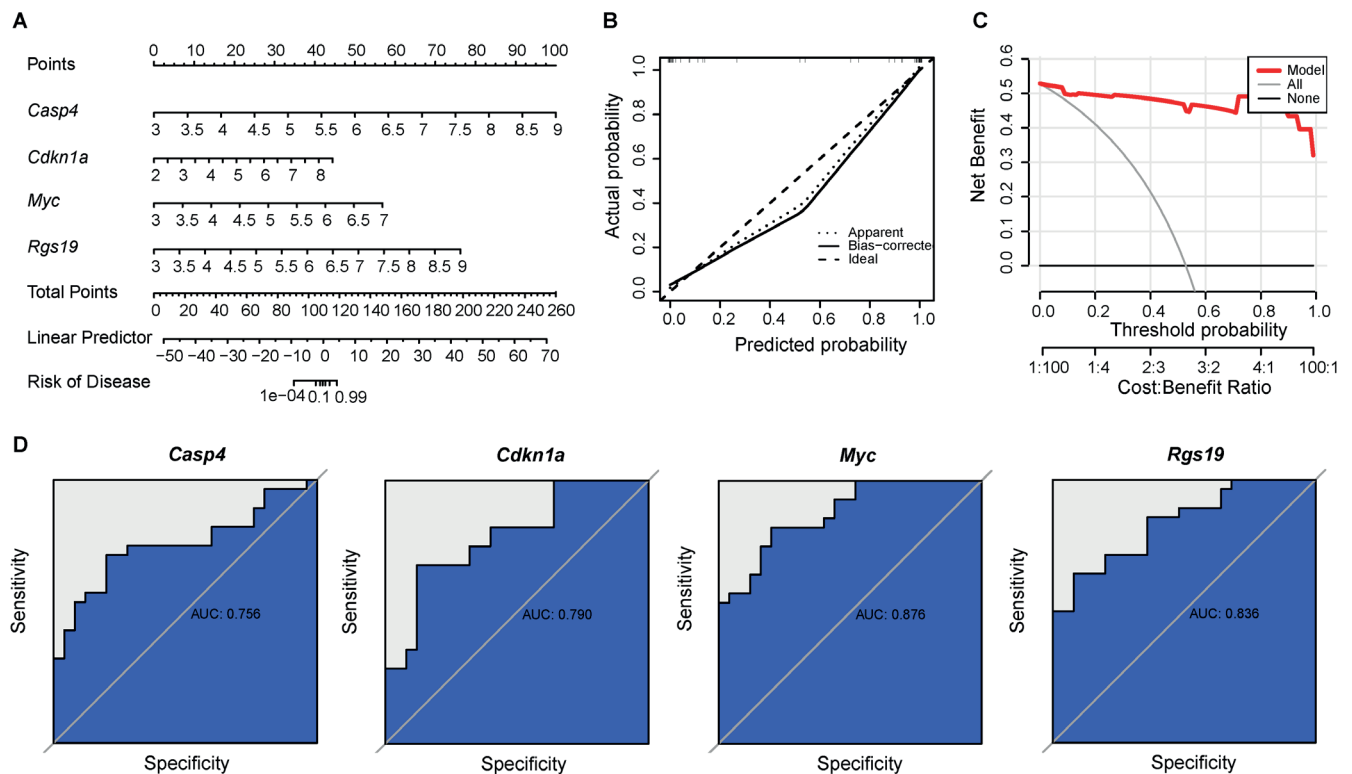


Fig. 4. Development of a nomogram for predicting myocardial ischemia/reperfusion (I/R) injury. A. Nomogram predicting the risk of myocardial I/R injury based on *Casp4*, *Cdkn1a*, *Myc*, and *Rgs19*; B. Calibration curve of the nomogram; C. Decision curve analysis of the nomogram; D. Receiver operating characteristic (ROC) curves of the 4 candidate biomarkers showing their diagnostic performance

tool (Fig. 4A). The calibration curve (Fig. 4B) demonstrated good agreement between the predicted and observed probabilities, indicating excellent calibration. In addition, decision curve analysis (Fig. 4C) demonstrated the potential clinical utility of the nomogram for the diagnosis of myocardial I/R injury. In the development cohort, the AUCs were 0.756 for *Casp4*, 0.790 for *Cdkn1a*, 0.876 for *Myc*, and 0.836 for *Rgs19* (Fig. 4D), indicating high diagnostic accuracy and strong discriminatory performance.

Single-gene GSEA analysis of hub biomarkers

To investigate the potential biological functions associated with the diagnostic biomarkers, single-gene GSEA was performed, and the top 5 positively and negatively enriched pathways were visualized. The analysis demonstrated that the 4 hub genes were predominantly associated with metabolic pathways, including lipoic acid metabolism and propanoate metabolism. In addition, all 4 genes were enriched in pathways related to valine, leucine, and isoleucine degradation, as well as extracellular matrix (ECM)-receptor interaction (Fig. 5).

Association between immune cell infiltration and hub genes

Additionally, the CIBERSORT algorithm was used to estimate immune cell infiltration in the I/R and sham groups. As shown in Fig. 6A, significant differences in immune cell composition were observed between the 2 groups. Compared with the sham group, the I/R group exhibited increased infiltration of naïve CD8⁺ T cells, M2 macrophages, and activated dendritic cells (DCs), whereas the sham group showed higher proportions of mast cells, plasma cells, memory CD8⁺ T cells, and Th17 cells. Furthermore, correlation analysis suggested that the identified biomarker genes may contribute to the progression of I/R injury through interactions with multiple immune cell populations, including T cells, activated DCs, macrophages, neutrophils, and B cells (Fig. 6B–E).

Exploring subtypes of I/R with biological molecular mechanisms

To further characterize heterogeneity within the I/R group, unsupervised clustering analysis was performed to classify I/R samples into distinct molecular subtypes. Using the expression profiles of the 7 shared genes, consensus clustering was applied to 28 I/R samples. Two optimal subtypes were identified based on the consensus matrix, CDF plot, and relative changes in the area under the CDF curve (Fig. 7A). After

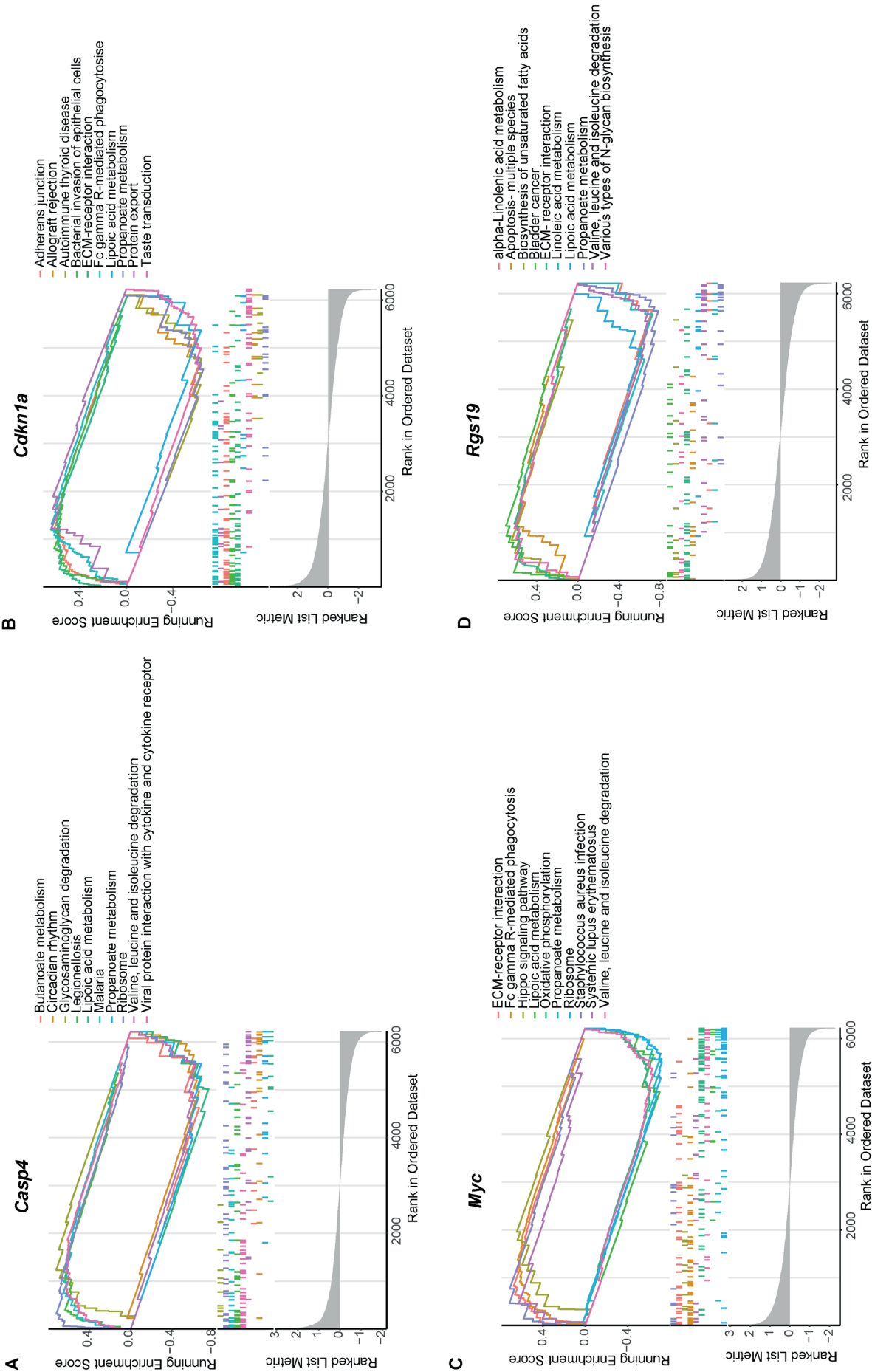


Fig. 5. Gene set enrichment analysis (GSEA) of the 4 signature genes. Enriched biological pathways associated with *Casp4* (A), *Cdkn1a* (B), *Myc* (C), and *Rgs19* (D) in myocardial ischemia/reperfusion (I/R)

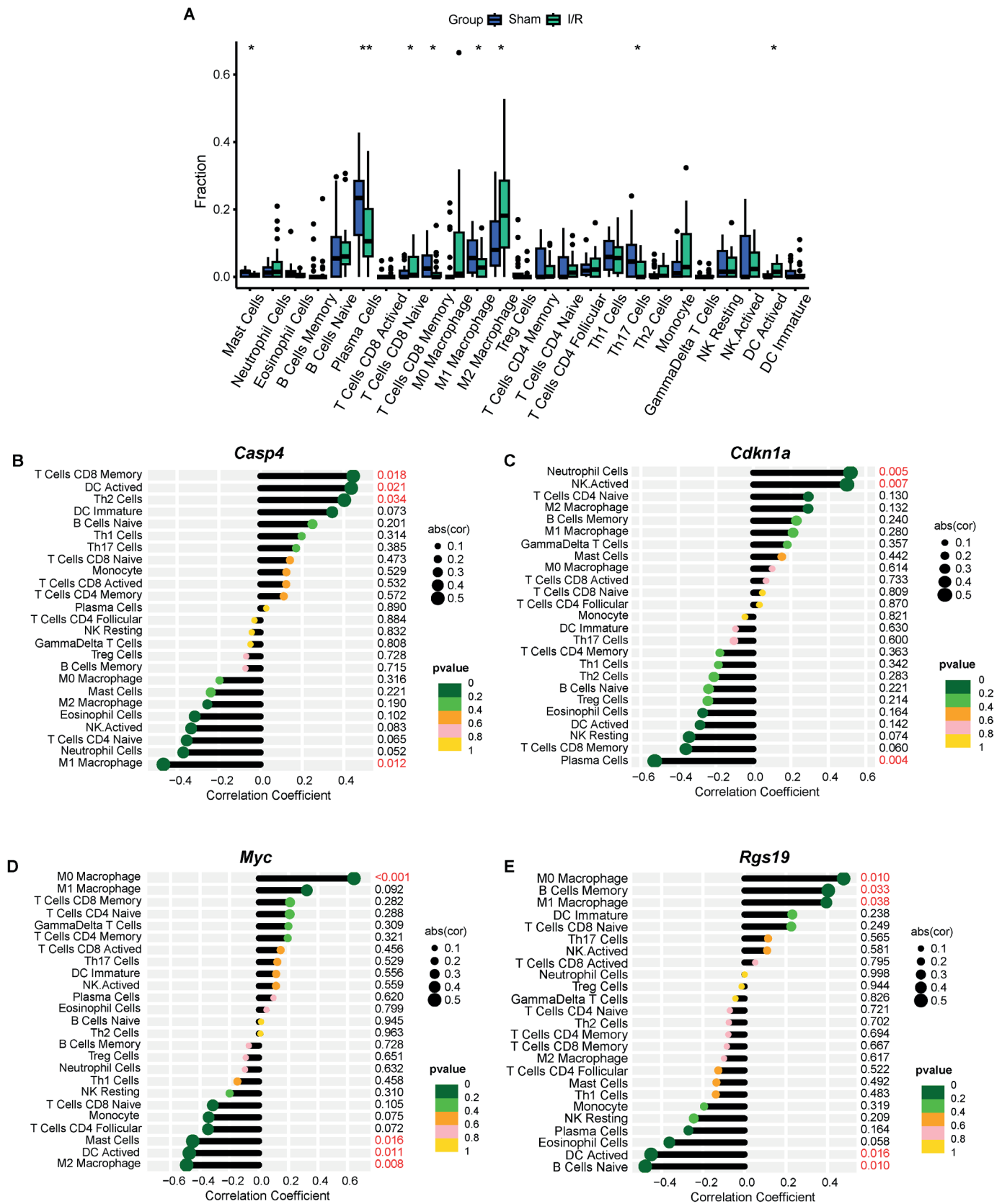


Fig. 6. Immune cell infiltration analysis. A. Distribution of 25 immune cell types between the sham and myocardial ischemia/reperfusion (I/R) groups; B–E. Correlations between immune cell abundance and the expression of the signature genes in I/R tissue (* $p < 0.05$, ** $p < 0.01$)

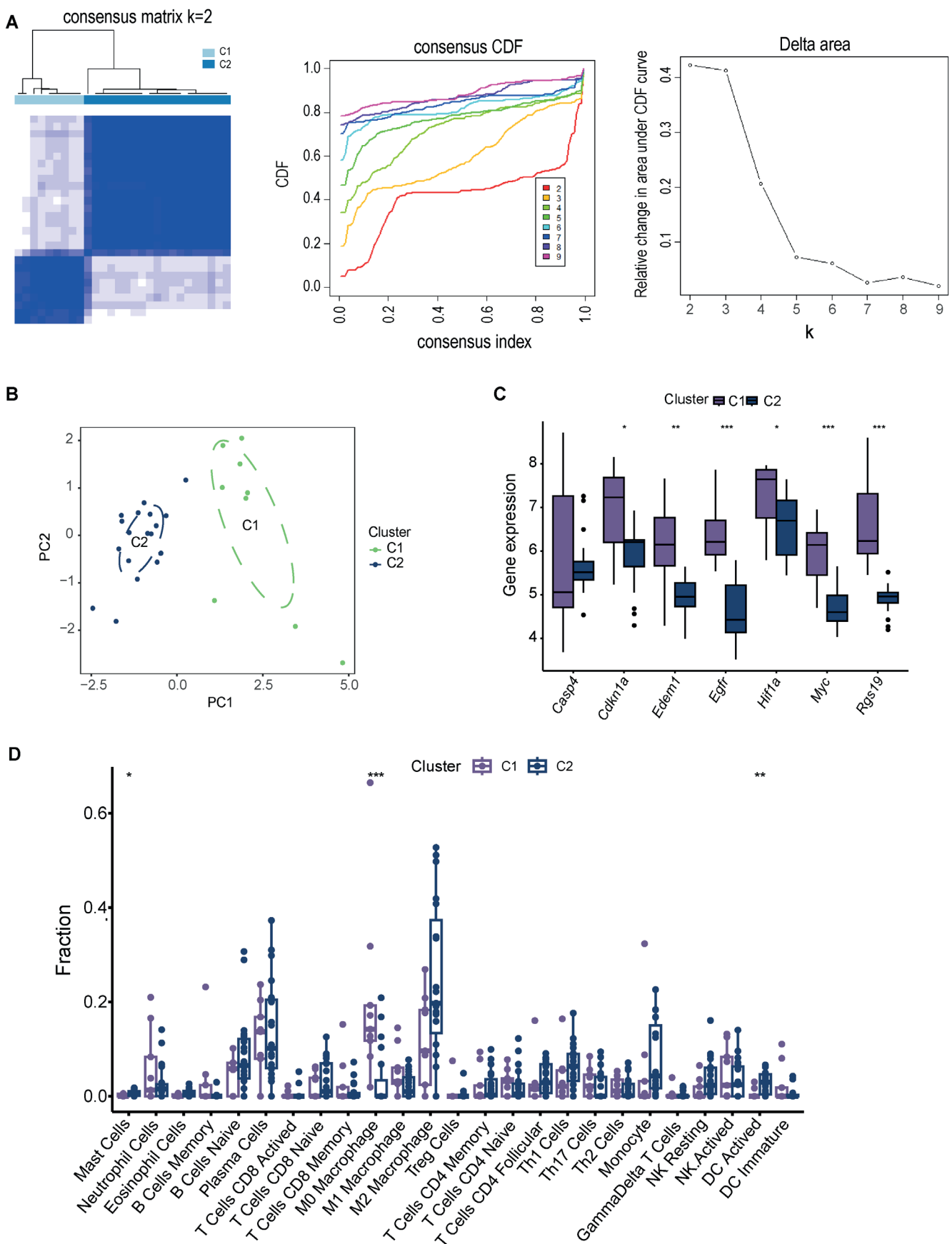


Fig. 7. Identification and characterization of distinct molecular subtypes in myocardial ischemia/reperfusion (I/R). **A.** Consensus clustering matrix, cumulative distribution function (CDF) curves, and CDF delta area plots identifying 2 clusters among the 28 I/R samples ($k = 2$); **B.** Principal component analysis (PCA) distinguishing the 2 subtypes based on the expression profiles of 7 autophagy-related differentially expressed genes (ARDEGs); **C.** Box plots showing the expression profiles of the 7 shared ARDEGs in the 2 subtypes; **D.** Distribution of immune cell infiltration across the 2 autophagy-related subtypes (* $p < 0.05$, ** $p < 0.01$)

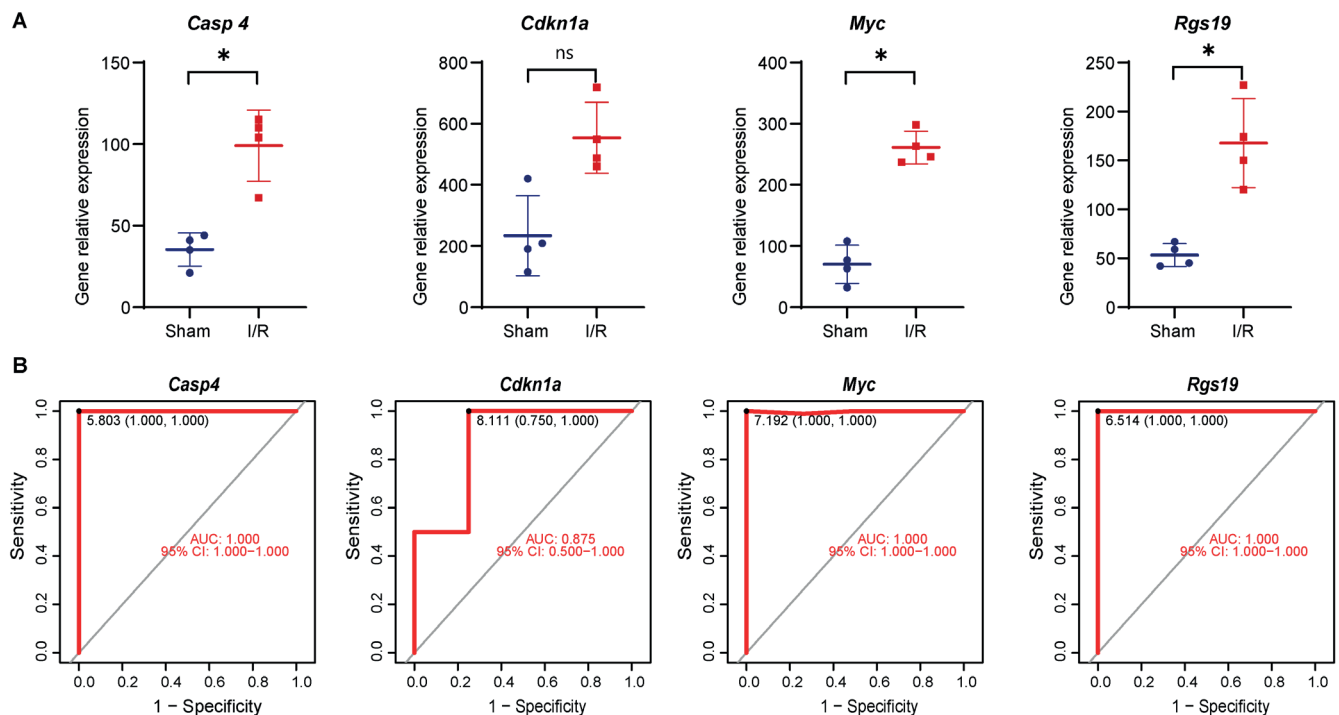


Fig. 8. Expression and diagnostic performance of the 4 hub genes in the GSE168610 dataset. A. Expression levels of *Casp4*, *Cdkn1a*, *Myc*, and *Rgs19* in the I/R group compared with the sham group. *Casp4*, *Myc*, and *Rgs19* were significantly increased ($p < 0.05$), whereas *Cdkn1a* was not significantly different ($p > 0.05$); B. Receiver operating characteristic (ROC) curves and areas under the curve (AUCs) for the 4 candidate biomarkers

classification into 2 subtypes (C1, $n = 9$; C2, $n = 19$), PCA demonstrated clear separation of the C1 and C2 groups based on gene expression profiles (Fig. 7B).

Further analysis of the shared genes revealed that, with the exception of *Casp4*, the C1 subtype exhibited significantly higher expression of *Cdkn1a*, *Edem1*, *Egfr*, *Hif1a*, *Myc*, and *Rgs19* than the C2 subtype (Fig. 7C). In addition, marked differences in immune cell abundance were observed between the 2 subtypes. The C2 subtype showed higher proportions of mast cells and activated DCs, whereas the C1 subtype exhibited greater infiltration of M0 macrophages (Fig. 7D).

To investigate functional differences between the 2 subtypes, KEGG and GO enrichment analyses were performed. As shown in Supplementary Fig. 3A,B, the DEGs between C1 and C2 were significantly enriched in pathways related to neurodegeneration, oxidative phosphorylation, Parkinson's disease, and cardiac muscle contraction. Gene Ontology enrichment analysis further demonstrated significant enrichment of biological processes related to cellular respiration, energy metabolism, mitochondrial protein complexes, structural constituents of the ribosome, and proton transmembrane transporter activity.

Hub genes expression and diagnostics in GSE168610

To validate the reliability of the bioinformatics analysis, the 4 key genes were independently evaluated in the external validation cohort (GSE168610). As shown in Fig. 8A, the expression levels of *Casp4*, *Myc*, and *Rgs19*

were significantly increased in I/R myocardial tissue compared with sham controls ($p < 0.05$), whereas *Cdkn1a* expression did not differ significantly (ns, $p > 0.05$). Furthermore, ROC curve analysis (Fig. 8B) demonstrated that *Casp4*, *Myc*, and *Rgs19* showed perfect diagnostic performance for distinguishing I/R injury (AUC = 1.000), whereas *Cdkn1a* also demonstrated excellent diagnostic performance (AUC = 0.875).

Preliminary validation of autophagy-related prognostic genes in myocardial I/R and CMECs H/R model

To validate the roles of *Casp4*, *Cdkn1a*, *Myc*, and *Rgs19* identified through bioinformatics analysis, an in vivo myocardial I/R-induced microvascular injury model and an in vitro H/R model were established to evaluate the expression of these candidate biomarkers. Electrocardiographic analysis revealed significant ST-segment elevation during myocardial ischemia, which decreased by more than 50% after reperfusion (Supplementary Fig. 1A). TTC/Evans blue staining demonstrated a marked increase in infarct size following I/R injury (Supplementary Fig. 1B and Supplementary Table 6). After I/R, cardiac function was significantly impaired, as evidenced by reduced LVEF and LVFS (Supplementary Fig. 1C,D and Supplementary Table 6). Gelatin-ink staining further confirmed impaired microvascular perfusion in myocardial tissue after I/R (Supplementary Fig. 1E and Supplementary Table 6). Myocardial tissue subjected to I/R exhibited significantly increased mRNA expression

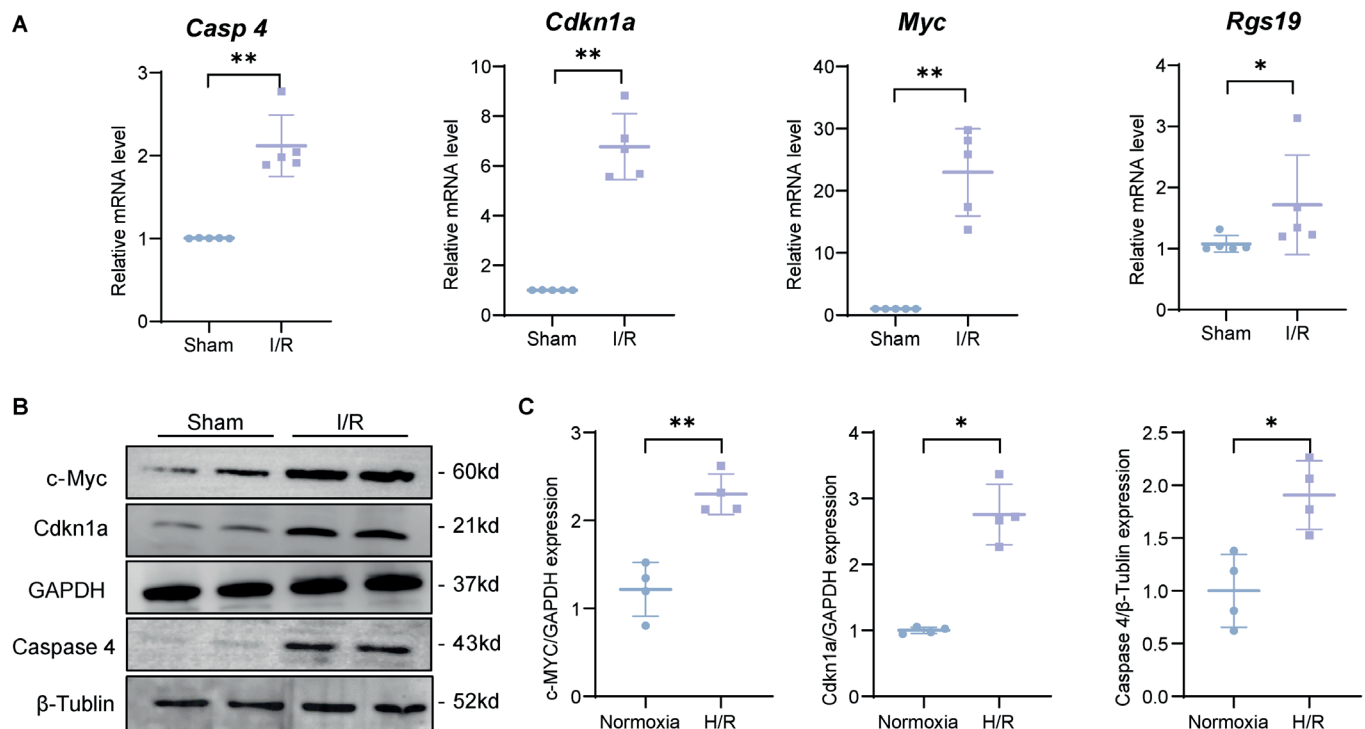


Fig. 9. Expression of 4 hub-gene mRNAs and 3 corresponding proteins in myocardial I/R injury. A. mRNA expression levels of *Myc*, *Casp4*, *Cdkn1a*, and *Rgs19* (Mann–Whitney U test; $n = 5$); B,C. Western blot analysis and quantification of c-Myc, Caspase-4, and p21 (Mann–Whitney U test; $n = 4$). RGS19 protein was not assessed (* $p < 0.05$, ** $p < 0.01$)

Table 1. The mRNA expression levels of *Casp4*, *Cdkn1a*, *Myc*, and *Rgs19* were compared between the sham and myocardial ischemia/reperfusion (I/R) groups and between the normoxia and hypoxia/reoxygenation (H/R) groups using Welch's t test or the Mann–Whitney U test (Fig. 9A and Supplementary Fig. 5A)

Genes	Groups	Samples (n)	Median (Q1, Q3)	U statistic	p-value
<i>Casp4</i>	sham	5	1.00 (1.00, 1.01)	U = 0	0.008**
	I/R	5	1.98 (1.90, 2.41)		
	normoxia	6	1.00 (1.00, 1.01)	U = 0	0.002**
	H/R	6	2.24 (2.14, 2.31)		
<i>Cdkn1a</i>	sham	5	1.01 (1.00, 1.01)	U = 0	0.008**
	I/R	5	5.56 (5.51, 5.68)		
	normoxia	6	1.00 (1.00, 1.01)	U = 0	0.002**
	H/R	6	2.27 (1.38, 3.18)		
<i>Myc</i>	sham	5	1.02 (1.01, 1.17)	U = 0	0.008**
	I/R	5	25.88 (16.49, 28.92)		
	normoxia	6	1.01 (1.001, 1.02)	U = 0	0.002**
	H/R	6	1.58 (1.32, 2.15)		
<i>Rgs19</i>	sham	5	1.02 (1.01, 1.04)	U = 2	0.032*
	I/R	5	1.35 (1.23, 1.23)		
	normoxia	6	1.01 (1.00, 1.03)	U = 0	0.002**
	H/R	6	2.72 (1.78, 3.80)		

* $p < 0.05$, ** $p < 0.01$.

of *Casp4*, *Cdkn1a*, *Myc*, and *Rgs19* compared with sham controls (Fig. 9A and Table 1), together with increased protein expression of caspase-4, p21, and c-Myc ($U = 0$, $p < 0.05$; Fig. 9B and Table 2). Immunohistochemistry confirmed enrichment of caspase-4 and c-Myc within areas of microvascular injury (Supplementary Fig. 4A,B).

In addition, RT-qPCR demonstrated H/R-induced up-regulation of *Casp4*, *Cdkn1a*, *Myc*, and *Rgs19* compared with normoxic controls, whereas increased protein expression of caspase-4, p21, and c-Myc was confirmed with western blot (WB) analysis (Supplementary Fig. 5A–C and Tables 1,2).

Table 2. Protein expression levels of caspase-4, Cdkn1a, and c-Myc were compared between the sham and myocardial ischemia/reperfusion (I/R) groups and between the normoxia and hypoxia/reoxygenation (H/R) groups using the Mann–Whitney U test (Fig. 9B,C and Supplementary Fig. 5B,C)

Genes	Group	Samples, n	Median (Q1, Q3)	U statistic	p-value
Caspase-4	sham	4	1.00 (0.90, 1.10)	U = 0	0.029*
	I/R	4	2.54 (2.29, 2.70)		
	normoxia	4	1.00 (0.77, 1.23)	U = 0	0.029*
	H/R	4	1.90 (1.69, 2.11)		
p21	sham	4	1.00 (0.94, 1.11)	U = 0	0.029*
	I/R	4	2.70 (2.59, 3.50)		
	normoxia	4	1.00 (0.96, 1.04)	U = 0	0.029*
	H/R	4	2.70 (2.47, 3.05)		
c-Myc	sham	4	1.12 (0.98, 1.17)	U = 0	0.029*
	I/R	4	2.45 (2.39, 2.58)		
	normoxia	4	1.27 (1.05, 1.43)	U = 0	0.029*
	H/R	4	2.22 (2.13, 2.47)		

*p < 0.05, **p < 0.01.

Myc knockout reduces apoptosis in H/R-induced CMECs

We performed TUNEL staining to detect apoptosis. As shown in Fig. 10A,B and Table 3, the apoptotic rate of vascular endothelial cells was significantly higher in the H/R group than in the normoxia group. This increase was almost completely reversed by si-Myc treatment. Western blot analysis demonstrated that H/R injury significantly upregulated Bax expression, downregulated Bcl-2 expression, and promoted LC3-II conversion, whereas Myc knockout largely normalized these alterations in protein expression ($p < 0.05$; Fig. 10C,D and Table 4). Transmission electron microscopy was then used to examine mitochondrial ultrastructure and autophagosome formation. In cells exposed to H/R injury, mitochondria exhibited marked swelling and disruption of the cristae, accompanied by abundant autophagic vacuoles. In contrast, si-Myc treatment markedly ameliorated these ultrastructural alterations (Supplementary Fig. 6).

Discussion

Despite clinical efforts to restore blood flow to the ischemic myocardium and limit myocardial necrosis, myocardial I/R continues to damage both cardiac tissue and the coronary microcirculation.²¹ Extensive microvascular injury impairs myocardial perfusion and limits the delivery of therapeutic agents to the distal microvasculature, thereby complicating clinical management.²² By integrating machine-learning approaches with multimodal validation, we identified a potential 4-biomarker panel (*Casp4*, *Cdkn1a*, *Myc*, and *Rgs19*) with high diagnostic accuracy (AUCs of 0.756 for *Casp4*, 0.790 for *Cdkn1a*, 0.876 for *Myc*, and 0.836 for *Rgs19*; Fig. 4D) for microvascular I/R injury. Importantly, c-Myc emerged as a dual-function regulator:

beyond its diagnostic value, c-Myc knockout significantly reduced endothelial apoptosis and autophagic dysregulation (Fig. 10A–D), highlighting its potential as a therapeutic target. This finding directly addresses a persistent challenge in reperfusion therapy. Although rapid revascularization remains essential for the treatment of ischemic cardiomyopathy, it paradoxically induces microvascular I/R injury that compromises treatment efficacy. The dual protective and detrimental roles of autophagy in cardiac I/R make this pathway an attractive therapeutic target. Our data indicate that dysregulated autophagy, particularly excessive c-Myc-mediated autophagy, is a key mechanism underlying this paradox. This mechanistic insight identifies a potential therapeutic target for mitigating the no-reflow phenomenon, which limits drug delivery and tissue perfusion.

Casp4 has been reported to mediate pyroptosis in microvascular endothelial cells and to play a critical role in the pathophysiology of myocardial I/R.¹⁸ Our findings are consistent with previous studies, demonstrating a significant upregulation of *Casp4* expression in both the I/R and H/R models. Previous research has also suggested that inhibition of *Casp4*-mediated cell death through activation of autophagy signaling pathways may represent a promising therapeutic strategy for myocardial reperfusion-related microvascular injury.¹⁸ Given its prognostic significance and overexpression in clear cell renal cell carcinoma, *Casp4* has also emerged as a candidate biomarker for disease monitoring and a potential molecular target for therapeutic intervention.²³ During cardiac I/R, cells are exposed to hypoxia, nutrient deprivation, inflammation, and oxidative stress, resulting in dysregulated cell-cycle progression and subsequent cellular dysfunction and injury. *Cdkn1a* (*p21*), a cyclin-dependent kinase inhibitor, plays a protective role in I/R injury by limiting cell-cycle progression and reducing oxidative stress.²⁴ Previous studies have shown that *Cdkn1a* interacts with the autophagy-related protein LC3B, thereby contributing to the attenuation of cardiac

Table 3. Differences in TUNEL-positive cell quantification among the experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (Fig. 10A,B)

Variable	Group comparison	Mean $\pm$ SD	ANOVA F-value	df	ANOVA p-value	Tukey's q-value	Tukey's p-value
TUNEL staining	normoxia vs H/R	0.21 $\pm$ 0.05 vs 32.50 $\pm$ 6.32	1.67	(3, 8)	0.449	6.59	0.007**
	H/R + siNC vs H/R + si-Myc	35.20 $\pm$ 4.65 vs 10.58 $\pm$ 2.02				5.05	0.030*

df – degrees of freedom; Tukey's q – Tukey's honestly significant difference (HSD) test statistic; I/R – ischemia/reperfusion; H/R – hypoxia/reoxygenation; SD – standard deviation; siNC – small interfering RNA negative control; si-Myc – small interfering RNA targeting Myc; *p < 0.05, **p < 0.01.

Table 4. Protein expression levels of c-Myc, Bax, Bcl-2, and LC3-II/I were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for the following comparisons: normoxia vs H/R and H/R + siNC vs H/R + si-Myc (Fig. 10C,D)

Genes	Group comparison	Mean $\pm$ SD	ANOVA F-value	df	ANOVA p-value	Tukey's q-value	Tukey's p-value
c-Myc	normoxia vs H/R	0.38 $\pm$ 0.01 vs 1.03 $\pm$ 0.25	1.67	(3, 8)	0.449	6.59	0.007**
	H/R + siNC vs H/R + si-Myc	0.92 $\pm$ 0.19 vs 0.42 $\pm$ 0.04				5.05	0.030*
Bax	normoxia vs H/R	0.45 $\pm$ 0.12 vs 1.04 $\pm$ 0.04	0.980	(3, 8)	0.208	5.74	0.015*
	H/R + siNC vs H/R + si-Myc	1.03 $\pm$ 0.07 vs 0.37 $\pm$ 0.03				6.08	0.011*
Bcl2	normoxia vs H/R	0.95 $\pm$ 0.21 vs 0.48 $\pm$ 0.20	1.90	(3, 8)	0.376	13.66	<0.001**
	H/R + siNC vs H/R + si-Myc	0.49 $\pm$ 0.21 vs 0.85 $\pm$ 0.33				9.29	<0.001**
LC3II/I	normoxia vs H/R	1.15 $\pm$ 0.46 vs 3.12 $\pm$ 0.73	2.25	(3, 8)	0.160	7.15	0.004**
	H/R + siNC vs H/R + si-Myc	2.97 $\pm$ 0.40 vs 1.46 $\pm$ 0.28				5.42	0.021*

df – degrees of freedom; Tukey's q – Tukey's honestly significant difference (HSD) test statistic; I/R – ischemia/reperfusion; H/R – hypoxia/reoxygenation; SD – standard deviation; siNC – small interfering RNA negative control; si-Myc – small interfering RNA targeting Myc; *p < 0.05, **p < 0.01.

dysfunction.²⁵ Moreover, activation of *Cdkn1a*-mediated cell-cycle arrest has been shown to alleviate injury in cardiac microvascular endothelial cells.²⁶ Multiomics data derived from human cardiac cells have also identified *Cdkn1a* as a potential therapeutic target for heart failure.²⁷ Our study demonstrated a marked increase in *Cdkn1a* expression in myocardial I/R tissue and, to our knowledge, is the first to document significant upregulation of *Cdkn1a* in H/R-treated CMECs. These findings underscore the pivotal role of *Cdkn1a* in the progression of myocardial I/R-induced microvascular injury and highlight its potential as a therapeutic target for this condition.

The *Myc* oncogene encodes a potent transcription factor that regulates nearly every cellular process through interactions with numerous transcriptional regulators and protein complexes.²⁸ The *Myc* family comprises 3 members: *Myc* (*c-Myc*), *Mycl*, and *Mycn*, with *c-Myc* being particularly relevant to cardiomyocytes. Previous studies have indicated that *c-Myc* overexpression may contribute to cardiomyocyte apoptosis following myocardial I/R.²⁹ Research has also suggested that regulation of *Myc* expression and its downstream signaling pathways is essential for maintaining cellular integrity and function under

cardiac stress.³⁰ Evidence further indicates that selective inhibition of *c-Myc* overexpression in cardiomyocytes may represent a novel therapeutic strategy for cardiac hypertrophy.³¹ In our study, *c-Myc* knockout alleviated H/R-induced microvascular injury, suggesting that *c-Myc* may represent a promising therapeutic target for myocardial I/R injury. Aberrant *Myc* expression in cardiac tissue has also been proposed as a potential biomarker for predicting outcomes in patients with septic cardiomyopathy.³²

The clinical relevance of *Myc* expression is further supported by human transcriptomic datasets. Analysis of GSE59867 (peripheral blood samples from 312 patients after myocardial infarction) demonstrated >2-fold upregulation of *Myc* compared with controls (p < 0.001), whereas GSE60993 (human ischemic myocardium) showed a significant correlation between *Myc* expression and infarct severity (r = 0.68, p < 0.01).^{33,34} These findings support the potential role of *Myc* as both a diagnostic biomarker and a therapeutic target in ischemic injury. However, previous studies have also shown that *Myc* downregulation may exacerbate cardiomyocyte apoptosis and oxidative stress, thereby aggravating myocardial I/R injury.³⁵ This apparent discrepancy may reflect differences in animal

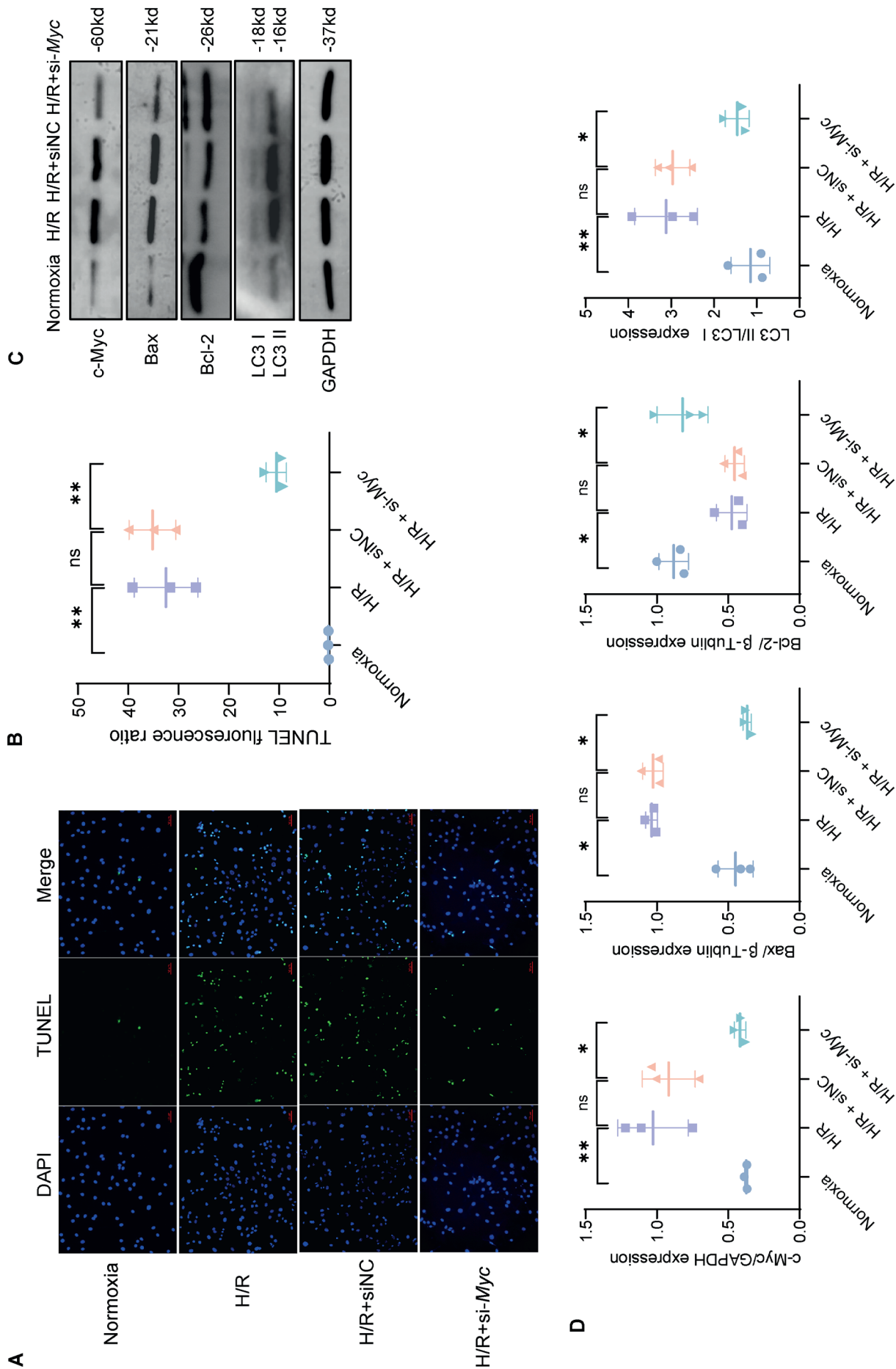


Fig. 10. Myc knockout attenuates hypoxia/reoxygenation (H/R)-induced apoptosis and excessive autophagy. A: TUNEL/DAPI immunofluorescence staining showing apoptotic cells (green) in normoxic, H/R-treated, and Myc knockdown H/R cells (n = 3); Scale bar: 20 μm; B: Quantification of TUNEL-positive cells (n = 3); C: Western blot (WB) analysis of c-Myc, Bax, Bcl-2, LC3-I, and LC3-II (n = 3); D: Quantification of the relative protein expression levels of c-Myc, Bax, Bcl-2, LC3-I, and LC3-II. Data are presented as the mean ± standard deviation (SD) (*p < 0.05, **p < 0.01); one-way analysis of variance (ANOVA) with Tukey's post hoc test

and cellular models, as well as variations in the duration and experimental protocols of I/R.

Rgs19, a member of the regulator of G protein signaling (RGS) family, modulates G protein-coupled receptor (GPCR) signaling by accelerating GTP hydrolysis of $G\alpha$ subunits, thereby acting as a negative regulator of intracellular signaling. According to previous studies, *Rgs19* influences cardiomyocyte differentiation and cardiac development through the Wnt/ β -catenin signaling pathway. Transgenic mice overexpressing *Rgs19* exhibit increased expression of heart failure markers, enhanced cell proliferation, and abnormal ventricular repolarization.³⁶ Overexpression of *Rgs19* has also been reported in several cancers and is associated with poor clinical outcomes.^{37,38} However, little is known about the role of *Rgs19* in myocardial I/R-induced microvascular dysfunction. Our study is the first to demonstrate significant upregulation of *Rgs19* in myocardial I/R-induced microvascular injury, suggesting that it may play an important role in the pathogenesis of this condition.

Furthermore, we developed a nomogram incorporating the 4 biomarker genes. This integrated model demonstrated a high AUC together with excellent calibration, indicating good diagnostic performance and reliability for detecting myocardial I/R injury. Implementation of this tool in clinical practice may improve the early identification of microvascular complications associated with myocardial I/R.

Further GSEA suggested that the pathogenesis of myocardial I/R may be associated with disturbances in citric acid cycle metabolism involving the 4 diagnostic genes. Substantial evidence indicates that myocardial I/R is closely associated with abnormalities in glucose and lipid metabolism.³⁹ These findings suggest that the 4 hub genes may participate in metabolic regulation during myocardial I/R and provide new insights into potential therapeutic targets. Myocardial I/R is also closely associated with immune regulation. During myocardial I/R injury, infiltration of immune cells, including macrophages and T lymphocytes, contributes to the inflammatory response.⁴⁰ In the early phase of reperfusion, activated neutrophils markedly increase reactive oxygen species (ROS) production, thereby exacerbating cardiomyocyte necrosis.⁴¹ Significant differences in immune cell abundance were observed between the 2 groups, and the expression of the biomarker genes was closely associated with the infiltration of multiple immune cell populations, including T cells, B cells, activated DCs, natural killer (NK) cells, and, in particular, macrophages. Macrophages, which play a pivotal role in the immune response to myocardial I/R, are commonly classified into M0, M1, and M2 subtypes. M2 macrophages exhibit anti-inflammatory and tissue-protective properties, whereas M1 macrophages promote inflammation and tissue injury.⁴² These findings suggest that the 4 hub genes may play important roles in immune regulation during myocardial I/R injury.

Moreover, an unsupervised clustering approach identified 2 molecular subtypes based on the expression profiles of 7 autophagy-related regulators. Functional enrichment analysis revealed that the C2 subtype was associated with oxidative phosphorylation, ROS generation, and the mitochondrial respiratory chain complex, all of which are involved in the regulation of autophagy.^{43,44} These findings suggest that the C2 subtype may be closely associated with autophagy dysregulation, which could have important implications for the early detection and treatment of myocardial I/R injury. Given the poor prognosis associated with the no-reflow phenomenon resulting from myocardial microvascular injury, the upregulation of the 4 autophagy-related hub genes identified in this study may represent potential therapeutic targets. These findings provide new insights into the molecular mechanisms of autophagy in myocardial I/R and may contribute to the development of improved strategies for the early diagnosis and treatment of this condition, ultimately improving patient outcomes.

Limitations of the study

However, this study has several limitations. First, evaluation of the therapeutic efficacy of *c-Myc* inhibition in animal models that more closely mimic human cardiac pathophysiology is needed. Second, the data analyzed in this study were obtained from the GEO database, and suitable human datasets were not available. Furthermore, the expression profiles of the 4 key genes were validated only in animal models and cell-based experiments and therefore require further validation in human cohorts. Future validation should include prospective studies evaluating serum biomarkers using enzyme-linked immunosorbent assay (ELISA) or Luminex assays in relation to cardiac magnetic resonance imaging (cMRI)-quantified microvascular obstruction (MVO) and the no-reflow phenomenon after percutaneous coronary intervention (PCI) in patients with ST-segment elevation myocardial infarction (STEMI). Such studies would establish the clinical utility of these biomarkers for predicting the no-reflow phenomenon and guiding adjuvant therapies. In addition, further investigation of the molecular mechanisms and signaling pathways involving these genes in myocardial I/R-induced microvascular injury is warranted.

Conclusions

This study is the first to identify autophagy-related biomarkers (*Myc*, *Casp4*, *Cdkn1a*, and *Rgs19*) associated with microvascular injury in cardiac I/R using integrated machine learning and experimental validation. We identified *c-Myc* as a pivotal regulator linking autophagic dysregulation to endothelial injury, with *c-Myc* knockout

demonstrating significant therapeutic potential. These findings highlight *c-Myc* inhibition as a novel treat-to-target therapeutic strategy for reperfusion injury, warranting clinical validation in STEMI cohorts and the development of point-of-care diagnostics. Our findings may also inform the future development of biomarker-guided strategies for the early detection and therapeutic modulation of cardiac microvascular I/R injury.

Supplementary data

The Supplementary materials are available at <https://doi.org/10.5281/zenodo.22122481>. The package contains the following files:

Supplementary Fig. 1. Cardiac I/R establishment and microvascular injury evaluation.

Supplementary Fig. 2. Bioinformatic analysis of GSE168610: PCA, differential expression, and autophagy gene overlap in I/R model.

Supplementary Fig. 3. GO and KEGG pathway analysis for distinct I/R subgroup.

Supplementary Fig. 4. Immunohistochemical (IHC) staining of *c-Myc* and *Caspase 4* in myocardial I/R.

Supplementary Fig. 5. Changes in mRNA and protein expression levels of the hub biomarkers in the H/R mode.

Supplementary Fig. 6. Observation of cellular autophagosome.

Supplementary Table 1. Specific information of datasets.

Supplementary Table 2. The primer sequences used for RT-qPCR.

Supplementary Table 3. Validation of key gene expression in the dataset GSE168610.

Supplementary Table 4. Power analysis of endpoint assays in myocardial I/R model.

Supplementary Table 5. Assessment of parametric test assumptions and selection of statistical methods for genes expression and TUNEL fluorescence intensity analysis.

Supplementary Table 6. Mann–Whitney U tests performed to compare 4 cardiac and functional parameters between sham and I/R groups (Supplementary Fig. 1B–E).

Data Availability Statement

The datasets supporting the findings of the current study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17137494>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Autoimmune disease influence on polycystic ovary syndrome: Insights from Mendelian randomization and multi-omics analysis

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Conflict of interest

None declared

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Abstract

Background. Polycystic ovary syndrome (PCOS) is a common endocrine disorder associated with a substantial health burden.

Objectives. Given the interplay between the immune and endocrine systems, this study aimed to investigate the potential causal relationship between autoimmune diseases and PCOS using Mendelian randomization (MR).

Materials and methods. A 2-sample MR analysis was conducted using genome-wide association study (GWAS) data from the FinnGen ($n = 118,870$) and European Bioinformatics Institute (EBI) ($n = 141,355$) cohorts. Instrumental variables were selected as single nucleotide polymorphisms (SNPs), and the inverse-variance weighted (IVW), weighted median, and MR-Egger methods were applied. Sensitivity analyses were performed to assess heterogeneity and horizontal pleiotropy. To validate the MR findings, transcriptomic analyses of Gene Expression Omnibus (GEO) datasets (GSE209596 for multiple sclerosis (MS) and GSE277906 for PCOS) were performed to identify shared differentially expressed genes (DEGs), followed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. Diagnostic nomograms were constructed based on the identified key genes.

Results. Mendelian randomization analysis suggested a potential inverse causal association between MS and PCOS (IVW: odds ratio (OR) = 0.906; 95% confidence interval (95% CI): 0.820–0.999; $p = 0.049$), which was consistent across both datasets and robust in the sensitivity analyses. No significant causal associations were identified for the other autoimmune diseases. Transcriptomic analysis identified 4 shared DEGs (*CDS2*, *ARHGD1B*, *GCHFR*, and *S100A9*), which were enriched in immune-related pathways. Nomogram models based on these genes accurately discriminated patients from controls in both cohorts, achieving areas under the curve (AUCs) of 82.8% for MS and 81.1% for PCOS.

Conclusions. This integrative analysis suggests a potential protective association between MS and PCOS mediated by immune-related mechanisms. These findings provide new insights into the immunopathology of PCOS and support the future development of diagnostic biomarkers.

Key words: transcriptomics, polycystic ovary syndrome (PCOS), multiple sclerosis (MS), Mendelian randomization, autoimmune diseases

Highlights

- Mendelian randomization (MR) reveals a protective link between multiple sclerosis (MS) and polycystic ovary syndrome (PCOS): Genetic evidence shows an inverse causal relationship, suggesting that MS-related immune mechanisms may reduce PCOS risk.
- Genome-wide MR analysis confirms robust associations using large FinnGen and EBI cohorts: Consistent findings across inverse-variance weighting (IVW), weighted median, and MR-Egger methods strengthen the evidence for a causal immune–endocrine connection.
- Shared immune-related biomarkers identified through transcriptomic profiling: Four overlapping DEGs – *CD52*, *ARHGDI1*, *GCHFR*, and *S100A9* – highlight common inflammatory pathways between MS and PCOS.
- Diagnostic nomograms achieve strong predictive accuracy for both diseases: Gene-based models demonstrated high discrimination performance, with AUC > 80%, supporting their potential for biomarker-guided diagnosis.

Background

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age, affecting approx. 6–12% of women worldwide.¹ It is characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology. It is a multifactorial disorder with a complex pathophysiology involving genetic predisposition, insulin resistance, and endocrine dysregulation. These factors contribute to reproductive complications, including infertility, menstrual irregularities, and pregnancy complications, as well as metabolic disturbances such as type 2 diabetes, dyslipidemia, and cardiovascular disease.^{2–6} Consequently, PCOS imposes a substantial burden on women's health and quality of life, increasing the risk of metabolic syndrome, type 1 diabetes (T1D), hypertension, and cardiovascular disease, thereby contributing to long-term morbidity and healthcare costs.⁷

Autoimmune diseases, including T1D, celiac disease, systemic lupus erythematosus, and autoimmune thyroid diseases,^{8–11} result from immune dysregulation, leading to chronic inflammation and tissue damage. Recent studies have shown that patients with PCOS often exhibit low-grade chronic inflammation, imbalances in immune cell subsets, and elevated levels of pro-inflammatory cytokines, suggesting that PCOS may have an underlying immunopathological component in addition to its endocrine and metabolic features.^{12,13} Evidence also indicates shared genetic susceptibility loci and immune-related pathways between PCOS and several autoimmune diseases, supporting the hypothesis of overlapping pathophysiological mechanisms.¹⁴

Among autoimmune diseases, multiple sclerosis (MS) is a prototypical disorder characterized by central nervous system demyelination and chronic neuroinflammation. It predominantly affects women of reproductive age and involves T-cell- and B-cell-mediated immune dysregulation and inflammatory signaling pathways.¹⁵ Recent studies have explored the genetic relationship among PCOS,

testosterone levels, sex hormone-binding globulin (SHBG), and MS. Although these studies did not support a protective causal effect of PCOS, as a hyperandrogenic phenotype, on the risk of MS in women, they identified several shared genetic loci involving testosterone, SHBG, and MS, providing new insights into the underlying biological mechanisms.¹⁶ These findings highlight the importance of considering both genetic and hormonal factors when investigating the relationship between PCOS and MS. Therefore, whether MS or other autoimmune diseases are causally associated with PCOS warrants further investigation and mechanistic validation.

Mendelian randomization (MR) is a genetics-based analytical approach that evaluates causal relationships between exposure factors and disease outcomes by using genetic variants as instrumental variables. For example, genetic variants associated with PCOS can be used to investigate their potential causal relationship with autoimmune diseases.¹⁷ This approach minimizes confounding and reverse causation, thereby allowing more robust causal inference. By leveraging genetic variants as naturally randomized instruments, MR provides stronger evidence for causality than conventional observational studies. Although previous studies have suggested potential associations between PCOS and autoimmune diseases, the causality and magnitude of these associations remain unclear.

Objectives

To clarify the direction of the causal relationship between autoimmune diseases and PCOS, we performed a 2-sample MR analysis using large-scale genome-wide association study (GWAS) data. To enhance the biological interpretability and clinical relevance of the MR findings, we further integrated transcriptomic analyses of publicly available gene expression datasets to identify shared differentially expressed genes (DEGs) between MS and PCOS. Functional enrichment analyses and predictive modeling

were subsequently performed. This integrative approach aims to provide a more comprehensive understanding of the immune-related mechanisms underlying the potential association between MS and PCOS and to identify candidate biomarkers for future risk assessment and therapeutic targeting.

Materials and methods

Study design and participants

The causal association between autoimmune diseases and PCOS was investigated using a 2-sample MR analysis. The analysis was based on GWAS datasets for autoimmune diseases and PCOS obtained from publicly available resources. Genetic variants represented by single nucleotide polymorphisms (SNPs) that were significantly associated with autoimmune diseases were selected as instrumental variables. The MR approach is based on 3 core assumptions: 1) the instrumental SNPs are strongly associated with the exposure (autoimmune diseases); 2) the selected SNPs are independent of potential confounding factors; and 3) the SNPs influence PCOS only through the exposure, with no alternative causal pathways.

A total of 8 autoimmune diseases were included as exposure variables: systemic lupus erythematosus, ankylosing spondylitis, MS, celiac disease, T1D, Crohn's disease, juvenile idiopathic arthritis, and primary biliary cholangitis. Polycystic ovary syndrome was analyzed as the outcome. Mendelian randomization analyses were performed using the inverse-variance weighted (IVW), weighted median, and MR-Egger methods. Sensitivity analyses were conducted to evaluate the robustness and validity of the findings. An overview of the study design is presented in Supplementary Fig. 1.

To enhance the biological interpretability and clinical relevance of the MR findings, we further integrated transcriptomic analyses, including differential gene expression analysis, functional enrichment analyses (GO and KEGG), and predictive nomogram construction.

Data sources and measurement

This study used data obtained from 2 large GWAS resources (<https://gwas.mrcieu.ac.uk>) to ensure adequate statistical power and reliability. PCOS-related genetic data were obtained from the FinnGen database (R8 release), which included 118,870 individuals with PCOS and covered 16,379,676 SNPs, and from the European Bioinformatics Institute (EBI) GWAS Catalog, which included 141,355 individuals with PCOS and covered 22,981,890 SNPs.

Genetic data for autoimmune diseases were obtained from multiple GWAS datasets covering systemic lupus erythematosus, ankylosing spondylitis, MS, celiac disease, T1D, Crohn's disease, juvenile idiopathic arthritis, and primary biliary cholangitis, with sample sizes ranging from 1,219 to 520,580 participants. All datasets included in this study were derived from populations of European ancestry to minimize potential bias due to population stratification. Detailed information on each dataset, including sample size and SNP coverage, is provided in Table 1.

To enhance the biological interpretability of the MR findings, we integrated transcriptomic data from 2 Gene Expression Omnibus (GEO) datasets: GSE209596 (memory regulatory T (mTreg) and memory effector T (mTeff) cells from 37 patients with MS and 40 healthy controls) and GSE277906 (cumulus cells from 23 patients with PCOS and 17 controls without PCOS). These datasets were used to identify and functionally characterize DEGs.

Variables

Selection of instrumental variables

Single nucleotide polymorphisms significantly associated with the exposure (autoimmune diseases) were selected as instrumental variables using a genome-wide significance threshold of $p < 5 \times 10^{-8}$.¹⁸ When no eligible SNPs met this criterion, a more relaxed threshold of $p < 5 \times 10^{-6}$ was applied. To ensure the independence of the selected SNPs, linkage disequilibrium (LD) pruning was performed using an LD threshold of $r^2 < 0.001$ within a 1,000-kb window. Single nucleotide polymorphisms with an F statistic ≤ 10

Table 1. Data sources and characteristics of the autoimmune disease datasets (all populations were of European ancestry)

GWAS ID	Exposure	Sample size	Number of SNPs
ebi-a-GCST90018917	systemic lupus erythematosus	482,911	24,198,877
ebi-a-GCST005529	ankylosing spondylitis	22,647	99,962
ieu-b-18	multiple sclerosis	115,803	6,304,359
ebi-a-GCST90061440	primary biliary cholangitis	24,510	5,004,018
ebi-a-GCST90020071	Crohn's disease	1,219	5,394,739
ebi-a-GCST90014023	type 1 diabetes	520,580	59,999,551
ebi-a-GCST005523	celiac disease	23,649	97,422
ebi-a-GCST005528	juvenile idiopathic arthritis	15,872	103,767

GWAS – genome-wide association study; SNP – single nucleotide polymorphism.

were considered weak instruments and excluded from the analysis, whereas those with an F statistic >10 were retained.

Mendelian randomization analysis

The TwoSampleMR package (v. 0.5.7; <https://github.com/MRCIEU/TwoSampleMR>) in R (R Foundation for Statistical Computing, Vienna, Austria) was used to perform MR analyses and investigate the causal relationship between autoimmune diseases and PCOS. The MR analyses included the IVW, weighted median, MR-Egger, simple mode, and weighted mode methods, allowing causal estimates to be evaluated using multiple complementary approaches.¹⁹ The IVW method combines the effects of multiple genetic variants using inverse-variance weighting, assuming that all instrumental variables are valid and unaffected by horizontal pleiotropy. Because the IVW method is sensitive to horizontal pleiotropy, sensitivity analyses were performed to assess the robustness of the findings. Applying all 5 MR methods enhances the robustness and reliability of the causal inference. Consistent findings across the different methods strengthen the evidence for a causal relationship, whereas inconsistent results warrant further investigation of potential pleiotropy or the validity of the selected instrumental variables. As the primary analytical method, IVW was used to assess the causal relationship between the exposure and the outcome, whereas the remaining methods provided supportive evidence. A positive result was defined as consistent directions of effect (all β coefficients either >0 or <0) across the IVW, weighted median, and MR-Egger methods, together with an IVW $p < 0.05$.

Sensitivity analyses

To validate the stability and credibility of MR results, extensive sensitivity analyses were carried out.²⁰ Cochran's Q test was used to assess heterogeneity, with a $Q_{pval} > 0.05$ indicating no heterogeneity. Heterogeneity prompted the use of either the weighted median method or the IVW random-effects model. In the absence of heterogeneity, the IVW random-effects model was used. Additionally, when sensitivity analyses indicated pleiotropy, the MR-PRESSO test was applied to identify and adjust for horizontal pleiotropy, enhancing the reliability of causal estimates. A pleiotropy $p > 0.05$ indicated no pleiotropy. If pleiotropy was detected, outliers were removed and the analysis was repeated; persistent pleiotropy led to exclusion of the result. The effect of each SNP on the overall estimate was assessed using a leave-one-out sensitivity analysis.

Differential expression analysis

Transcriptomic data were obtained from the GEO database, specifically GSE209596 (MS) and GSE277906

(PCOS). Differential expression analysis was carried out using the R package limma (<https://bioconductor.org/packages/release/bioc/html/limma.html>). After log₂ normalization of the expression matrices, a design matrix was constructed, and differential expression was evaluated using a linear model with empirical Bayes (eBayes) moderation. Genes with $|\log_2FC| > 0.585$ and a false discovery rate (FDR) <0.05 were considered significantly DEGs. The distribution and expression patterns of the DEGs were visualized using volcano plots and heatmaps.

Enrichment analysis

In order to explore the potential biological functions of disease-related target genes, target genes were subjected to GO and KEGG pathway enrichment analysis. First, the gene symbols were converted to Entrez IDs using the org.Hs.eg.db data package, and the clusterProfiler package was used to perform annotation analysis after removing genes that could not be mapped. In the GO analysis, the 3 major ontology categories (Biological Process, Cellular Component, and Molecular Function) were selected, and the thresholds for the p-value and q-value were set at 0.05. The KEGG pathway enrichment analysis was performed using the enrichKEGG function, and the significance thresholds were also set at $p < 0.05$ and $q < 0.05$.

Nomogram construction

A logistic regression-based nomogram model was constructed using key DEGs as predictive variables. Receiver operating characteristic (ROC) curve analysis was used to assess the model's discriminative ability, and decision curve analysis (DCA) was conducted to evaluate its net clinical benefit, highlighting its clinical utility.

Statistical analyses

Data analyses were executed using R v. 4.3.3 and GraphPad Prism v. 10.0.2 (GraphPad Software, San Diego, USA). Continuous variables were expressed as the mean $\pm$ standard deviation (SD) for normally distributed data and as the median with the interquartile range (IQR) for non-normally distributed data. The Shapiro–Wilk test was used to assess normality. For comparisons between 2 groups, either Student's t test or the Mann–Whitney U test was applied, as appropriate. For comparisons among 3 or more groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or the Kruskal–Wallis test followed by Dunn's multiple comparisons test was used. Correlation analyses were conducted using Pearson's or Spearman's methods, depending on data distribution. A 2-tailed $p < 0.05$ was considered statistically significant.

Results

Effect of autoimmune diseases on PCOS from the FinnGen database

Using the FinnGen dataset, MR analysis estimated a weak protective causal relationship between MS and PCOS, with the IVW method yielding an odds ratio (OR) of 0.906 (95% confidence interval (95% CI): 0.820–0.999, $p = 0.049$) (Fig. 1). MR-Egger, IVW, and weighted median analyses revealed consistent trends (Fig. 2). Sensitivity analyses showed no statistically significant heterogeneity ($Q_{pval} > 0.05$) or pleiotropy ($pval > 0.05$) (Table 2), indicating that the observed association was not driven by horizontal pleiotropy. No significant causal associations were observed between PCOS and the other autoimmune diseases included in the analysis, namely ankylosing spondylitis, celiac disease, Crohn's disease, juvenile idiopathic arthritis, primary biliary cholangitis,

Table 2. Sensitivity analysis results for PCOS from the FinnGen database

Exposure	Heterogeneity		Pleiotropy
	Q	Q_pval	pval
Ankylosing spondylitis	28.4877	0.160	0.647
Celiac disease	48.3491	0.053	0.585
Crohn's disease	8.5035	0.668	0.658
Juvenile idiopathic arthritis	0.9972	0.607	0.644
Multiple sclerosis	71.7435	0.211	0.064
Primary biliary cholangitis	29.7738	0.827	0.311
Systemic lupus erythematosus	1.9736	0.578	0.708
Type 1 diabetes	80.6416	0.279	0.693

The outcome is PCOS; PCOS – polycystic ovary syndrome.

systemic lupus erythematosus, and T1D (all $p > 0.05$), as summarized in Tables 2,3. To account for multiple testing across all autoimmune diseases, we applied the Benjamini–Hochberg FDR correction to the IVW

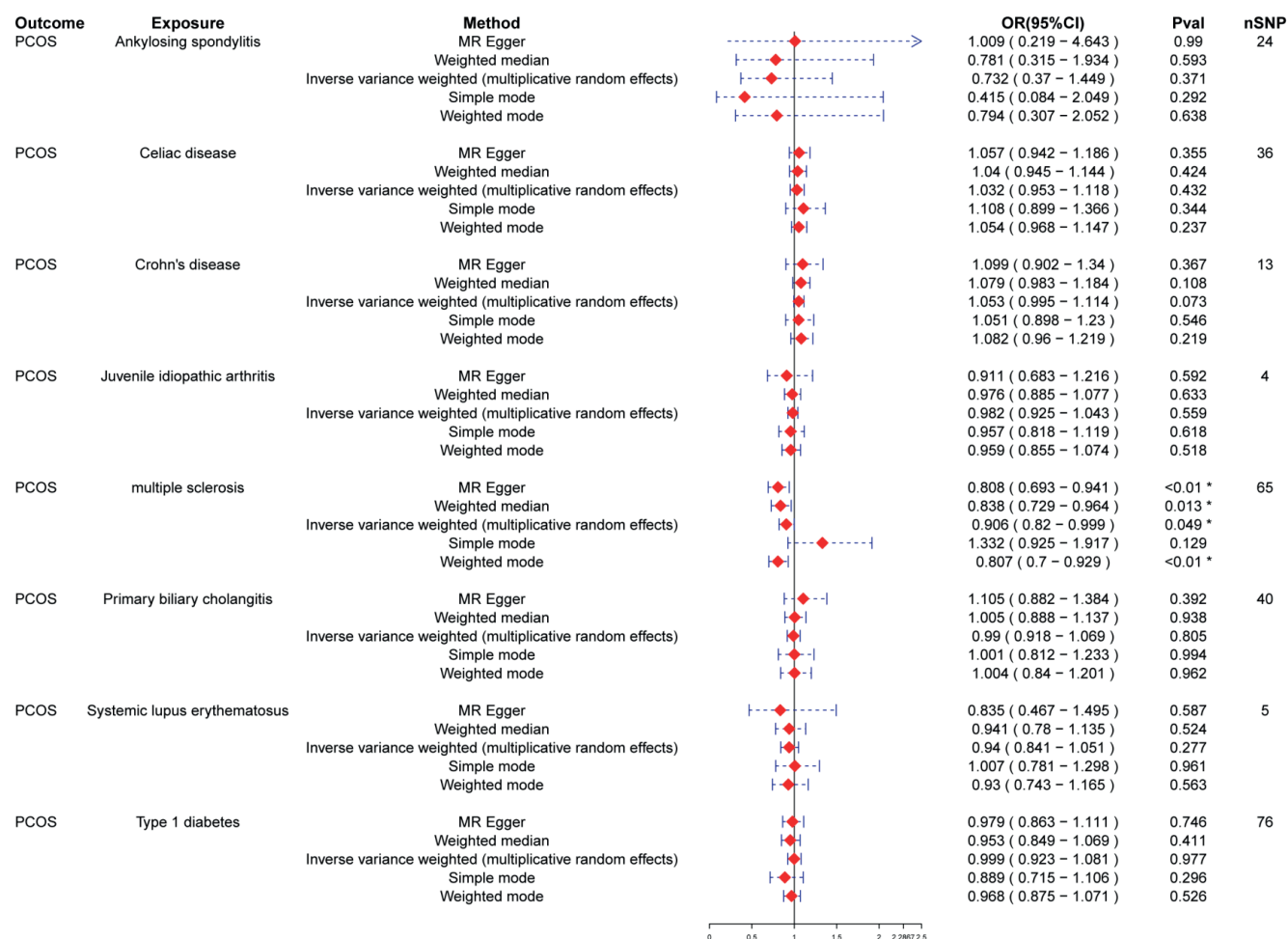


Fig. 1. Forest plot of the 2-sample Mendelian randomization (MR) analysis of autoimmune diseases and polycystic ovary syndrome (PCOS) using the FinnGen database. Rows illustrate distinct exposures or outcomes, showing the strength and direction of the associations. The red square represents the odds ratio (OR), and the horizontal line represents the 95% confidence interval (95% CI). The number of single nucleotide polymorphisms (SNPs) included in each analysis is shown in the far-right column. The x-axis represents the OR and its 95% CI for each association tested in the MR analysis. An OR of 1.0 (often marked by a vertical line) indicates no effect. Values greater than 1 indicate a positive association (increased odds), whereas values less than 1 indicate a negative association (decreased odds)

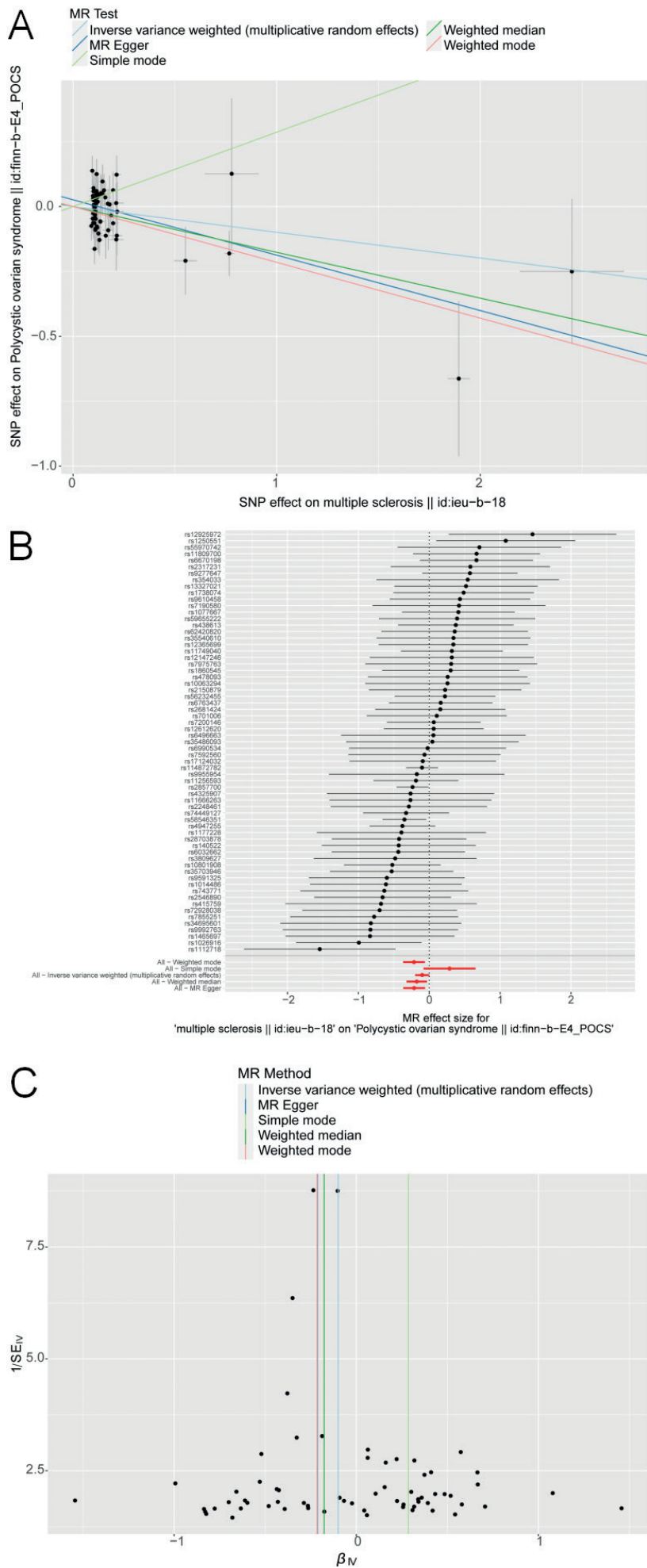


Fig. 2. Scatter plot of the 2-sample Mendelian randomization (MR) analysis using the FinnGen dataset, showing the effect of autoimmune diseases on polycystic ovary syndrome (PCOS). Each dot represents a single nucleotide polymorphism (SNP). A. Scatter plot – the x-axis shows the SNP effect sizes for autoimmune diseases, whereas the y-axis shows the SNP effect sizes for PCOS; B. Forest plot; C. Funnel plot

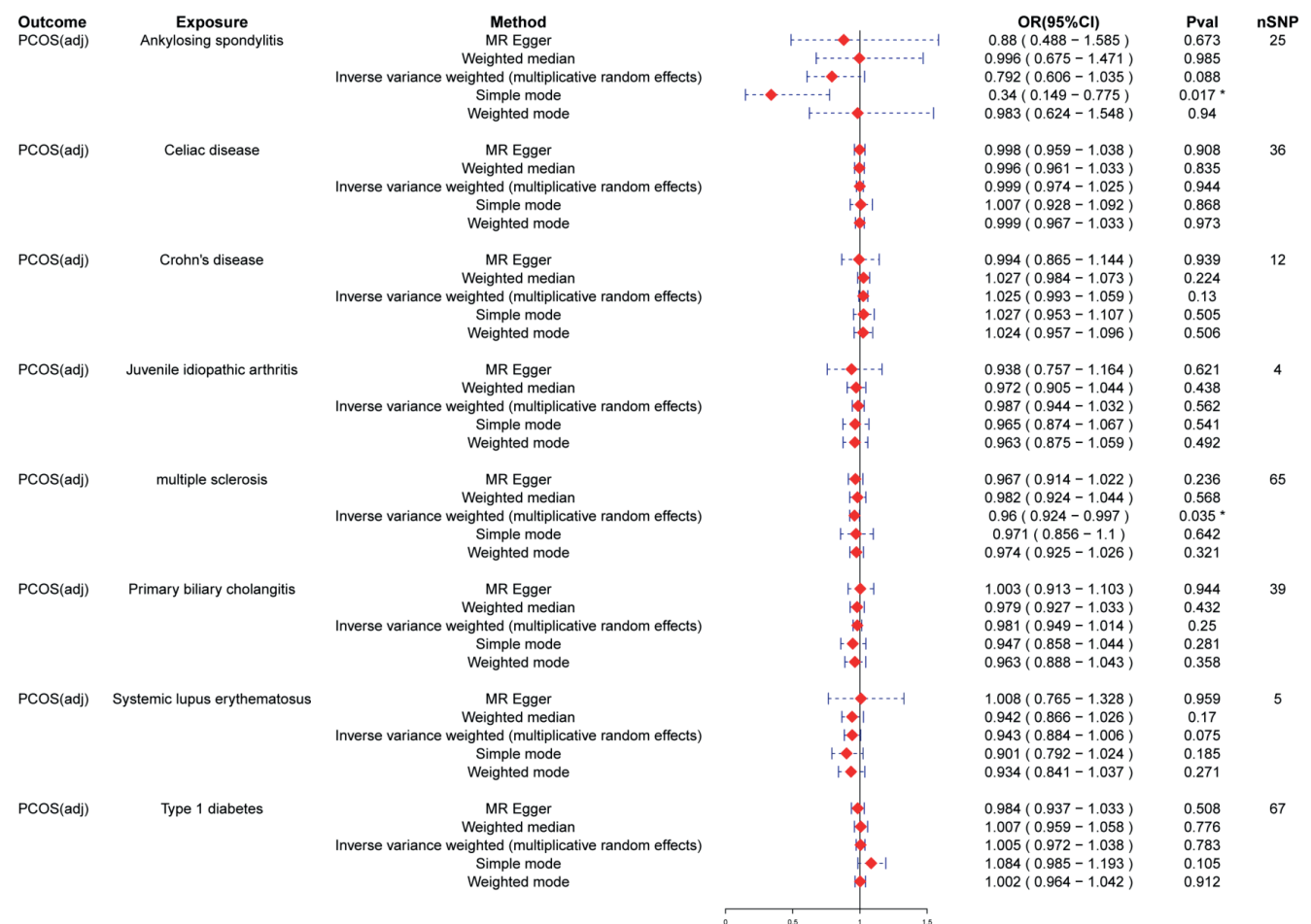


Fig. 3. Forest plot of the 2-sample Mendelian randomization (MR) analysis of autoimmune diseases and adjusted polycystic ovary syndrome (PCOS) using the European Bioinformatics Institute (EBI) database. Rows represent different exposures or outcomes, indicating the strength and direction of the associations. The red square represents the odds ratio (OR), and the horizontal line represents the 95% confidence interval (95% CI). The rightmost column indicates the number of single nucleotide polymorphisms (SNPs) used in each analysis. The x-axis represents the OR and its 95% CI for each association tested in the MR analysis

Table 3. Sensitivity analysis results for PCOS from the EBI database

Exposure	Heterogeneity		Pleiotropy
	Q	Q_pval	pval
Ankylosing spondylitis	25.1237	0.344	0.697
Celiac disease	28.9441	0.714	0.920
Crohn's disease	10.939	0.362	0.670
Juvenile idiopathic arthritis	1.4275	0.490	0.680
Multiple sclerosis	67.4216	0.329	0.745
Primary biliary cholangitis	30.4041	0.770	0.611
Systemic lupus erythematosus	3.5193	0.318	0.657
Type 1 diabetes	72.7314	0.239	0.253

The outcome is PCOS (adjusted). PCOS – polycystic ovary syndrome; EBI – European Bioinformatics Institute.

p-values; however, the MS–PCOS association did not remain significant after adjustment (Supplementary Table 1). These findings suggest that, if autoimmune factors contribute to PCOS, their effects are likely disease-specific rather than generalizable across autoimmune disorders.

Effect of autoimmune diseases on PCOS from the EBI database

To further investigate the observed relationship and eliminate potential confounding by age, an additional MR analysis was conducted using age-standardized PCOS data from the EBI database. The results remained consistent, with the IVW method estimating an OR = 0.960 (95% CI: 0.924–0.997, $p = 0.035$) (Fig. 3) for the protective effect of MS on PCOS. MR-Egger, IVW, and weighted median analyses revealed consistent trends (Fig. 4). Sensitivity analyses showed no statistically significant heterogeneity ($Q_{pval} > 0.05$) or pleiotropy ($pval > 0.05$) (Table 3), reinforcing the robustness of the observed association. After applying FDR correction, the inverse association between MS and PCOS was no longer significant in the EBI analysis (Supplementary Table 1).

Differential expression analysis and identification of key genes in MS and PCOS

In the MS dataset, a total of 3,316 significantly DEGs were identified through differential analysis between

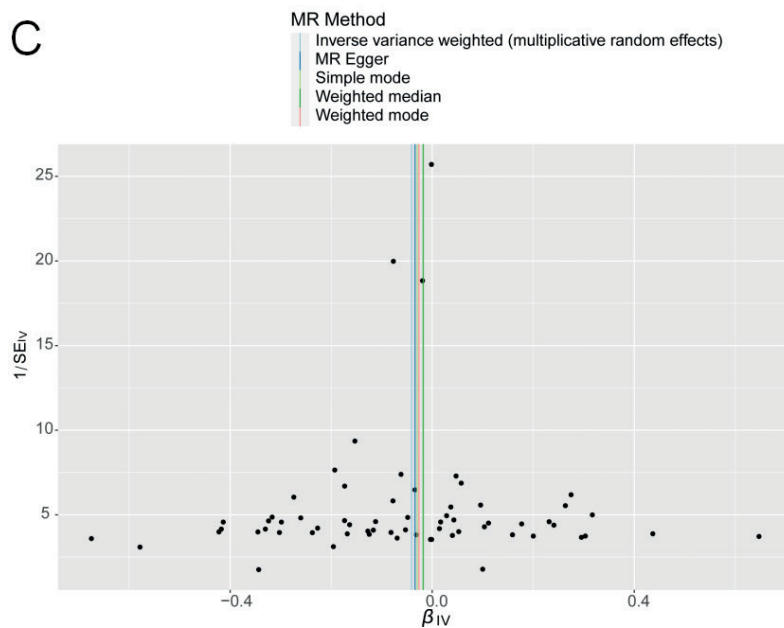
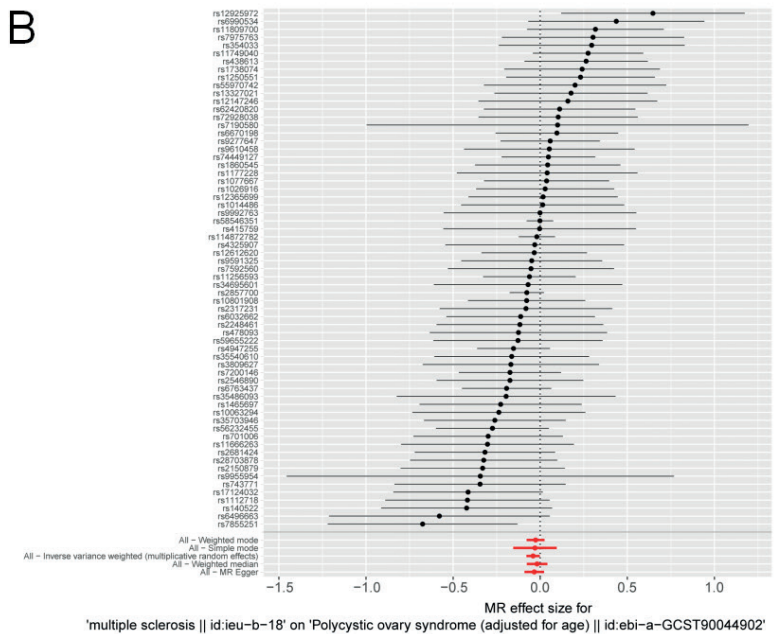
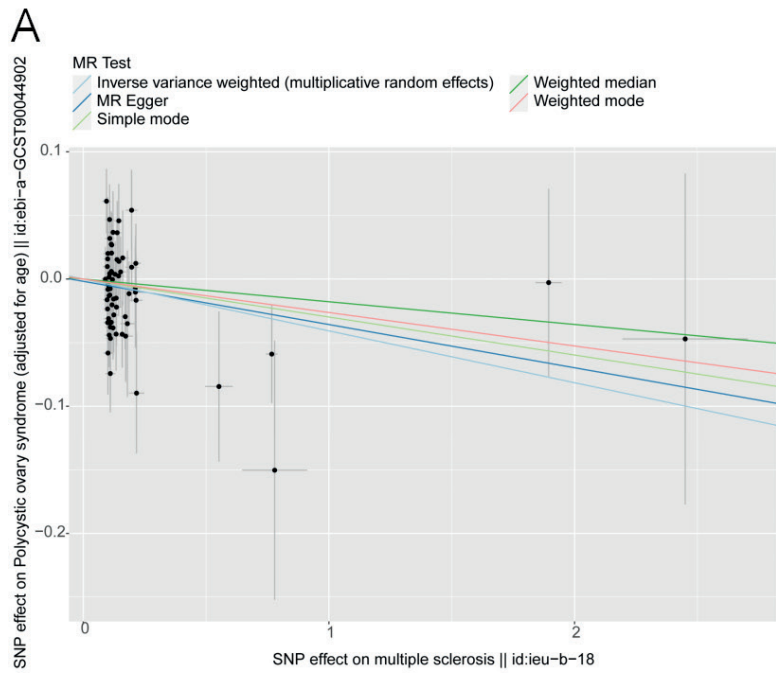


Fig. 4. Scatter plot of the 2-sample Mendelian randomization (MR) analysis using the European Bioinformatics Institute (EBI) dataset, showing the effect of autoimmune diseases on polycystic ovary syndrome (PCOS). Each dot represents a single nucleotide polymorphism (SNP). A. Scatter plot – the x-axis shows SNP effect sizes for autoimmune diseases, whereas the y-axis shows SNP effect sizes for PCOS; B. Forest plot; C. Funnel plot

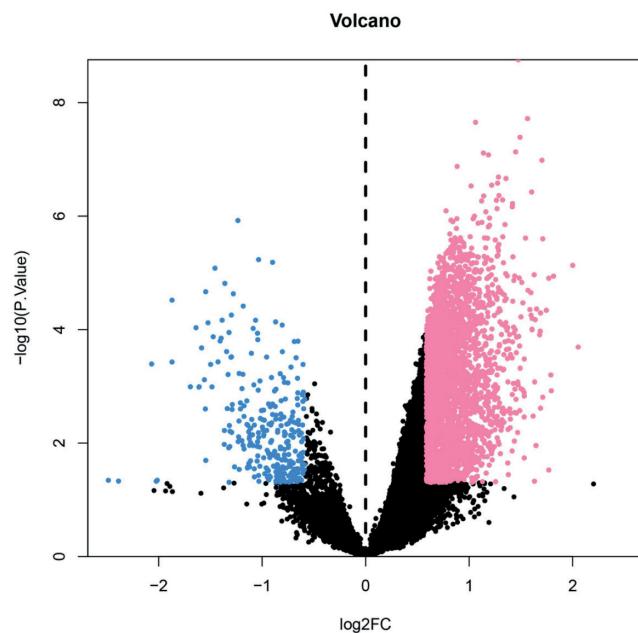


Fig. 5. Volcano plot of differentially expressed genes (DEGs) between multiple sclerosis (MS) patients and healthy controls in dataset GSE209596. Red and blue dots indicate significantly upregulated and downregulated genes, respectively ($|\log_2FC| > 0.585$, false discovery rate (FDR) < 0.05)

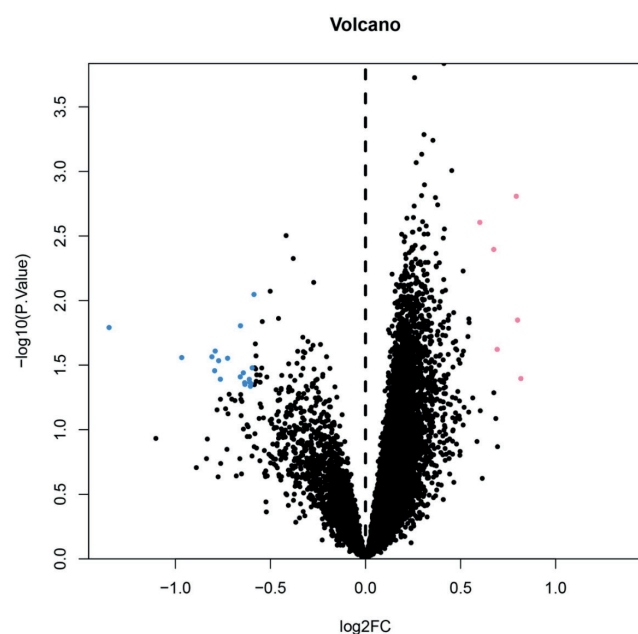


Fig. 6. Volcano plot of differentially expressed genes (DEGs) between polycystic ovary syndrome (PCOS) patients and non-PCOS controls in dataset GSE277906. Red and blue dots indicate significantly upregulated and downregulated genes, respectively ($|\log_2FC| > 0.585$, false discovery rate (FDR) < 0.05)

the disease and control groups, including 3,028 upregulated and 288 downregulated genes (Supplementary Table 2). A volcano plot clearly illustrates the distribution of significantly upregulated (red) and downregulated (blue) DEGs (Fig. 5). In the PCOS dataset, 25 significant DEGs were identified, comprising 6 upregulated and 19 downregulated genes (Supplementary Table 3), as visualized

in Fig. 6. Subsequently, the intersection of the 2 DEG sets revealed 4 shared DEGs: *CD52*, *ARHGDIB*, *GCHFR*, and *S100A9* (Supplementary Fig. 2).

Functional enrichment analysis of key DEGs via GO and KEGG

Gene Ontology (GO) enrichment analysis showed that these genes were significantly enriched in multiple immune- and inflammation-related biological processes. For example, the enriched biological processes included regulation of the immune response, leukocyte migration, cell chemotaxis, and T-cell activation. These findings suggest that the key genes may be involved in regulating the host immune response, particularly the immune imbalance observed in inflammatory disease states. Gene Ontology analysis also identified pathways involved in cytokine-mediated signal transduction and endocrine-immune interactions, further supporting their close association with disease-related immune mechanisms (Fig. 7A; Supplementary Table 4).

In the KEGG pathway enrichment analysis, the key genes were significantly enriched in several well-established signaling pathways, including the neurotrophin signaling pathway, interleukin (IL)-17 signaling pathway, and vasopressin-regulated water reabsorption. Among these, the IL-17 signaling pathway is closely associated with a variety of autoimmune and metabolic diseases, whereas the neurotrophin signaling pathway is involved in inflammatory regulation and cell survival. These results suggest that the key genes may contribute to immune regulation and participate in disease development through the neuroimmune axis (Fig. 7B; Supplementary Table 5).

Construction and evaluation of predictive nomogram models based on key DEGs

To evaluate the diagnostic utility of the 4 shared hub genes (*CD52*, *ARHGDIB*, *GCHFR*, and *S100A9*), we constructed multivariable logistic regression-based nomogram models for both MS and PCOS. The predictive performance of each model was assessed using ROC and DCA. In the MS cohort, the model demonstrated excellent discriminative ability, with an area under the curve (AUC) of 82.8% (95% CI: 73.4–92.1%). Decision curve analysis further confirmed a net clinical benefit across a wide range of threshold probabilities, highlighting the model's potential clinical applicability (Fig. 8). Similarly, the nomogram constructed for the PCOS cohort achieved an AUC of 81.1% (95% CI: 67.2–94.9%), accurately discriminating patients from controls and demonstrating strong diagnostic performance. The DCA curve showed a favorable net benefit, reinforcing its applicability for risk prediction and early clinical intervention (Fig. 9). The combined expression profile of the 4 hub genes may serve as a practical biomarker panel for individualized risk assessment in both MS and PCOS populations.

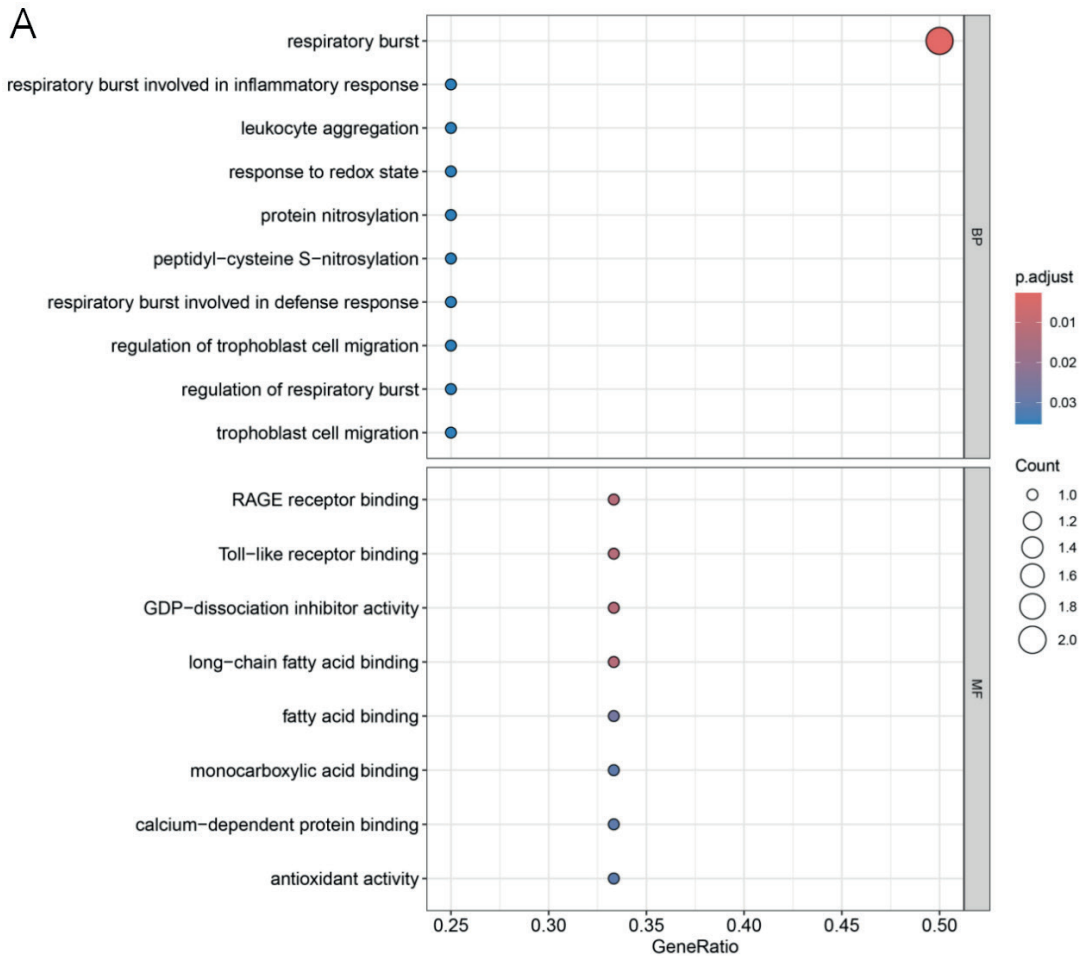


Fig. 7. Functional enrichment analysis of the 4 hub genes (*CD52*, *ARHGD1B*, *GCHFR*, and *S100A9*) identified from overlapping differentially expressed genes (DEGs) in multiple sclerosis (MS) and polycystic ovary syndrome (PCOS). A. Gene Ontology (GO) analysis showed significant enrichment in immune-related biological processes, including immune response regulation, leukocyte migration, chemotaxis, and T-cell activation; B. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed enrichment in pathways such as interleukin (IL)-17 signaling, neurotrophin signaling, and vasopressin-regulated water reabsorption, suggesting potential roles in immune modulation and neuroimmune interactions in MS and PCOS



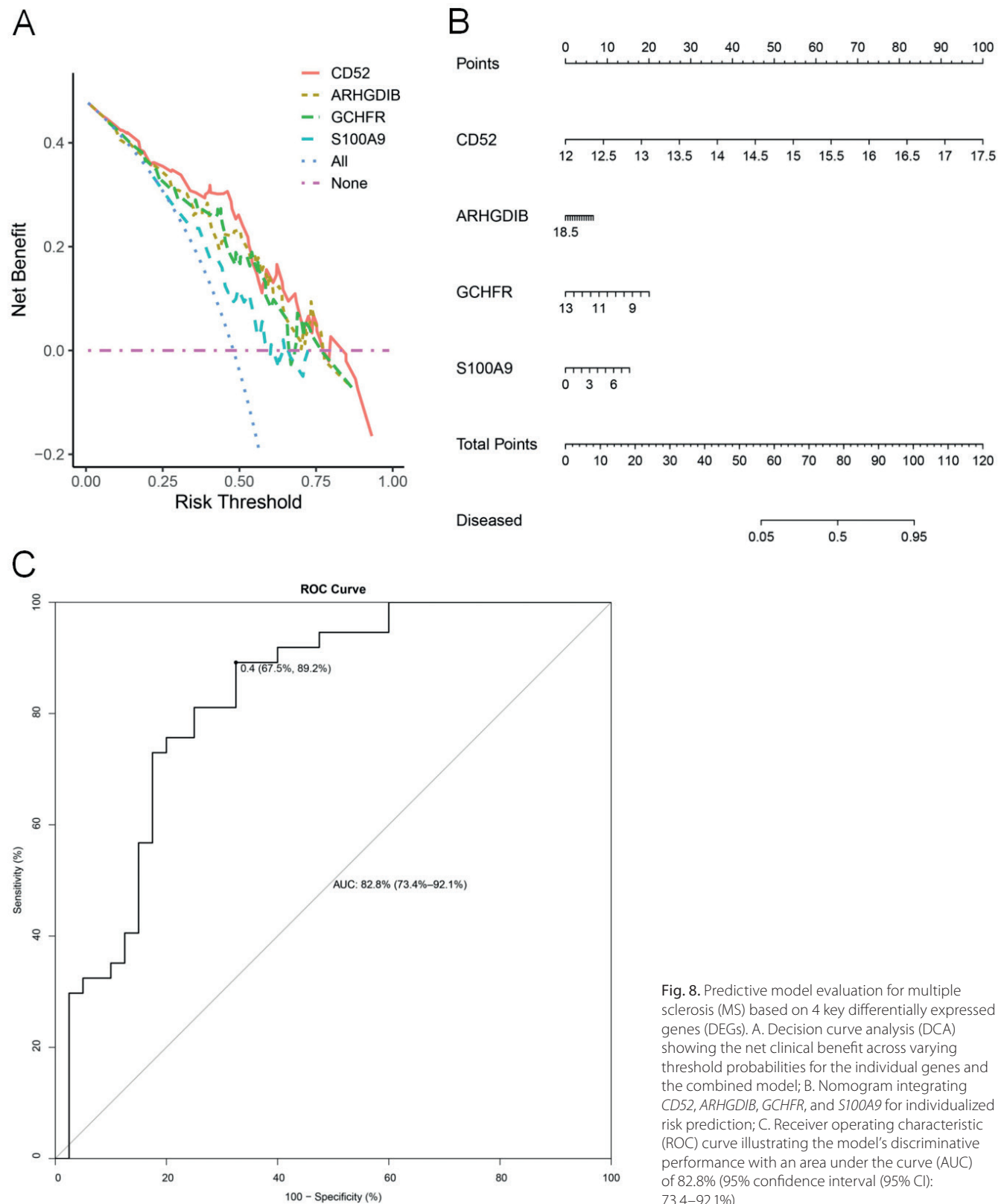


Fig. 8. Predictive model evaluation for multiple sclerosis (MS) based on 4 key differentially expressed genes (DEGs). A. Decision curve analysis (DCA) showing the net clinical benefit across varying threshold probabilities for the individual genes and the combined model; B. Nomogram integrating *CD52*, *ARHGDIB*, *GCHFR*, and *S100A9* for individualized risk prediction; C. Receiver operating characteristic (ROC) curve illustrating the model's discriminative performance with an area under the curve (AUC) of 82.8% (95% confidence interval (95% CI): 73.4–92.1%)

Discussion

Polycystic ovary syndrome is a common endocrine and metabolic disorder with a multifactorial pathogenesis involving hormonal imbalance, insulin resistance, and low-grade chronic inflammation.^{1,21} In this study,

we identified a novel inverse causal relationship between MS and PCOS using 2-sample MR. This finding was consistently observed across 2 large GWAS datasets and remained robust under multiple analytical frameworks. To further explore the underlying mechanisms, we integrated transcriptomic profiling and identified 4 shared

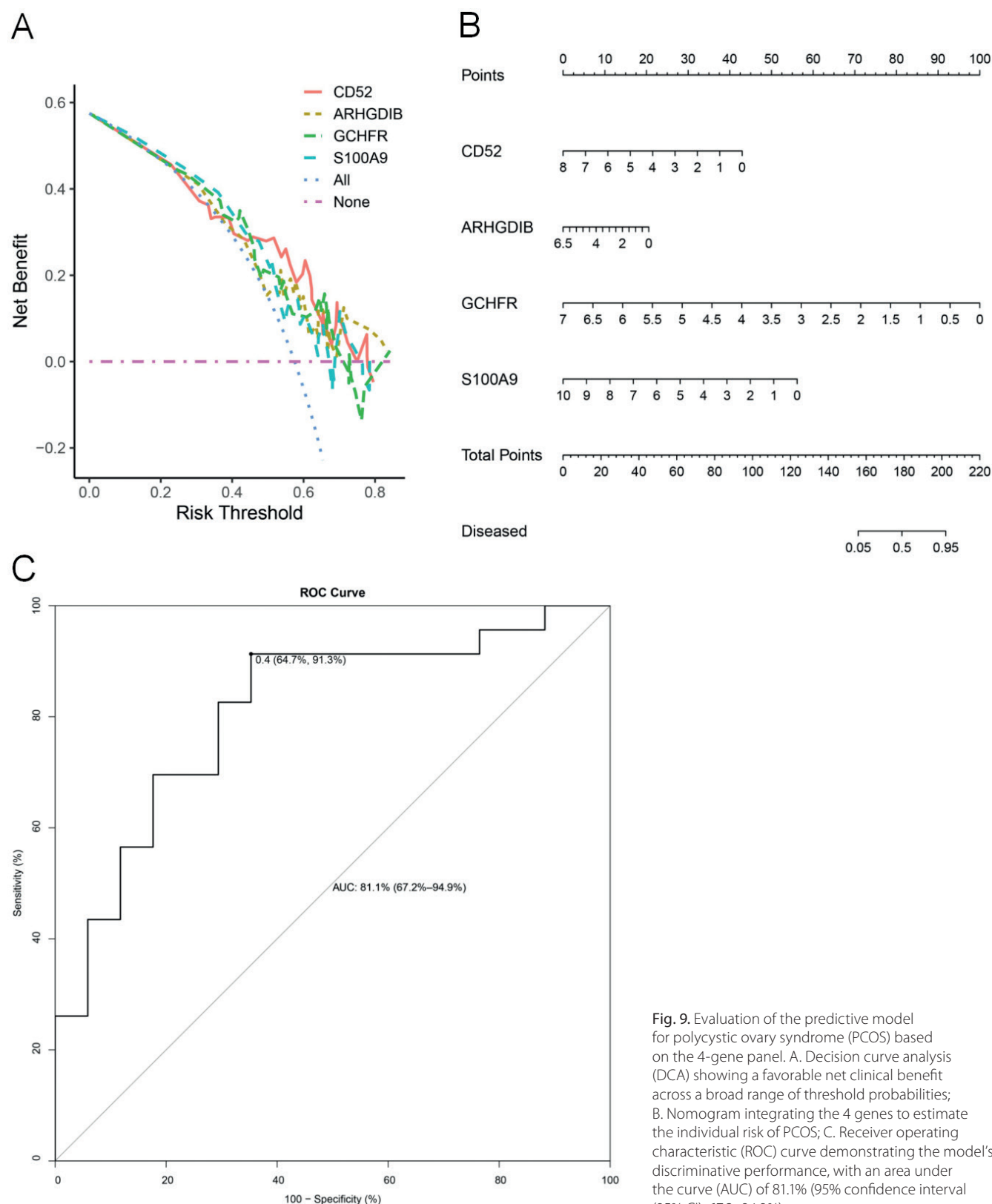


Fig. 9. Evaluation of the predictive model for polycystic ovary syndrome (PCOS) based on the 4-gene panel. A. Decision curve analysis (DCA) showing a favorable net clinical benefit across a broad range of threshold probabilities; B. Nomogram integrating the 4 genes to estimate the individual risk of PCOS; C. Receiver operating characteristic (ROC) curve demonstrating the model's discriminative performance, with an area under the curve (AUC) of 81.1% (95% confidence interval (95% CI): 67.2–94.9%)

hub genes (*CD52*, *ARHGDIB*, *GCHFR*, and *S100A9*) that exhibited opposite expression patterns in MS and PCOS. Functional enrichment analyses implicated these genes in immune and neuroendocrine pathways. Together, our findings provide evidence for immunological divergence

between MS and PCOS and suggest candidate molecular targets for future investigation.

The marked difference in the number of DEGs between MS and PCOS may result from variations in sample size, tissue type, and analytical thresholds. The MS dataset,

derived from immune cells, reflects broad inflammatory changes, whereas the PCOS dataset, based on cumulus cells, represents a localized endocrine environment with subtler transcriptomic alterations. Despite the smaller DEG set in the PCOS dataset, the shared genes likely indicate biologically robust immune–endocrine interactions.

It is also noteworthy that no significant causal associations were found between PCOS and the other autoimmune diseases. This may be explained by limited statistical power in some GWAS datasets, weak genetic correlations between PCOS and most autoimmune traits, or biological heterogeneity among different autoimmune disorders. These factors suggest that the observed association between MS and PCOS is likely disease-specific and may reflect distinct immune and endocrine interactions rather than a generalized autoimmune effect. Although the MS–PCOS association reached nominal significance, it did not remain significant after FDR correction, indicating that this result should be interpreted with caution and validated in larger cohorts.

The downstream transcriptomic analysis identified 4 hub genes (*CD52*, *ARHGDIB*, *GCHFR*, and *S100A9*) that were differentially expressed in MS and PCOS but in opposite directions, highlighting a shared yet antagonistically regulated immune signature. *CD52*, a lymphocyte surface antigen and the therapeutic target of alemtuzumab in MS, is known to suppress T-cell activation.²² Interestingly, downregulation of *CD52* has been observed in granulosa and cumulus cells from patients with PCOS, potentially contributing to impaired immune privilege and altered folliculogenesis.²³

S100A9 is a calcium-binding protein that typically forms a heterodimer with *S100A8*, known as calprotectin. It acts as a damage-associated molecular pattern (DAMP) molecule in various inflammatory diseases.²⁴ Studies have shown that serum levels of the *S100A8/S100A9* complex are significantly elevated in patients with MS, potentially contributing to central nervous system inflammation and demyelination by activating microglia and promoting the release of pro-inflammatory cytokines.²⁵ In PCOS, upregulation of *S100A9* has been associated with insulin resistance and low-grade chronic inflammation, suggesting that it may contribute to the pathogenesis of both diseases through distinct mechanisms, reflecting its multifunctional role in immune and metabolic regulation.²⁶

ARHGDIB has been linked to neutrophil function and blood–brain barrier regulation,²⁷ while *GCHFR* may influence neurotransmitter balance in MS and nitric oxide availability in ovarian tissue in PCOS by regulating tetrahydrobiopterin metabolism.²⁸ Additionally, previous studies have also reported downregulation of *CD52* and *ARHGDIB* in immune cells from PCOS patients, accompanied by increased pro-inflammatory cytokines, indicating a shift toward a pro-inflammatory immune profile.²⁹ The nomogram model constructed based on these 4 key genes demonstrated strong predictive performance in both MS and PCOS populations, suggesting their potential as disease-specific immune biomarkers to guide future stratified immunotherapeutic strategies.

In addition to gene-level signals, our functional enrichment analyses further confirmed mechanistic coherence between the transcriptomic and genetic findings. The 4 hub genes were enriched in IL-17 signaling and neurotrophin pathways – both pivotal in MS pathogenesis and increasingly implicated in PCOS. IL-17-producing Th17 cells are elevated in active MS and contribute to neuroinflammatory damage,³⁰ while reduced IL-17 signaling in PCOS is linked to disrupted ovarian angiogenesis and insulin signaling.³¹ Likewise, neurotrophins such as BDNF (brain-derived neurotrophic factor) and NGF (nerve growth factor) are upregulated in MS and aid in remyelination, but their deficiency in PCOS is associated with follicular dysmaturation and ovulatory failure.³² These findings suggest that shared immune–endocrine axes may be differently tuned across autoimmune and metabolic disease contexts, thus reinforcing the MR-based inference with biological plausibility.

Based on MR analysis, a potential inverse causal relationship between MS and PCOS was identified. Integrative transcriptomic analysis identified 4 immune-related hub genes (*CD52*, *ARHGDIB*, *GCHFR*, *S100A9*) with opposing expression trends in MS and PCOS, implicating divergent immune regulatory mechanisms. These findings provide preliminary evidence for disease-specific immunopathogenesis and offer potential molecular targets for future investigation.

Limitations of the study

While this integrative approach provides valuable insights, several limitations should be acknowledged. First, the GWAS and transcriptomic datasets were predominantly derived from European populations, which may limit the generalizability of our findings. Second, gene expression data were obtained from peripheral blood and cumulus cells rather than disease-specific tissues, such as the central nervous system or ovarian stroma. Third, reverse MR analysis was not performed. Finally, neither external nor internal validation of the transcriptomic or nomogram analyses was conducted because of the limited sample size, which may increase the risk of overfitting and affect reproducibility. Nonetheless, the consistency observed across the genetic and transcriptomic data supports the robustness of our conclusions. Future studies should include functional validation of the identified genes, broader multiethnic analyses, and bidirectional MR approaches to more definitively support or exclude the possibility of reciprocal causality between MS and PCOS.

Conclusions

This study suggests a possible inverse causal relationship between MS and polycystic ovary syndrome, although the association was not sustained after correction for multiple testing. Transcriptomic integration identified 4 shared

immune-related genes with opposite expression patterns, indicating distinct immune regulatory features in the 2 conditions. These findings provide preliminary biological support for disease-specific immune involvement in PCOS and highlight targets for further mechanistic validation. Larger and more diverse studies are required to confirm these results.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.17462656>. The package contains the following files:

Supplementary Table 1. FDR-adjusted MR results for autoimmune diseases and PCOS.

Supplementary Table 2. DEGs found between MS patients and healthy controls in the GSE209596 dataset.

Supplementary Table 3. DEGs identified between PCOS patients and non-PCOS controls in the GSE277906 dataset.

Supplementary Table 4. GO enrichment analysis results for the 4 shared hub genes.

Supplementary Table 5. KEGG pathway enrichment analysis of the 4 shared hub genes.

Supplementary Fig. 1. Research design.

Supplementary Fig. 2. Venn diagram showing the overlap of DEGs between MS and PCOS. Four common genes – *CD52*, *ARHGDI*B, *GCHFR*, and *S100A9* – were identified as shared DEGs between the 2 conditions.

Data Availability Statement

Data sharing does not apply to this article, as all data are already included in the manuscript and its supplementary files.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Neutrophil-related gene signature predicts prognosis and immune microenvironment patterns in thyroid cancer

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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. Thyroid carcinoma (THCA) represents the most prevalent malignancy of the endocrine system. Although the majority of patients exhibit favorable clinical outcomes, a subset still faces significant clinical challenges, including distant metastasis, recurrence, and poor response to therapeutic interventions, all of which substantially compromise quality of life and overall survival (OS).

Objectives. Neutrophils, as pivotal components of the innate immune system, exhibit dichotomous roles in cancer biology, exerting both tumor-promoting and tumor-suppressive functions. However, the prognostic value of neutrophil-related genes (NRGs) and their contribution to the molecular stratification of THCA remain poorly defined. Our aim was to identify and evaluate NRG biomarkers with the potential to inform personalized therapeutic strategies for patients with THCA.

Materials and methods. We first identified differentially expressed genes (DEGs) between THCA tumor and normal samples. Prognosis-associated DEGs were filtered using univariate Cox regression analysis. Subsequently, least absolute shrinkage and selection operator (LASSO) regression and multivariate Cox analysis were employed to identify a core set of prognostic NRGs. A risk model was constructed based on these core genes and validated in independent testing cohorts. Furthermore, consensus clustering was applied to delineate THCA molecular subtypes based on NRG expression profiles, followed by comprehensive analyses of functional enrichment and immune microenvironment characteristics.

Results. The prognostic signature derived from NRGs demonstrated robust and consistent predictive performance across the training and validation datasets. Patients with high NRG-based risk scores exhibited significantly poorer OS and a more immunologically complex tumor microenvironment (TME) than their low-risk counterparts. Consensus clustering further revealed 2 distinct molecular subtypes of THCA, each characterized by divergent immune infiltration patterns and functional pathway enrichment, suggesting biological heterogeneity with potential therapeutic implications.

Conclusions. Our study highlights NRGs as promising prognostic biomarkers in THCA, uncovering distinct molecular subtypes with potential relevance for personalized therapeutic stratification. These findings underscore the clinical value of NRGs and emphasize the need for further mechanistic investigations to advance our understanding of THCA biology and refine precision treatment strategies.

Key words: thyroid neoplasms, neutrophils, prognostic biomarkers, tumor microenvironment, gene expression profiling

Highlights

- Neutrophil-related genes (NRGs) predict prognosis and overall survival in thyroid carcinoma.
- An NRG-based risk model identifies thyroid cancer patients with significantly poorer overall survival.
- Two molecular subtypes of thyroid carcinoma show distinct immune infiltration and tumor microenvironment profiles.
- NRG biomarkers may support personalized risk stratification and precision treatment strategies in thyroid cancer.

Background

Thyroid carcinoma (THCA) is the most prevalent endocrine tumor.^{1,2} It is generally categorized into 3 pathological subtypes: differentiated thyroid cancer (DTC), medullary thyroid cancer (MTC), and anaplastic thyroid cancer (ATC).³ Its incidence is influenced by a variety of factors, most notably geographic region⁴ and sex, with women exhibiting nearly threefold higher incidence rates compared to men.⁵ Most cases of THCA exhibit an indolent clinical course and are amenable to conventional treatment strategies, including surgical resection and radioactive iodine therapy.⁶ While surgery is the standard therapy for MTC and is curative only in intrathyroidal disease, ATC and poorly differentiated thyroid cancer (PDTC) exhibit the most unfavorable prognosis.⁷ A subset of patients presents with aggressive clinical features, such as extrathyroidal extension,⁸ vascular invasion, or distant metastases, and consequently faces a markedly poorer prognosis.⁹ These clinical challenges underscore the pressing need to elucidate the molecular mechanisms driving THCA progression and to identify robust biomarkers that can not only predict therapeutic responses but also stratify patients according to their risk, thereby guiding the development of more precise and effective therapeutic strategies.

Within the tumor microenvironment (TME), a heterogeneous and dynamic network composed of malignant cells, stromal elements, and infiltrating immune populations collectively governs tumor initiation, progression, and therapeutic response.^{10,11} Among these, neutrophils represent the most abundant phagocytic leukocyte population, exhibiting both pro-tumorigenic and antitumor functions.¹² Tumor-associated neutrophils (TANs) have been shown to possess a spectrum of tumor-suppressive capacities. Neutrophil infiltration has been associated with enhanced CD8⁺ T-cell recruitment and improved prognosis in colorectal cancer, suggesting their role in promoting antitumor immunity. Conversely, TANs can also contribute to malignant progression by secreting pro-inflammatory chemokines such as interleukin-8 (IL-8) and potent angiogenic factors such as vascular endothelial growth factor (VEGF), thereby promoting neovascularization and tumor expansion.¹³ Neutrophils also release proteolytic enzymes that degrade components

of the extracellular matrix (ECM) and facilitate cancer cell invasion and metastasis.¹⁴ This functional duality underscores the complex role of neutrophils in cancer biology; however, their prognostic relevance and immunological context in THCA remain incompletely defined.

Objectives

The primary objective of this study was to identify and evaluate neutrophil-related gene (NRG) biomarkers with the potential to inform personalized therapeutic strategies for patients with THCA. Leveraging transcriptomic data from The Cancer Genome Atlas (TCGA), we sought to elucidate the prognostic value of NRG signatures in THCA. To this end, we constructed a prognostic model centered on a core set of NRGs, capable of stratifying patients by risk and predicting clinical outcomes.

Material and methods

Data collection

The mRNA expression profiles, somatic mutation data, and corresponding clinical information for patients with THCA were retrieved from the TCGA database (<https://portal.gdc.cancer.gov>). To ensure the reliability of the survival analyses, patients with an overall survival (OS) of less than 30 days were excluded, resulting in a final cohort of 502 cases. The cohort was randomly partitioned into a training and a validation set (7 : 3). Neutrophil-related genes were curated based on previously published literature and further refined using gene sets annotated in the Molecular Signatures Database (<https://www.gsea-msigdb.org/gsea/msigdb>).

Identification of differentially expressed NRGs

Differentially expressed genes (DEGs) between tumor and normal samples in the TCGA-THCA cohort were identified using the limma package in R (<https://bioconductor.org/packages/release/bioc/html/limma.html>) ($|\log_2FC| > 1$, adjusted $p < 0.05$). The intersection of the DEGs and NRGs

was then computed to obtain differentially expressed NRGs (DENRGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on the DENRGs using the clusterProfiler R package (<https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>).

Selection of prognostic features and construction of the THCA prognostic model

Univariate Cox regression was performed on the TCGA-THCA cohort to identify candidate genes ($p < 0.05$). Least absolute shrinkage and selection operator (LASSO) regression, implemented via the glmnet package in R (<https://cran.r-project.org/web/packages/glmnet/index.html>), was used to minimize overfitting and select genes by cross-validation with an optimal penalty parameter (λ). Multivariate Cox regression was then applied to construct a prognostic model using the survival package (<https://cran.r-project.org/web/packages/survival/index.html>). Risk scores were calculated based on gene expression levels and the corresponding coefficients. Patients were stratified into 2 groups according to the median risk score. Survival curves were plotted, and the model's predictive accuracy was evaluated using time-dependent receiver operating characteristic (ROC) curves with the timeROC package (<https://cran.r-project.org/web/packages/timeROC/index.html>). Risk score distributions, survival status, and heatmaps of gene expression were generated for visual comparison between the groups.

Construction of a nomogram for independent prognostic analysis

To assess the independent prognostic value of the constructed risk model, a nomogram was developed using the rms package in R (<https://cran.r-project.org/web/packages/rms/index.html>), integrating the prognostic risk score and clinical factors to predict the survival probabilities of patients with THCA. Calibration curves were plotted to evaluate the accuracy of the nomogram's predictions by comparing the predicted and observed survival rates.

Immune infiltration analysis

Malignant Tumours using Expression data (ESTIMATE) scores for patients with TCGA-THCA were calculated using Single Sample Gene Set Enrichment Analysis (ssGSEA) with the estimate and GSVA R packages (<https://estimate.r-forge.r-project.org>; <https://www.bioconductor.org/packages/release/bioc/html/GSVA.html>). Immune infiltration levels were assessed using the CIBERSORT algorithm (<https://cibersortx.stanford.edu>). Immune checkpoint expression across different

groups was analyzed and visualized using box plots. The association between immune subtypes and risk groups (high vs low) was evaluated. Immune subtype scoring data for THCA were downloaded from The Cancer Immunome Atlas (TCIA), and differences in scores between subtypes were analyzed.

Gene set enrichment analysis

The gene set enrichment analysis (GSEA) was performed using the GSEA software (<https://www.gsea-msigdb.org/gsea/downloads.jsp>) to identify enriched pathways between risk groups. Differential analysis between the high- and low-risk groups was conducted using the limma package ($|\log_2FC| > 0.585$, $p < 0.05$). The GO enrichment analysis was performed on differential genes.

THCA subtype analysis

Based on the NRGs from the constructed model, consensus clustering was performed on THCA samples from the TCGA-THCA dataset using the ConsensusClusterPlus package (<https://www.rdocumentation.org/packages/ConsensusClusterPlus/versions/1.36.0/topics/ConsensusClusterPlus>). A total of 205 patients were assigned to Cluster 1 and 297 to Cluster 2. Kaplan–Meier survival curves were generated using the survminer package (<https://cran.r-project.org/web/packages/survminer/index.html>) to compare OS between subtypes. Immune infiltration analysis was subsequently conducted for both subtypes, with differential analysis performed using the limma package and GO enrichment analysis.

Drug sensitivity analysis and regulatory network construction

To identify potential therapeutic targets and effective drugs, we utilized the CellMiner database (<https://discover.nci.nih.gov/cellmineradb>) to screen for antitumor drugs whose sensitivity is significantly associated with prognostic genes. The pRRophetic R package (<https://github.com/paulgeeleher/pRRophetic>) was used to predict the half-maximal inhibitory concentration (IC_{50}) of different drugs in risk groups.

Statistical analyses

Statistical significance was determined using R v 4.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Spearman analysis was utilized to compute the correlation coefficient. The difference between groups was assessed for statistical significance using Wilcoxon rank sum test. Two-way analysis of variance (ANOVA) test was utilized for comparing differences among the 3 groups. R v. 4.4.2 software was employed for analyzing all bioinformatics data. All Cox regression analyses, including

model fitting and assumption verification, were performed using the survival R package. The ROC curves were used to evaluate the predictive performance of candidate genes used to construct predictive models.

Results

Development of a neutrophil-related prognostic model for THCA

The overall research workflow is illustrated in Fig. 1. Differential expression analysis between tumor and normal tissues identified DEGs (Supplementary Fig. 1A). Intersecting the DEGs with the NRGs gen set yielded 91 DENRGs (Supplementary Table 1), which were subjected to GO and KEGG enrichment analyses. These genes were significantly enriched in immune-related processes and pathways, including “leukocyte migration”, “cell chemotaxis”, “cytoplasmic vesicle lumen”, and “cytokine–cytokine

receptor interaction” (Supplementary Fig. 1B). Univariate Cox regression identified prognostic DENRGs (Fig. 2A), followed by LASSO regression to reduce redundancy and prevent overfitting, ultimately selecting 4 candidate genes (Fig. 2B,C). Multivariate Cox regression refined the model to 3 signature genes (Fig. 2D): Risk score = $0.583 \times CCL17 - 0.467 \times NPC2 - 0.564 \times DPP4$. Schoenfeld and Martingale residuals indicated that the proportional hazards and linearity assumptions of the Cox model were satisfied, and all variance inflation factors were below 3 (Supplementary Tables 2,3 and Supplementary Fig. 2), supporting the validity of the analysis. Spearman’s correlation among the 3 genes was low (all $p < 0.05$, Supplementary Fig. 3), confirming the independence of the predictors.

Patients were stratified into risk groups based on the median risk score. Time-dependent ROC analysis showed that the area under the curve (AUC) values for 1-, 3-, and 5-year survival all exceeded 0.7 in the training, validation, and full TCGA cohorts, suggesting strong predictive performance (Fig. 3A). Compared with other transcriptome-based

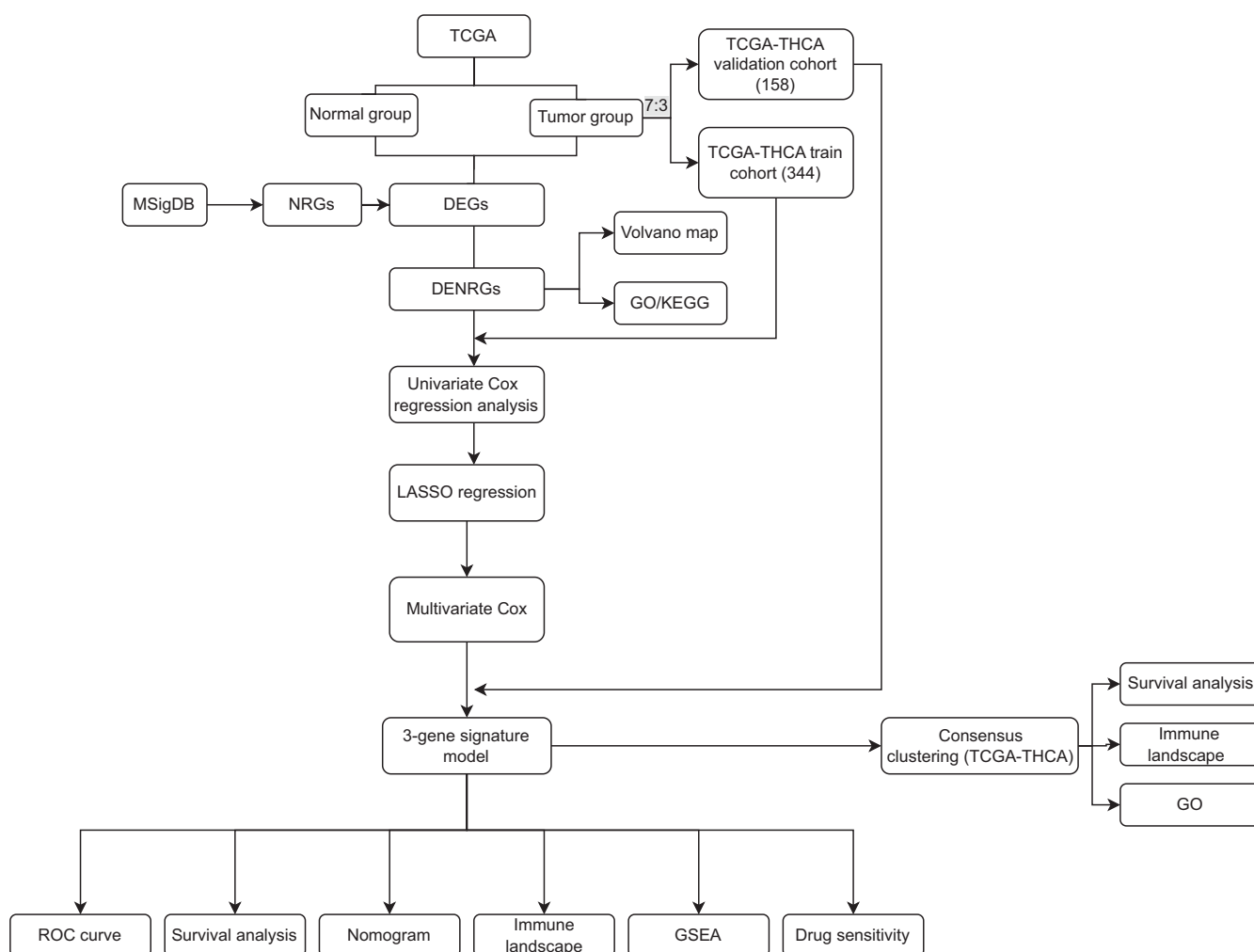


Fig. 1. Flowchart of the research process

THCA – thyroid carcinoma; TCGA – The Cancer Genome Atlas; KEGG – Kyoto Encyclopedia of Genes and Genomes; GO – Gene Ontology; GSEA – gene set enrichment analysis; DEGs – differentially expressed genes; NRGs – neutrophil-related genes; DENRGs – differentially expressed NRGs; ROC – receiver operating characteristic; LASSO – least absolute shrinkage and selection operator.

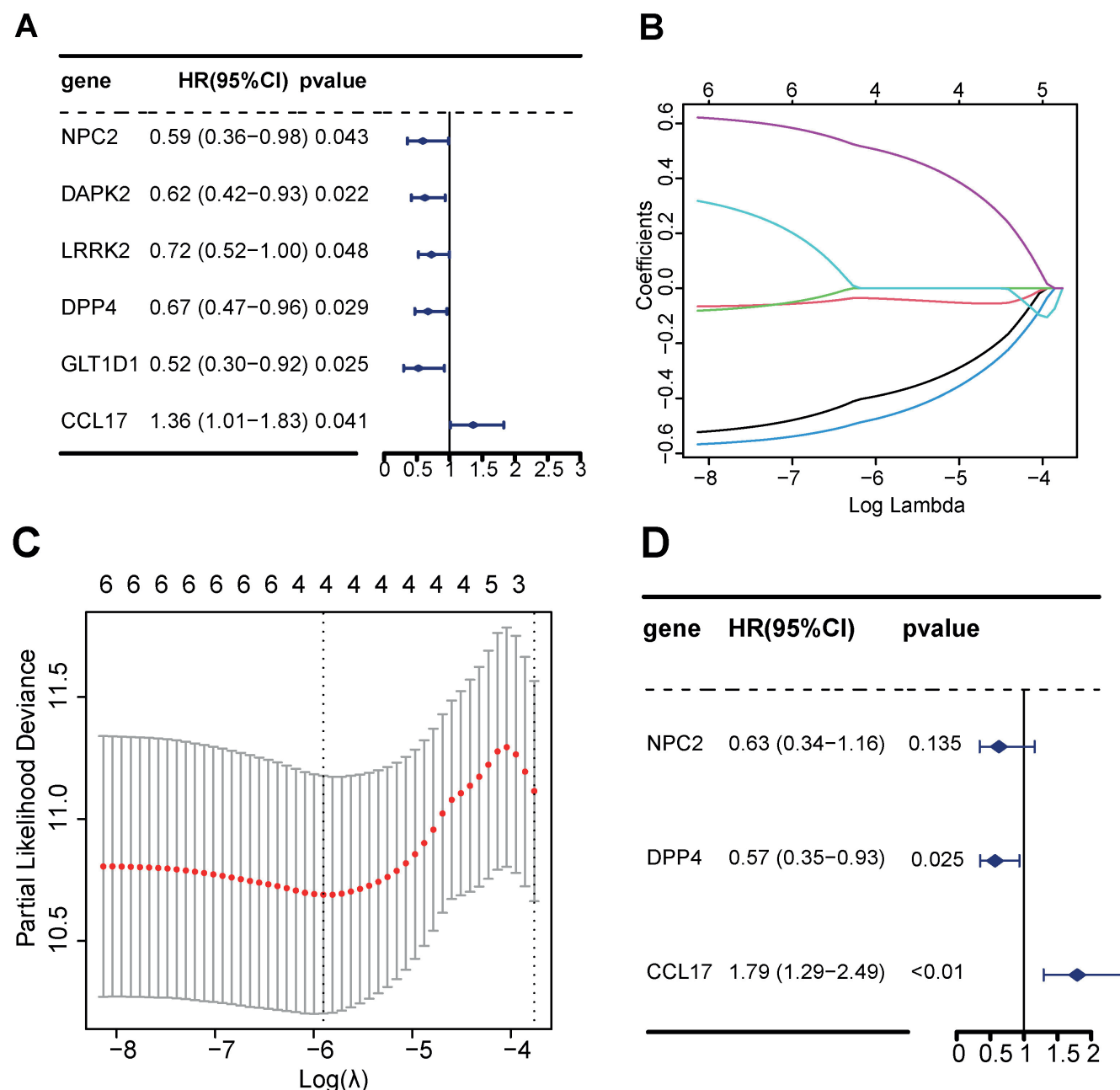


Fig. 2. Development and validation of the prognostic model. **A.** Univariate Cox regression analysis of prognosis-related differentially expressed neutrophil-related genes (DENRGs); **B.** Feature selection using least absolute shrinkage and selection operator (LASSO) regression analysis of candidate neutrophil-related genes (NRGs); **C.** Coefficient profiles from LASSO regression of the 4 prognostic NRGs; **D.** Multivariate Cox regression results for the 3-gene NRG signature

HR – hazard ratio; 95% CI – 95% confidence interval.

prognostic models, our model achieved relatively high AUC values (Supplementary Fig. 4). Kaplan–Meier analysis demonstrated significantly worse survival in the high-risk group across all cohorts (Fig. 3B). Risk score distributions, survival status plots, and heatmaps of gene expression further illustrated clear distinctions between the 2 risk groups (Fig. 3C). Among the 3 signature genes, *CCL17* was significantly upregulated in the high-risk group, whereas *NPC2* and *DPP4* were downregulated (Fig. 3D). Expression levels of all 3 genes were significantly elevated in tumor tissues compared with normal controls (Fig. 3E). Moreover,

survival analysis based on gene expression stratification revealed that high *NPC2* expression was associated with a better prognosis (Fig. 3F).

Independent prognostic value and nomogram construction

Univariate and multivariate Cox regression analyses, including clinical parameters and the risk score, indicated that both age and the risk score were independently associated with OS (Fig. 4A,B). Notably, the risk score remained

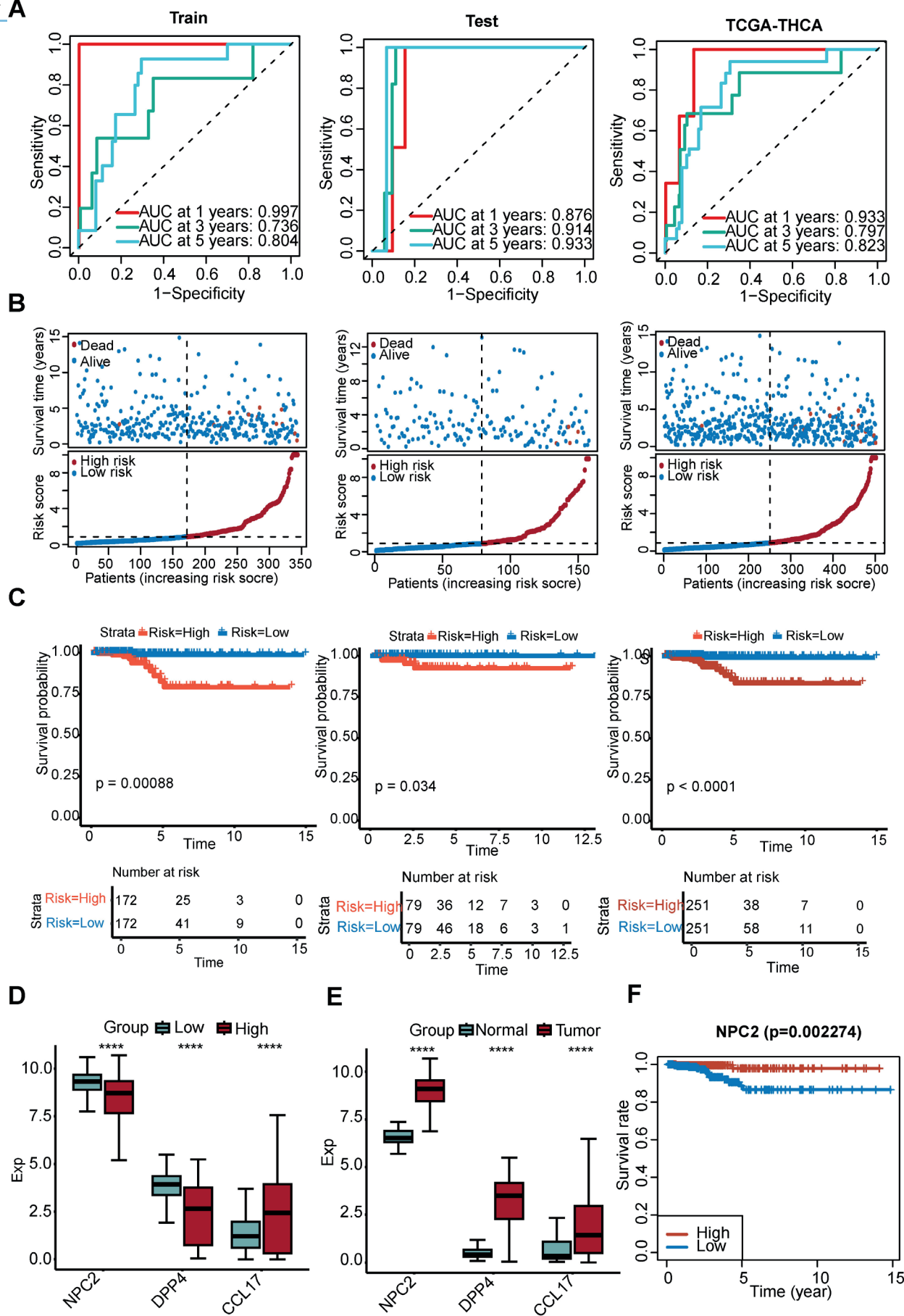


Fig. 3. Prognostic performance and expression patterns of the neutrophil-related gene (NRG)-based signature. **A.** Receiver operating characteristic (ROC) analysis in train, test, and The Cancer Genome Atlas (TCGA)-thyroid carcinoma (THCA) cohort; **B.** Risk score distribution, and survival status stratified by risk score for the training, test and TCGA-THCA cohort; **C.** The difference in expression of the 3 NRGs between high- and low-risk in TCGA dataset; **D.** The difference in expression of the 3 NRGs between normal and tumor tissues in TCGA dataset; **E.** Signature gene expression across risk groups

* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$; **** $p < 0.0001$; ns – not significant.

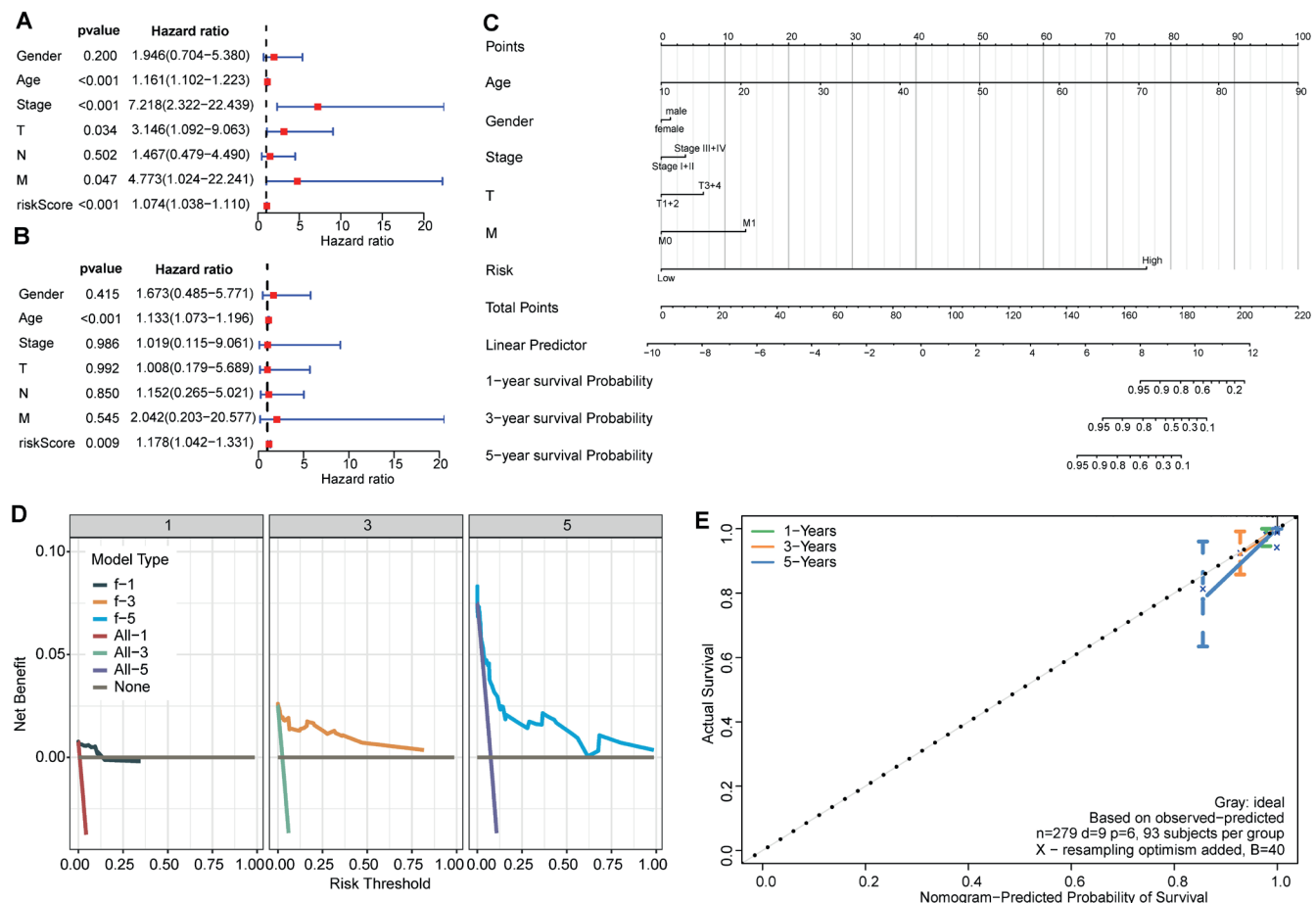


Fig. 4. Comprehensive prognostic evaluation of 3 signature genes and nomogram in thyroid carcinoma (THCA). Forest plot of univariate Cox (A) and (B) multivariate Cox regression analysis; C. Nomogram for predicting 1-, 3-, and 5-year survival; D. Decision curve analysis for 1-, 3-, and 5-year overall survival (OS); E. Calibration plots for 1-year, 3-year, and 5-year survival rates

a significant adverse prognostic factor ($p < 0.01$, hazard ratio (HR) >1), confirming its independence and robustness. A prognostic nomogram integrating clinical variables and the risk score was developed to predict survival probabilities in patients with THCA (Fig. 4C). Calibration plots demonstrated strong concordance between the predicted and observed survival outcomes (Fig. 4D,E), indicating good predictive performance of the model. Moreover, patients with advanced-stage disease (stage III–IV) exhibited significantly higher risk scores, aligning with the stratified prognostic potential of the risk model (Supplementary Fig. 5).

Immune infiltration landscape between high- and low-risk groups

Immune phenotyping based on the Immunophenoscore (IPS) revealed significantly higher scores across most immune categories in the low-risk group, except for the IPS_CTLA4_pos_PD1_pos subgroup, which showed no significant difference (Fig. 5A, Supplementary Table 4). These results suggest enhanced predicted responsiveness to immune checkpoint blockade in the low-risk cohort. We next assessed the expression of immune checkpoint (ICP)-related genes between the 2 groups (Fig. 5B, Supplementary

Table 5). Most ICP genes, including *CD200*, *CD11*, and *TNFSF15*, were downregulated in the high-risk group. Notably, *IDO1* and *TNFSF4* were significantly upregulated in the high-risk group, suggesting potential immune evasion mechanisms. Although the high-risk group exhibited a significantly elevated StromalScore, analysis of the IMvigor210 cohort revealed a reduced immune response compared with the low-risk group (Fig. 5C, Supplementary Tables 6,7). ssGSEA revealed distinct immune functional differences: aDCs and type II IFN response were enriched in the low-risk group, whereas B cells, dendritic cells (DCs), and T follicular helper (Tfh) cells were more abundant in the high-risk group (Fig. 6A,B, Supplementary Table 8). To further dissect immune infiltration, we applied the CIBERSORT algorithm to estimate the abundance of 22 immune cell types. Significant differences were observed in specific populations (Fig. 6C, Supplementary Table 9): monocytes were increased in the high-risk group, whereas M0 macrophages and resting mast cells were more enriched in the low-risk group.

Pathway enrichment analysis with GSEA

Gene set enrichment analysis was performed to identify KEGG pathways differentially enriched between

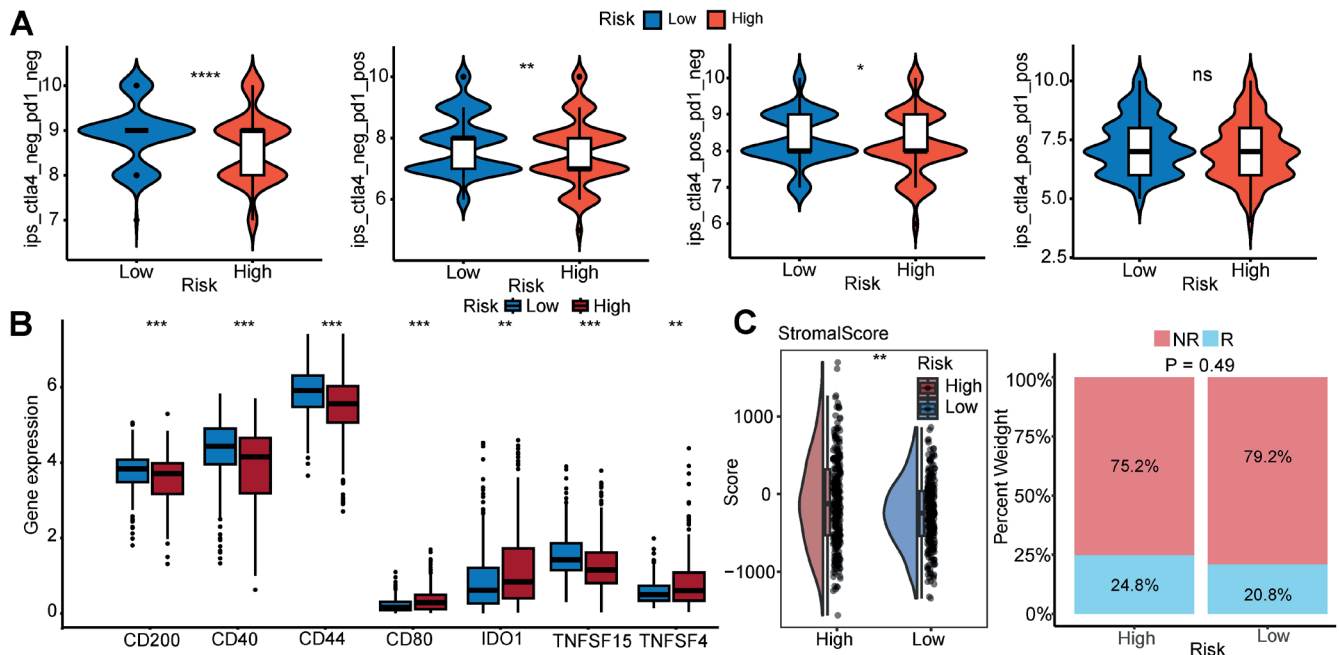


Fig. 5. Immune landscape and stromal characteristics in the risk subgroup. A. Immunophenoscore (IPS); B. Risk-stratified expression patterns of immune checkpoint molecules; C. ESTIMATE algorithm-derived stromal scores comparison

* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$; ns – not significant. NR – no response; R – response.

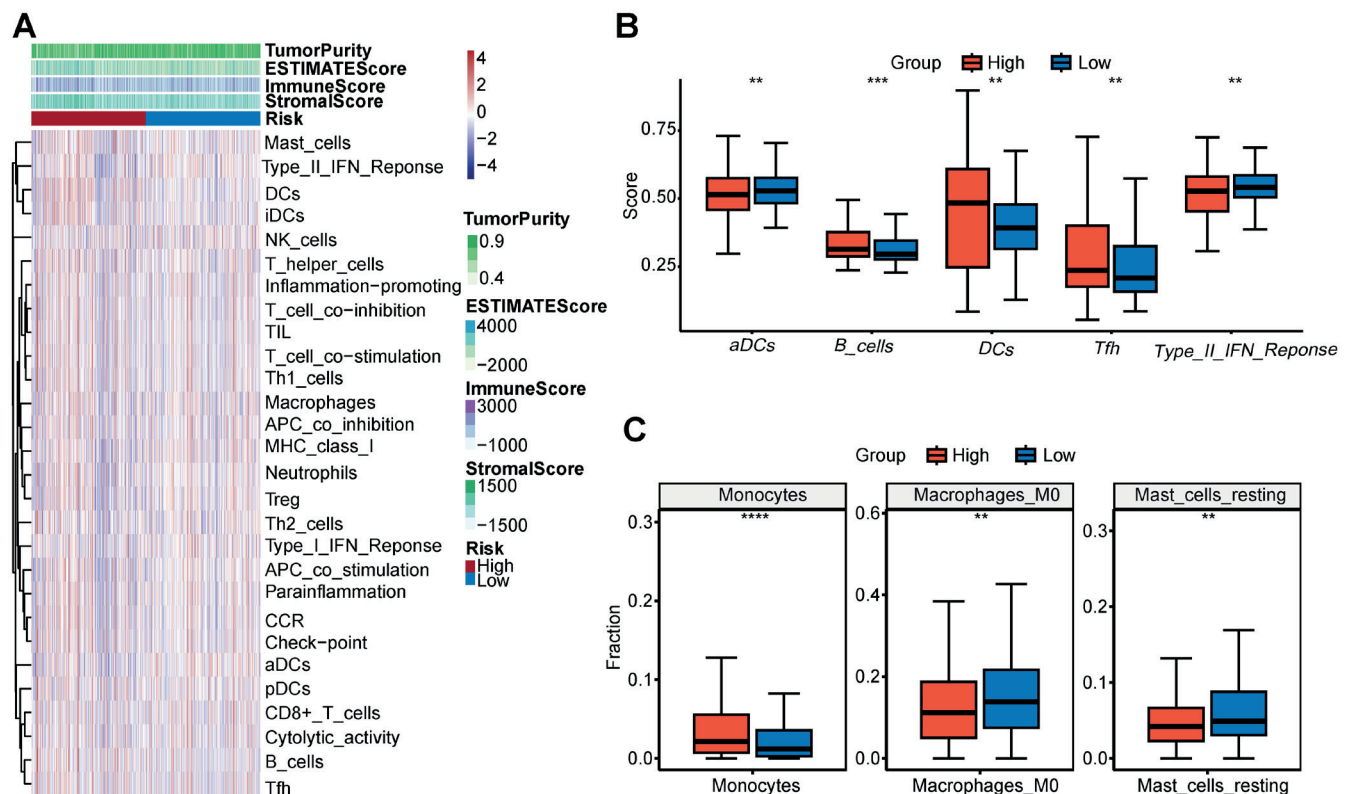


Fig. 6. Immune microenvironment and infiltration analysis in risk subgroups. A. The heatmap showed the immune cell infiltration and immune functions; B. Box plots of Single Sample Gene Set Enrichment Analysis (ssGSEA) immune checkpoint scores; C. CIBERSORT algorithm analysis of immune infiltration

* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$; ns – not significant.

the risk groups. In the high-risk group, significantly enriched pathways included cardiac muscle contraction, oxidative phosphorylation, Parkinson's disease, and propanoate metabolism (Supplementary Fig. 6). In contrast,

the low-risk group showed enrichment in pathways such as adherens junction, chronic myeloid leukemia, DNA replication, and lysosome (Supplementary Fig. 7). Subsequent differential expression analysis between the 2 risk

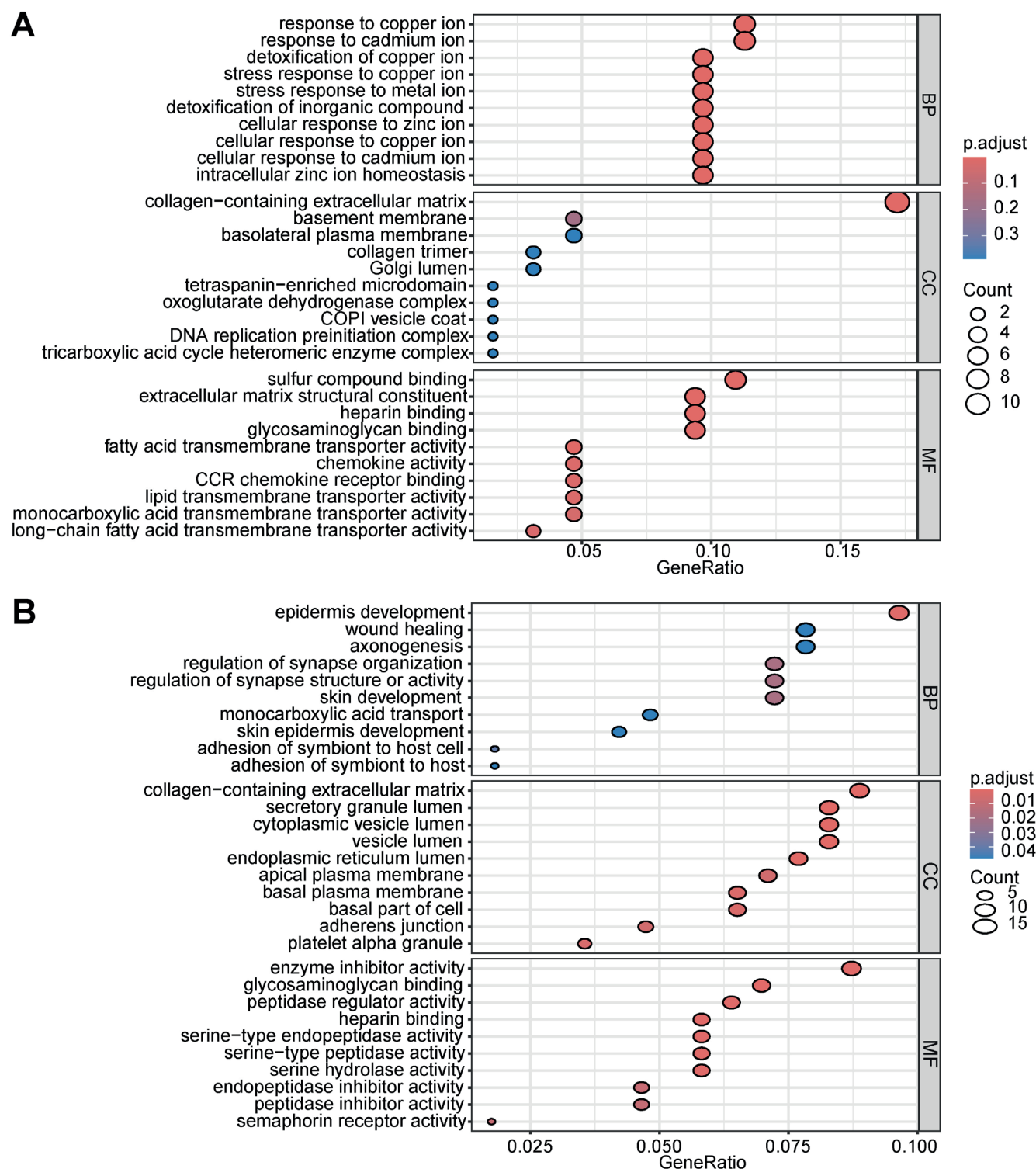


Fig. 7. Enrichment analysis between risk groups. A. Gene Ontology (GO) enrichment of upregulated genes in the high-risk group; B. GO enrichment of downregulated genes in the low-risk group

groups ($|\log_2FC| > 0.585$, adjusted $p < 0.05$) identified 67 upregulated and 176 downregulated genes. Gene Ontology enrichment analysis revealed that the upregulated genes were primarily associated with response to copper ion and response to cadmium ion (BP), basement membrane (CC), and ECM structural constituent and heparin

binding (MF) (Fig. 7A). Conversely, downregulated genes were enriched in epidermis development, wound healing, and axonogenesis (BP); collagen-containing ECM and cytoplasmic vesicle lumen (CC); and enzyme inhibitor activity, glycosaminoglycan binding, and peptidase regulator activity (MF) (Fig. 7B).

Molecular subtype analysis of THCA based on prognostic genes

Unsupervised consensus clustering based on the expression of the 3 prognostic genes identified 2 molecular subtypes of THCA: Cluster 1 ($n = 205$) and Cluster 2 ($n = 297$) (Fig. 8A). Kaplan–Meier survival analysis revealed significantly poorer OS in Cluster 1 (Fig. 8B). Expression levels of the 3 genes differed markedly between the 2 subtypes (Fig. 8C and Supplementary Table 10), with Cluster 1 exhibiting a significantly higher risk score (Fig. 8D and Supplementary Table 11). Immune profiling using the CIBERSORT algorithm indicated distinct immune infiltration patterns between the subtypes. Notably, monocytes were enriched in Cluster 1, whereas M0 macrophages were more abundant in Cluster 2 (Fig. 8E and Supplementary Table 12). ssGSEA revealed that Cluster 1 exhibited generally higher immune cell infiltration and immune-related functional scores than Cluster 2 (Fig. 8F and Supplementary Table 13). Moreover, tumor purity was higher in Cluster 2, whereas stromal and ESTIMATE scores were significantly higher in Cluster 1 (Fig. 9A and Supplementary Table 14). Analysis of the relationship between the subtypes and the 6 immune subtypes (C1–C6) from the TCGA Pan-Cancer immune classification revealed that both THCA clusters were predominantly enriched in the inflammatory subtype C3 (Fig. 9B). Immune checkpoint gene expression analysis showed significantly higher levels of *CD40*, *CD44*, *CD200*, and *TNFSF15* in Cluster 2, whereas *CCL19*, *GZMB*, *CTLA4*, and *IDO1* were more abundant in Cluster 1 (Fig. 9C and Supplementary Table 15). Differential expression analysis between the 2 subtypes ($|\log_2FC| > 0.585$, adjusted $p < 0.05$) identified 189 DEGs, including 47 upregulated and 142 downregulated genes in Cluster 1. Gene Ontology enrichment analysis of the upregulated genes highlighted terms such as Wnt-protein binding and CCR chemokine receptor binding, whereas the downregulated genes were enriched in enzyme inhibitor activity (Fig. 9D,E). Tumor mutational burden (TMB) analysis showed that the TMB of Cluster 1 was significantly higher than that of Cluster 2 (Supplementary Fig. 8).

Drug sensitivity analysis and network construction

We estimated the IC_{50} values of commonly used chemotherapeutic agents (docetaxel, cisplatin, fluorouracil, vinorelbine, doxorubicin, and paclitaxel) in the risk groups. IC_{50} was significantly higher in the high-risk group, suggesting reduced drug sensitivity (Fig. 10 and Supplementary Table 16). To further explore potential therapeutic targets, we analyzed the correlation between prognostic gene expression and drug sensitivity using data from the CellMiner database. *DPP4* expression was positively correlated with sensitivity to Midostaurin and ENMD-2076 precursor ($r = 0.523$ and 0.494 , respectively). *CCL17*

showed a significant negative correlation with 7-hydroxytaurosporine ($r = -0.450$) and a positive correlation with PLX-4720 ($r = 0.465$). *NPC2* expression was negatively correlated with des-fluoro-TAK-960 and docetaxel ($r = -0.407$ and -0.437 , respectively) (Supplementary Fig. 9 and Supplementary Table 17).

Discussion

Through comprehensive bioinformatics analysis of neutrophil-related genes, this study uncovers novel insights of THCA. It highlights the intricate relationship between neutrophils and THCA progression. Neutrophils are critical regulators of tumor progression. Our research confirms existing literature by demonstrating that high NRGs scores are associated with poor prognosis in THCA patients.^{15,16} The neutrophil-to-lymphocyte ratio (NLR) has prognostic value, with elevated NLR correlating with significantly worse OS.¹⁷ Inflammatory stimuli induce the release of neutrophil extracellular traps (NETs), which have been implicated in promoting tumor growth and progression.¹⁸ Additionally, *SLCO4A1* may effect progression-free survival (PFS) in THCA patients by modulating neutrophil-mediated immune pathways.¹⁹

The 3 core NRGs (*CCL17*, *NPC2*, and *DPP4*) are associated with THCA has been established. *CCL17*, a CCR4 ligand expressed in M2 macrophages and thymus,^{20,21} has been implicated in tumor progression across cancers.^{22–25} Consistent with previous findings in THCA,²⁶ our data show that high *CCL17* expression is associated with poorer survival. *NPC2* which encodes a lysosomal cholesterol transporter,²⁷ has been implicated in several malignancies including colon,²⁸ lung cancer,^{29,30} and thyroid cancer.³¹ In THCA, *NPC2* knockdown promotes tumor cell apoptosis in vitro. *DPP4* (*CD26*) is a cell surface glycoprotein.³² *DPP4* acts as either an oncogene or tumor suppressor depending on context.³³ Its high expression is linked to poor prognosis in clear cell carcinoma,³⁴ pancreatic cancer,³⁵ and colorectal cancer,³⁶ while exhibiting tumor-suppressive roles in ovarian, endometrial,³⁷ and prostate cancer.³⁸ *DPP4* overexpression in THCA can be used as a potential prognostic marker.^{39,40} Compared to models from other transcriptomic studies, our risk model demonstrated superior predictive performance, with AUCs ranging from 0.797 to 0.933. While direct comparisons with genomic markers such as *BRAF* mutation status are constrained by differences in data type and clinical context, our integrated nomogram – combining the risk score with key clinical parameters – offers a practical and interpretable tool for personalized patient management.

We analyzed DEGs between risk groups to explore how risk scores influence THCA progression. High-risk tumors showed enrichment of oxidative phosphorylation and myocardial contraction pathways, consistent with cardiovascular complications often observed in aggressive

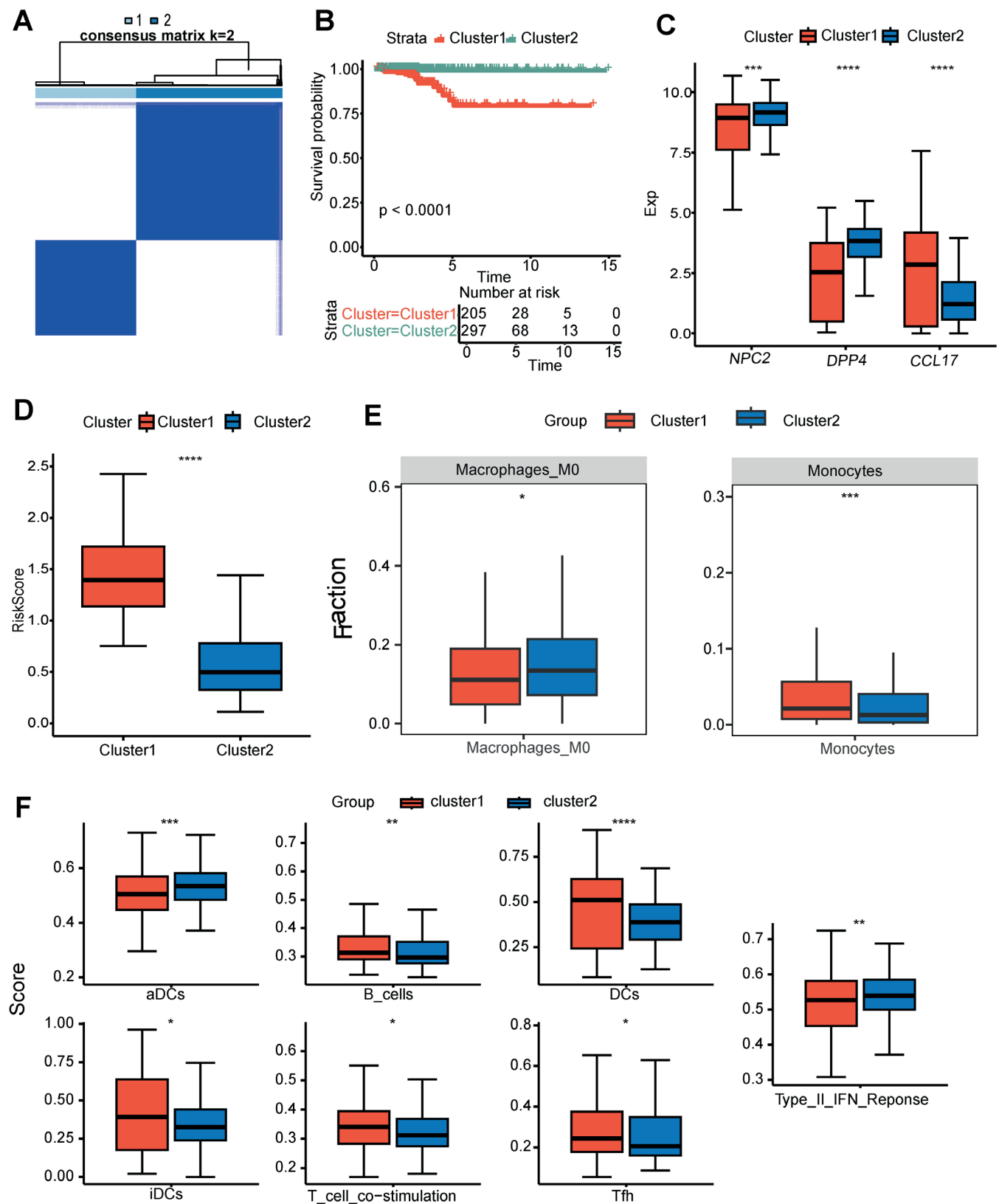


Fig. 8. Molecular subtyping and associated immune characteristics. A. Consensus clustering analysis heatmap with 2 subgroups ($k = 2$); B. Kaplan–Meier survival curve among subtypes; C. Differential expression of signature genes across subtypes; D. Boxplot of risk scores between subtypes; E. CIBERSORT algorithm analysis of immune infiltration; F. Boxplots displaying difference of immune cell infiltration profiles between clusters

* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$; **** $p < 0.0001$; ns –not significant.

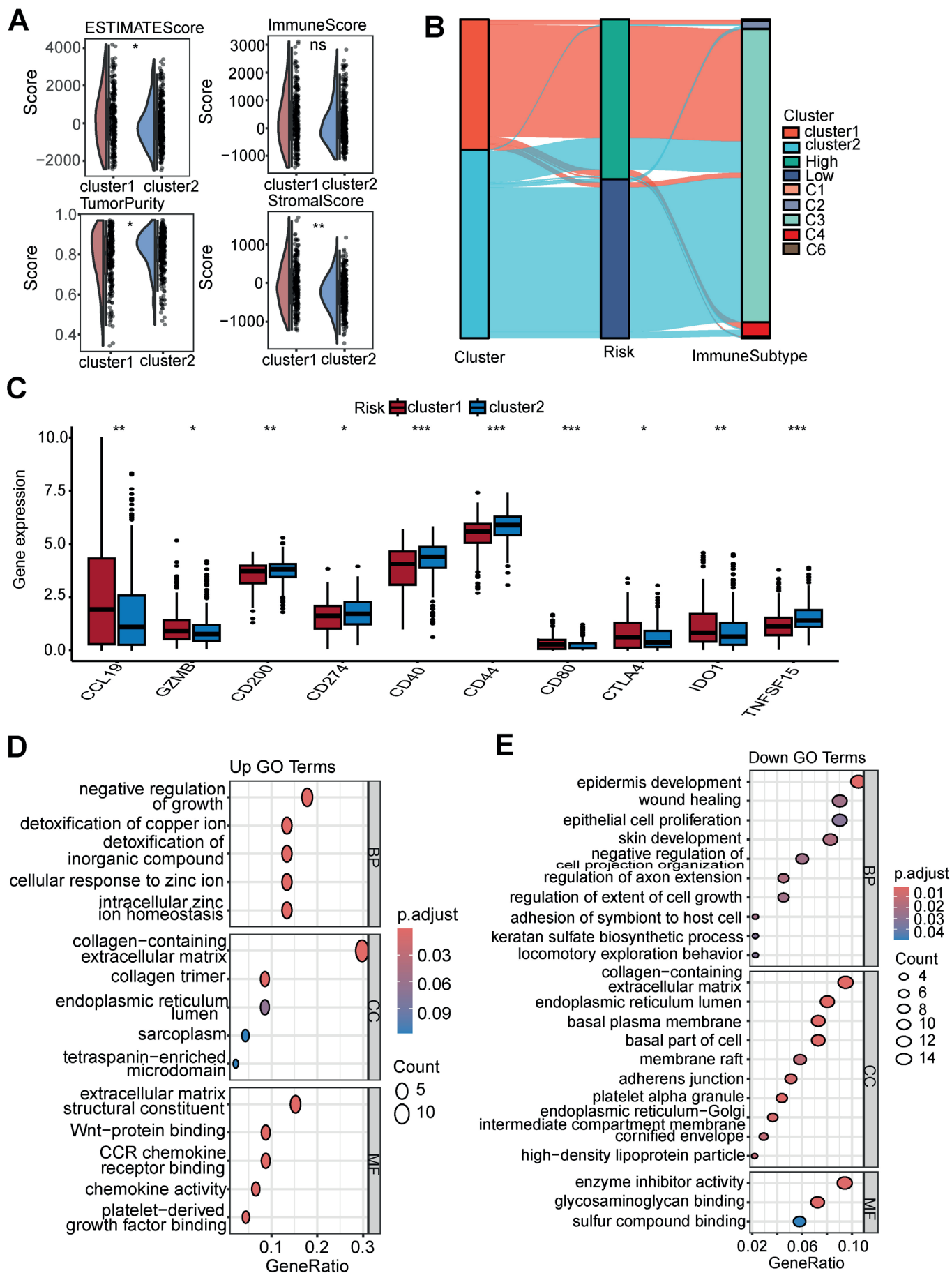


Fig. 9. Immune landscape and functional enrichment across molecular subtypes. A. Boxplots of stromal immune, ESTIMATE scores, and tumor purity among subtypes; B. The interaction between immune subtypes and 2 risk groups was depicted as an alluvial diagram; C. Immune checkpoint gene expression among subtypes; D. Upregulated Gene Ontology (GO) enriched bubble chart; E. Downregulated GO enriched bubble chart

* $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$; **** $p < 0.0001$; ns – not significant. C1 – wound healing, C2 – interferon gamma (IFN- γ)-dominant, C3 – inflammatory, and C4 – lymphocyte-depleted; C6 – tumor growth factor beta (TGF- β)-dominant.

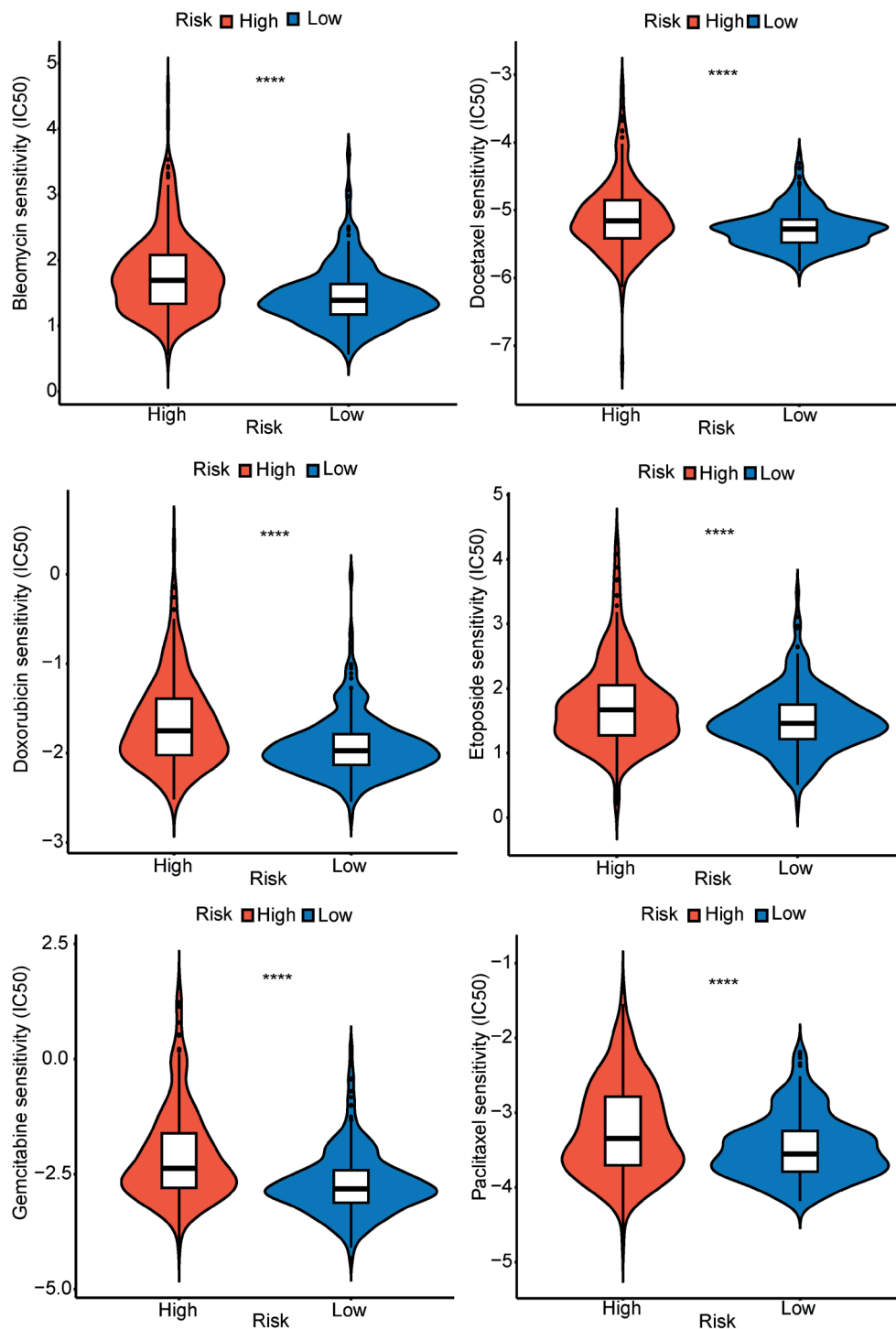


Fig. 10. Drug sensitivity prediction in thyroid carcinoma (THCA). Violin plot of half maximal inhibitory concentration (IC₅₀) values in risk groups

*p < 0.050; **p < 0.010; ***p < 0.001; ****p < 0.0001; ns – not significant.

THCA under thyrotoxic conditions.⁴¹ OXPHOS also promotes resistance to apoptosis and persistence of TH17 cells in the TME.⁴² Co-upregulation of copper ion response and collagen remodeling genes indicates a pro-metastatic phenotype, as copper drives tumor growth via RTK/ERK signaling and mediates cuproptosis.^{43,44} Increased matrix stiffness may trigger matrix autophagy, further supporting tumor progression.⁴⁵ These findings reflect the connective tissue proliferative stroma and metabolic plasticity observed in advanced THCA.⁴⁶ In contrast, low-risk tumors

preserve adherens junctions and DNA replication fidelity.⁴⁷ Suppression of axonogenesis and epidermal development genes may reduce perineural invasion.^{48,49} These findings provide mechanistic rationale for exploring targeted or repurposed therapies in aggressive THCA. Notably, bergapten may serve as a natural therapeutic agent for papillary thyroid carcinoma (PTC), as it has been shown to inhibit PTC cell growth through suppression of PI3K/AKT and GSK-3 β signaling, highlighting the potential of pathway-specific interventions.⁵⁰

Previous studies demonstrate that dynamic interactions within the TME drive tumor progression.^{51,52} In this study, we systematically profiled the immune landscape of THCA by integrating risk stratification and molecular subtyping. Immunophenoscore analysis revealed significantly higher immune scores in the low-risk group across most categories, except for the CTLA4+PD1+ subgroup. This corresponded with higher expression of most immune checkpoint genes, suggesting greater immunotherapy responsiveness in this group. These results are aligned with prior observations that immunotherapy can improve outcomes in THCA patients.⁵³ Moreover, we uncovered distinct immune infiltration patterns linked to our novel molecular subtypes. Cluster 1 exhibited higher infiltration of M0 macrophages, along with elevated CTLA4 expression and higher TMB. These features suggest an immunologically active phenotype potentially responsive to immune checkpoint inhibitors, particularly CTLA4 blockade.^{54,55} In contrast, Cluster 2 displayed lower immune infiltration, reduced TMB, and frequent *BRAF* mutations, consistent with an immune-cold, genomically stable phenotype that may benefit more from mitogen-activated protein kinase (MAPK)-targeted therapies.⁵⁶ While previous THCA immune profiling studies have highlighted macrophage and T-cell infiltration,^{39,57} our subtype-specific immune landscapes – especially the integration of neutrophil-related risk stratification – have not been previously reported. This framework provides novel insights into THCA immunobiology and may aid in identifying patients more likely to benefit from immunotherapy.

High-risk THCA patients exhibited broadly elevated IC₅₀ values, suggesting low-risk group is more sensitive to chemotherapy drugs than the high-risk group. This pattern may reflect intrinsic resistance mechanisms and underscores the limited benefit of cytotoxic regimens in aggressive THCA. To inform alternative strategies, we integrated gene expression with CellMiner drug response data to identify actionable associations. *DPP4* expression correlated with sensitivity to midostaurin and ENMD-2076, suggesting vulnerability to multi-kinase inhibition.⁵⁸ *CCL17* expression was associated with opposing sensitivities to 7-hydroxystaurosporine and PLX-4720, reflecting pathway-specific therapeutic windows.^{59,60} *NPC2* was linked to resistance against des-fluoro-TAK-960 and docetaxel, implying a role in mitotic drug evasion.⁶¹ These associations highlight candidate compounds for drug repurposing in high-risk THCA and warrant further translational exploration.

Limitations of the study

Despite the comprehensive analysis of NRGs in THCA, several limitations remain. Owing to current experimental constraints, functional and drug response validation could not be performed and will be pursued in future studies to support clinical translation. Future application of single-cell RNA sequencing and spatial transcriptomics could

uncover spatially confined immune niches or cell–cell interactions shaping the TME. Moreover, functional validation of the prognostic genes and subtype-specific immune features through in vitro perturbation and in vivo tumor models will be essential to confirm their mechanistic relevance and therapeutic potential.

Conclusions

This study elucidates the role of NRGs in shaping the THCA TME and proposes a clinically applicable biomarker system for prognostic stratification and therapeutic guidance. The 3-gene NRG signature predicts patient survival and facilitates risk-adapted treatment strategies involving chemotherapy and immunotherapy. Incorporating the signature into a nomogram alongside clinical variables enhances prognostic accuracy and supports its translational applicability. This model may assist in stratifying patients according to treatment intensity and tailoring follow-up schedules based on individualized risk profiles. Furthermore, subtype-specific immune landscapes underscore actionable therapeutic opportunities and may inform individualized approaches to immunotherapy and drug repurposing.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.17730267>. The package contains the following files:

Supplementary Fig. 1. Differential gene expression and enrichment analysis.

Supplementary Fig. 2. Martingale residuals of multivariate Cox model.

Supplementary Fig. 3. Spearman correlation analysis of 3 signature genes.

Supplementary Fig. 4. Comparison of AUC values for multiple models.

Supplementary Fig. 5. Risk scores for different cancer stages.

Supplementary Fig. 6. GSEA results of high-risk groups.

Supplementary Fig. 7. GSEA results of low-risk groups.

Supplementary Fig. 8. TMB of subtype.

Supplementary Fig. 9. Correlation plot of drug sensitivity predictions using the CellMiner database.

Supplementary Table 1. Differentially expressed neutrophil-related genes identified between tumor and normal samples.

Supplementary Table 2. Schoenfeld residual test for proportional hazards assumption.

Supplementary Table 3. Variance inflation factor (VIF) for multicollinearity assessment in the Cox regression model.

Supplementary Table 4. Comparison of immune phenotype scores between risk groups using Wilcoxon test.

Supplementary Table 5. Comparison of immune checkpoint-related gene expression between high- and low-risk groups using Wilcoxon test.

Supplementary Table 6. Comparison of ESTIMATE scores between high- and low-risk groups using Wilcoxon test.

Supplementary Table 7. χ^2 test results for the distribution of no response (NR) and response (R) across high- and low-risk groups.

Supplementary Table 8. Wilcoxon test of immune cell composition and immune function between high- and low-risk groups.

Supplementary Table 9. Comparison of immune cell type proportions between high- and low-risk groups using Wilcoxon test.

Supplementary Table 10. Wilcoxon test of signature gene expression between clusters.

Supplementary Table 11. Wilcoxon test of risk scores between clusters.

Supplementary Table 12. Wilcoxon test of immune cell composition between clusters.

Supplementary Table 13. Wilcoxon test of immune cell composition and immune function between clusters.

Supplementary Table 14. Wilcoxon test of ESTIMATE scores and tumor purity between clusters.

Supplementary Table 15. Wilcoxon test of immune checkpoint gene expression between clusters.

Supplementary Table 16. Wilcoxon test of drug response between risk groups.

Supplementary Table 17. Gene–drug correlation analysis.

Data Availability Statement

The datasets supporting the findings of the current study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17734513>.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Integrated single-cell transcriptomics, Mendelian randomization, and machine learning identify *CEBPZ* as an immune-related biomarker in oral lichen planus

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Abstract

Background. Oral lichen planus (OLP) is a chronic, immune-mediated oral mucosal disease with complex pathophysiology and potential for malignant transformation. Understanding its molecular basis is critical for the development of precise diagnostic and therapeutic strategies.

Objectives. We aimed to identify key immune-related biomarkers and characterize cellular dynamics in OLP, with a particular focus on the role of *CEBPZ* in disease pathogenesis.

Materials and methods. We analyzed single-cell RNA sequencing (scRNA-seq) data from OLP lamina propria samples (GSE211630) to identify disease-specific T-cell subpopulations using high-dimensional weighted gene co-expression network analysis (hdWGCNA) for oxidative stress-related gene modules. Summary-data-based Mendelian randomization (SMR) integrated FinnGen genome-wide association study (GWAS; 342,499 Europeans) data with Genotype-Tissue Expression (GTEx) expression quantitative trait loci (eQTL) data to identify causal genes. Machine learning (ML) models (least absolute shrinkage and selection operator (LASSO) and convolutional neural network (CNN)) were developed using bulk RNA-seq datasets (GSE52130 and GSE38616) for diagnostic purposes.

Results. We identified OLP-specific T-cell populations (clusters 0, 3, 5, 7, 13, and 15) with enhanced migration inhibition factor (MIF) pathway signaling toward B cells and monocytes. Two oxidative stress-associated modules contained hub genes, including *CEBPZ*. Summary-data-based Mendelian randomization analysis identified 231 OLP-associated genes, with *CEBPZ* uniquely intersecting LASSO-selected markers (odds ratio (OR) = 1.057, 95% confidence interval (95% CI) = 1.013–1.102, $p = 0.010$). Machine learning models achieved area under the curve (AUC) values ranging from 0.653 to 0.745, with the CNN model reaching a validation accuracy of 0.735. *CEBPZ* showed elevated expression in OLP T cells and correlated with enhanced MIF-(CD74+CXCR4) signaling.

Conclusions. This integrative approach identifies *CEBPZ* as a pivotal biomarker linking genetic susceptibility, oxidative stress, and immune dysregulation in OLP. Our diagnostic models offer promising tools for OLP management.

Key words: oral lichen planus, machine learning, *CEBPZ*, single-cell transcriptome sequencing, Mendelian randomization

Highlights

- Single-cell RNA sequencing identifies disease-specific T-cell subpopulations and immune signaling pathways involved in oral lichen planus (OLP).
- Integrative genomic analyses reveal *CEBPZ* as a key biomarker linking oxidative stress, immune dysregulation, and genetic susceptibility in OLP.
- Enhanced MIF-mediated signaling between T cells, B cells, and monocytes suggests a central mechanism in OLP pathogenesis.
- Machine learning diagnostic models based on transcriptomic data show promising accuracy in identifying OLP-associated molecular signatures.

Introduction

Oral lichen planus (OLP) represents a significant clinical challenge in oral medicine, affecting millions of people worldwide with chronic, painful lesions that impair quality of life and carry a risk of malignant transformation. It is a T-cell-mediated chronic inflammatory disease of the oral mucosa with no recognized cause, presenting as plaques, papules, white striations, erosions, erythema, or blisters, primarily affecting the gingiva, tongue, and buccal mucosa.^{1,2} The disease affects 1–2% of the adult population.³ Oral lichen planus diagnosis relies on histological and clinical characteristics; a biopsy is recommended to confirm the diagnosis and rule out malignancy.^{1,2} Annual surveillance is strongly recommended, as research indicates that 1.63% of OLP cases develop into oral squamous cell carcinoma (OSCC) within 7 years.^{4,5} However, there is no recognized gold standard treatment, and both systemic and local therapeutic approaches are used. Some clinical manifestations are particularly difficult to treat, significantly reducing patients' quality of life.^{2,6} Therefore, there is an urgent need to develop more accurate methods for the early diagnosis and treatment of OLP.

Oral lichen planus may be caused by several factors, including hypersensitivity reactions, psychological stress, viral infections, and autoimmune responses.^{7,8} Numerous immune cell types have been implicated in the development of OLP. The disease is characterized by liquefaction degeneration of basal keratinocytes mediated by T lymphocytes. CD8⁺ T lymphocytes produce granzymes and perforin, which can destroy oral mucosal keratinocytes and accelerate epithelial degradation.⁹ Natural killer (NK) cells in OLP lesions damage living cells and produce tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ).¹⁰ B cells are also frequently observed in OLP lesions.¹¹ The basement membrane of the oral mucosa in OLP may be damaged by particles and inflammatory mediators released by mast cells, as well as by matrix metalloproteinases (MMPs) produced by T cells.¹² Within complex microenvironments, immune cells interact to produce inflammatory mediators, such as chemokines and cytokines, which contribute to the development and progression of OLP. However, the complete single-cell characteristics of the immune response in OLP have not yet been fully characterized.¹³

Recent advances in single-cell RNA sequencing (scRNA-seq) have revolutionized our understanding of cellular heterogeneity in disease states. While previous scRNA-seq studies identified differences in T-cell composition between OLP and normal tissues,^{14–16} our study integrates multiple cutting-edge analytical approaches. We combined scRNA-seq with causal inference using summary data-based Mendelian randomization (SMR), network analysis via high-dimensional weighted gene co-expression network analysis (hdWGCNA), and machine learning (ML) to create a comprehensive framework for biomarker discovery and diagnostic tool development. This multimodal approach allows us not only to identify disease-associated cellular changes but also to establish their causal relationships and clinical utility.

Summary data-based Mendelian randomization is a powerful integrative analytical technique that uses genetic variants as instrumental variables to assess causal relationships between gene expression and disease outcomes.¹⁷ By combining summary-level expression quantitative trait loci (eQTL) data with genome-wide association study (GWAS) results, SMR identifies genes whose expression levels are causally linked to disease risk while minimizing the influence of confounding factors.

Using scRNA-seq analysis, we identified a distinct increase in specific T-cell subgroups in patients with OLP, designated as OLP-specific T cells (OLP_T cells). Through hdWGCNA, we identified T-cell gene modules associated with oxidative stress and linked to immune profiles. We developed diagnostic models using ML and constructed a gene-immune convolutional neural network deep learning model incorporating 10 immune cell types and 9 key genes. Summary data-based Mendelian randomization analysis of GWAS and eQTL data further identified risk genes, which were subsequently evaluated through scRNA-seq analysis and cellular communication analysis.

Objectives

This study aimed to identify key immune-related biomarkers and characterize the cellular dynamics involved

in OLP, with a particular focus on understanding the role of *CEBPZ* in disease pathogenesis, by integrating single-cell transcriptomics, genetic causal inference, and ML approaches to uncover novel diagnostic markers and therapeutic targets.

Materials and methods

Ethical approval and consent to participate

This study did not involve direct human or animal experimentation. All analyses were performed using publicly available datasets, including scRNA-seq data from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo>) (GSE211630, GSE52130, and GSE38616), GWAS summary data from the FinnGen database (<https://www.finnngen.fi>), and eQTL data from the Genotype-Tissue Expression (GTEx) database (<https://gtexportal.org>). Therefore, no ethical approval or informed consent was required. All data were handled in accordance with the ethical guidelines and data-use policies of the respective repositories.

Data acquisition

The GEO database provided the scRNA-seq dataset GSE211630, which was processed using the standard Seurat methodology.¹⁸ The Harmony algorithm was used to eliminate batch effects between samples. Bulk RNA-seq datasets GSE52130 and GSE38616 were also obtained from GEO.^{19,20} For SMR analysis, GWAS summary statistics were obtained from the FinnGen database (release 8), specifically using endpoint K11_LICHEORAL, comprising 342,499 samples of European ancestry with 20,169,554 single-nucleotide polymorphisms (SNPs). Esophageal mucosa eQTL summary data were obtained from the GTEx database (version 8) and selected as a proxy for oral squamous epithelium because of shared squamous epithelial characteristics and the lack of adequately powered oral tissue eQTL datasets. No sample overlap existed between the FinnGen and GTEx cohorts, and all genomic coordinates were harmonized to the GRCh38/hg38 reference build. Reference genotype data for linkage disequilibrium (LD) calculations were obtained from the Phase 3 European panel of the 1000 Genomes Project (EUR, $n = 503$). Protein interaction data were obtained from the STRING database.²¹

Single-cell sequencing data processing

Dataset GSE211630 was processed using the standard Seurat pipeline, with quality control parameters including a ribosomal gene percentage threshold of 10%, a mitochondrial gene proportion threshold of 20%, and between 500 and 3,000 detected genes per cell. Highly variable genes

were identified using Seurat's FindVariableFeatures function.²² Cell clustering was performed using the FindNeighbors and FindClusters functions,²³ and cell types were manually annotated using established cell markers.²⁴

The pipeline of high dimensional WGCNA

The hdWGCNA package extends traditional WGCNA to single-cell data by accounting for sparsity and high dimensionality.²⁵ The pipeline involves 5 key steps: pre-processing to eliminate batch effects while preserving biological variation; construction of a gene co-expression network using metacells to overcome sparsity; identification of gene modules with eigengene calculation; module preservation analysis to evaluate robustness; and functional enrichment analysis to determine associated biological pathways.

Pseudotime analysis

Pseudotime analysis was performed using the Monocle2 R package (<https://github.com/MaxMeieran/monocle2>) to investigate the potential effects of blue and brown module genes on T-cell development in the OLP microenvironment.^{26–29} The plot_cell_trajectory function was used to depict the cell differentiation trajectory, and the DDRTree method implemented through the reduceDimension function was used to calculate differentiation states.^{30,31} Additionally, the Velocity package (<https://github.com/velocity-team/velocity.py>) was used to infer cell developmental vectors based on the balance between spliced and unspliced mRNA, with the developmental stage linked to the quantile polarization of cell principal component values.³²

Cell–cell interaction analysis

Ligand–receptor-mediated interactions and inter-cellular communication networks were assessed using the R package CellChat (<https://github.com/sqjin/CellChat>). The volume and intensity of cell-to-cell communication were graphically represented in Fig.1. The migration inhibition factor (MIF) signaling pathway, which mediates inflammatory responses through the receptors CD74, CXCR4, and CD44, was specifically examined because of its role in chronic inflammation. The principal signaling inputs and outputs of each OLP cell subpopulation were assessed using CellChatDB.³³

Functional enrichment analysis

Potential interaction networks among target module proteins were identified using GeneMANIA.^{34,35} Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the Metascape platform,³⁶ with a p-value threshold of 0.05.

Immune infiltration analysis based on individualized CIBERSORTx signature or MCP_counter

CIBERSORTx was used to deconvolve complex gene expression data into cell type-specific expression patterns.³⁷ An OLP-specific gene expression signature matrix was generated from GSE211630 for the deconvolution of bulk RNA-seq datasets. The MCP-counter algorithm was used to assess the abundance of 10 cell types.³⁸

RNA-seq bulk NMF

Patients with OLP were divided into subgroups using non-negative matrix factorization based on blue and brown module gene expression levels.³⁹ The cophenetic coefficient was used to determine the optimal number of clusters.

Machine learning and deep learning

Key diagnostic genes were identified using univariate logistic regression. Least absolute shrinkage and selection operator (LASSO) regression was used for variable selection.⁴⁰ Ten ML models were evaluated using the mlr3 package (<https://cran.r-project.org/web/packages/mlr3/index.html>): KNN, LR, LDA, NB, Ranger, ABESS, Glmnet, SVM, XGBoost, and Multinom.⁴¹ GSE52130 served as the training set and GSE38616 served as the validation set, with performance assessed using 10 iterations of five-fold cross-validation. For deep learning, images of 10 immune cell types and 9 genes were generated using the formula $N_{j,i} = \text{immune}_i / \text{gene}_j$ to investigate the association between immune cells and target genes. Convolutional neural network (CNN) models were developed using the Keras and TensorFlow frameworks.^{42,43}

Summary-data-based Mendelian randomization analysis

Comprehensive SMR analysis was conducted using SMR software v. 1.3.1 (<https://yanglab.westlake.edu.cn/software/smr>) with FinnGen GWAS summary statistics, GTEx esophageal mucosa eQTL data, and the 1000 Genomes European reference panel for LD calculation (workflow shown in Supplementary Fig. 1). All data were harmonized to GRCh38/hg38, alleles were aligned, and palindromic SNPs with a minor allele frequency (MAF) of 0.4–0.6 were removed. Instrument selection used a cis-eQTL window of ± 1 Mb with clumping parameters of $r^2 < 0.1$ within 500 kb. An eQTL was considered significant when the p-value of the top associated cis-acting single-nucleotide polymorphism (cis-SNP) was $< 5.00 \times 10^{-8}$. The significance threshold for SMR analysis was determined using Bonferroni correction (0.05/N). Oral lichen

planus-associated risk genes were defined as those with PSMR (the p-value from SMR analysis indicating a pleiotropic association) < 0.05 and PHEIDI (the p-value from the Heterogeneity in Dependent Instruments test) > 0.05 . The HEIDI test examines whether an association is due to a shared causal variant (PHEIDI > 0.05) or linkage (PHEIDI ≤ 0.05). Default settings were maintained for the minimum number of instruments (≥ 3 SNPs) and instrument strength (F-statistic > 10).

Statistical analyses

Three groups were compared using the Kruskal–Wallis test, and 2 groups were compared using the Wilcoxon test. Pearson's correlation analysis was used to investigate the relationships between 10 immune cell types and 9 key genes. Statistical significance was set at $p < 0.05$. All analyses were performed using R v. 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

The change in the composition of T cell subtypes in OLP lamina propria

We acquired the scRNA-seq dataset GSE211630 and used Harmony integration, dimensionality reduction, and clustering to analyze the OLP-specific cellular composition (Supplementary Fig. 2). The Uniform Manifold Approximation and Projection (UMAP) scatter plot showed the distribution of cell subtypes in the OLP lamina propria (LP) (Fig. 1A), while the stacked bar chart displayed the altered proportions of these cell populations (Fig. 1B). T cells showed the most pronounced shift between OLP and normal tissues (Fig. 1A,B). We focused on T cells and performed dimensionality reduction and clustering of T-cell subpopulations. T-cell clusters 0, 3, 5, 7, 13, and 15 showed significant increases in the OLP LP (Fig. 1C,D), which were not observed in normal tissues. Given that these adjacent clusters were distant from other T-cell subpopulations in UMAP space and exhibited specific upregulation in OLP, we designated them as OLP_T cells.

Cell–cell communication analysis revealed that OLP_T cells exhibited more numerous and stronger interactions with other cell types, including B cells, other T cells, and monocytes (Fig. 1E,F). We further investigated the specific pathways through which certain cell types interacted more strongly with one another. OLP_T cells demonstrated enhanced communication through MIF-related pathways compared with other T cells, transmitting more signals to B cells and monocytes (Supplementary Fig. 3A,B). The MIF pathway mediates inflammatory responses through its receptors CD74, CXCR4, and CD44 and plays a crucial role in chronic inflammation. Overall, scRNA-seq

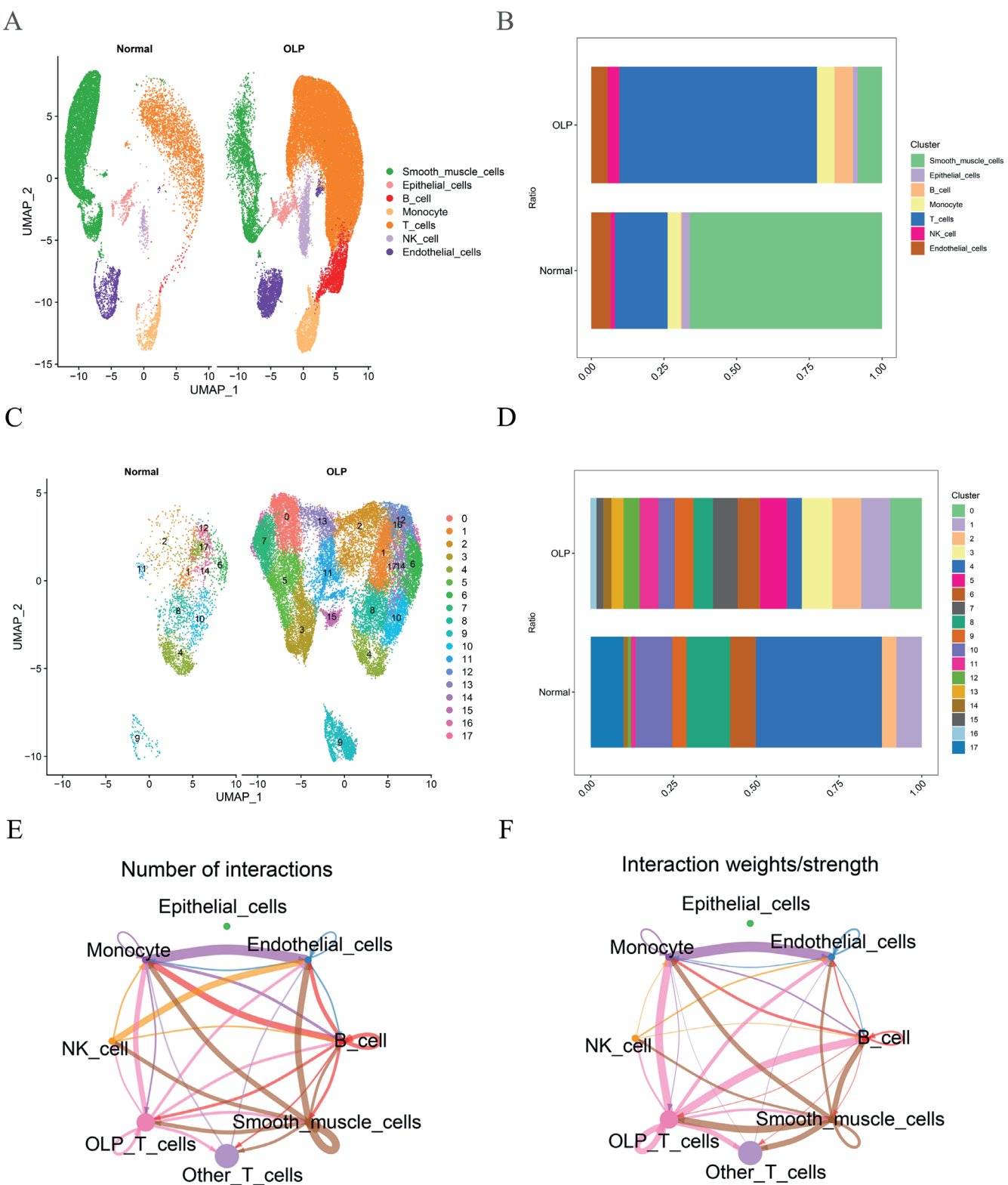


Fig. 1. T-cell composition and interactions in the oral lichen planus (OLP) lamina propria (LP). **A.** Uniform Manifold Approximation and Projection (UMAP) scatter plot showing the distribution of cell subtypes in the OLP LP; **B.** Stacked bar chart depicting the proportion of each cell type; **C.** UMAP scatter plot showing the distribution of T-cell subtypes; **D.** Stacked bar chart showing T-cell subtype proportions; **E,F.** Circle plots illustrating the number and strength of cell–cell interactions between T-cell subtypes and other cell types, with colors indicating the source cell populations

NC – normal control; OLP_T – OLP-specific T cells; NK cells – natural killer cells.

analysis revealed an increase in OLP-specific T-cell subtypes in the OLP LP. These cells exhibited close interactions with smooth muscle cells, B cells, and monocytes,

suggesting that this population of OLP-specific T-cell subtypes may be involved in the immune-inflammatory process.

hdWGCNA revealed that OLP_T cells were characterized by the blue and brown modules

We examined OLP_T cells using hdWGCNA. A power value of 9 was selected after considering the scale-free topology model fit, median connectivity, and mean connectivity (Fig. 2A). Four modules were identified (Fig. 2B). The blue and brown modules showed specific expression in OLP_T cells (Fig. 2C), with a positive correlation observed between the modules (Fig. 2D). Hub genes, including *CYTIP*, *RPL19*, *SELENOK*, *RPS28*, *FOSL2*, and *CXCR4*, were identified (Fig. 2E). Module correlations are shown in Fig. 2F.

Gene enrichment analysis revealed that the blue and brown module genes were primarily involved in oxidative phosphorylation, adenosine triphosphate (ATP) synthesis coupled with electron transport, and nicotinamide adenine dinucleotide (NADH) dehydrogenase activity (Fig. 3A). KEGG enrichment analysis showed that these genes participated in cellular responses to stress, implying a strong association with oxidative stress (Fig. 3B). This association between *CEBPZ* and oxidative stress is particularly relevant because oxidative stress drives inflammatory responses in chronic diseases such as OLP through the production of reactive oxygen species (ROS), which can damage cellular components and perpetuate the inflammatory cycle. Further analysis of cell–cell communication confirmed strong interactions between OLP_T cells and B cells and monocytes, particularly through the MIF signaling pathway (Fig. 3C–E; Supplementary Fig. 4A). OLP_T cells demonstrated higher signal transmission than other cell types, except in the CXCL signaling pathway (Supplementary Fig. 4B,C), where OLP_T cells were the most potent signal receivers.

Pseudotime analysis reveals early developmental stages of OLP_T cells

Pseudotime analysis revealed that OLP_T cells (clusters 0, 3, 5, 7, 13, and 15) were predominantly in stages 1 or 2 of T-cell development (Fig. 4). This finding suggests that the pathological milieu of the OLP LP may exert an educational effect on OLP_T cells, potentially priming them toward a pro-inflammatory phenotype early in their developmental trajectory. The enrichment of OLP_T cells in early developmental stages contrasts with the more differentiated state of T cells in normal tissues, supporting the hypothesis that the OLP microenvironment actively shapes T-cell differentiation toward pathogenic phenotypes.

Development of a machine learning diagnostic model based on OLP_T cell-specific genes

The LASSO regression analysis reduced the candidate genes to 9: *SRRM1*, *RPS11*, *CEBPZ*, *RAB9A*, *KLF3*,

CNOT6L, *RPL18*, *ARID4B*, and *IRF2BP2* (Fig. 5A,B). The UMAP diagram in Supplementary Fig. 5 displays the differential expression of these 9 genes between normal and OLP tissues. These selected genes were incorporated into multiple ML models, including support vector machine (SVM), naïve Bayes, K-nearest neighbors (KNN), linear discriminant analysis (LDA), and XGBoost. GSE52130 served as the training set and GSE38616 served as the validation set. Model performance was assessed using 10 iterations of five-fold cross-validation. Logistic regression (Logreg), Adaptive Best Subset Selection (ABESS), and LDA showed balanced precision and sensitivity in differentiating OLP tissues from normal tissues (Fig. 5C,D). The final ML framework included these 3 techniques, which demonstrated good predictive accuracy in the external validation set (area under the ROC curve (AUC) = 0.745, 0.694, and 0.653, respectively; Supplementary Fig. 6A–C).

Oxidative stress genes in OLP_T cells correlate with immune profiles and enable OLP patient subtyping

Using non-negative matrix factorization (NMF) based on oxidative stress genes, patients with OLP were stratified into 2 subgroups to investigate the clinical significance of these genes in patient classification (Fig. 6A). The consensus matrix heatmap displayed OLP patient subtypes when $k = 2$ (Fig. 6B). Further analysis at the bulk RNA-seq level revealed strong associations between immune infiltration and patient heterogeneity.

Using CIBERSORTx to generate an OLP-specific signature derived from scRNA-seq data, we determined the abundance of cellular components in the OLP LP and assessed correlations with OLP_T-specific oxidative stress gene expression patterns. Most of these genes showed strong associations with different cell types, suggesting their involvement in modulating the OLP microenvironment. Notably, most oxidative stress genes specific to OLP_T exhibited robust positive correlations with lymphocyte infiltration (Fig. 6C). Subtype 2 showed stronger lymphocyte activation (Fig. 6D) and higher proportions of most immune cell populations (Supplementary Fig. 7A), further supporting stronger immune activation in this subtype.

Establishment of the gene–immune convolutional neural network model

Considering the established relationship between the immunological landscape and oxidative stress genes in OLP_T cells, we developed a gene–immune CNN classifier to create a general diagnostic model while minimizing batch effects. We generated a unique gene–immune heatmap (9 units wide $\times$ 10 units long), in which the value of each square for a given OLP patient represents the proportion of a specific immune cell divided by the expression level of the indicated

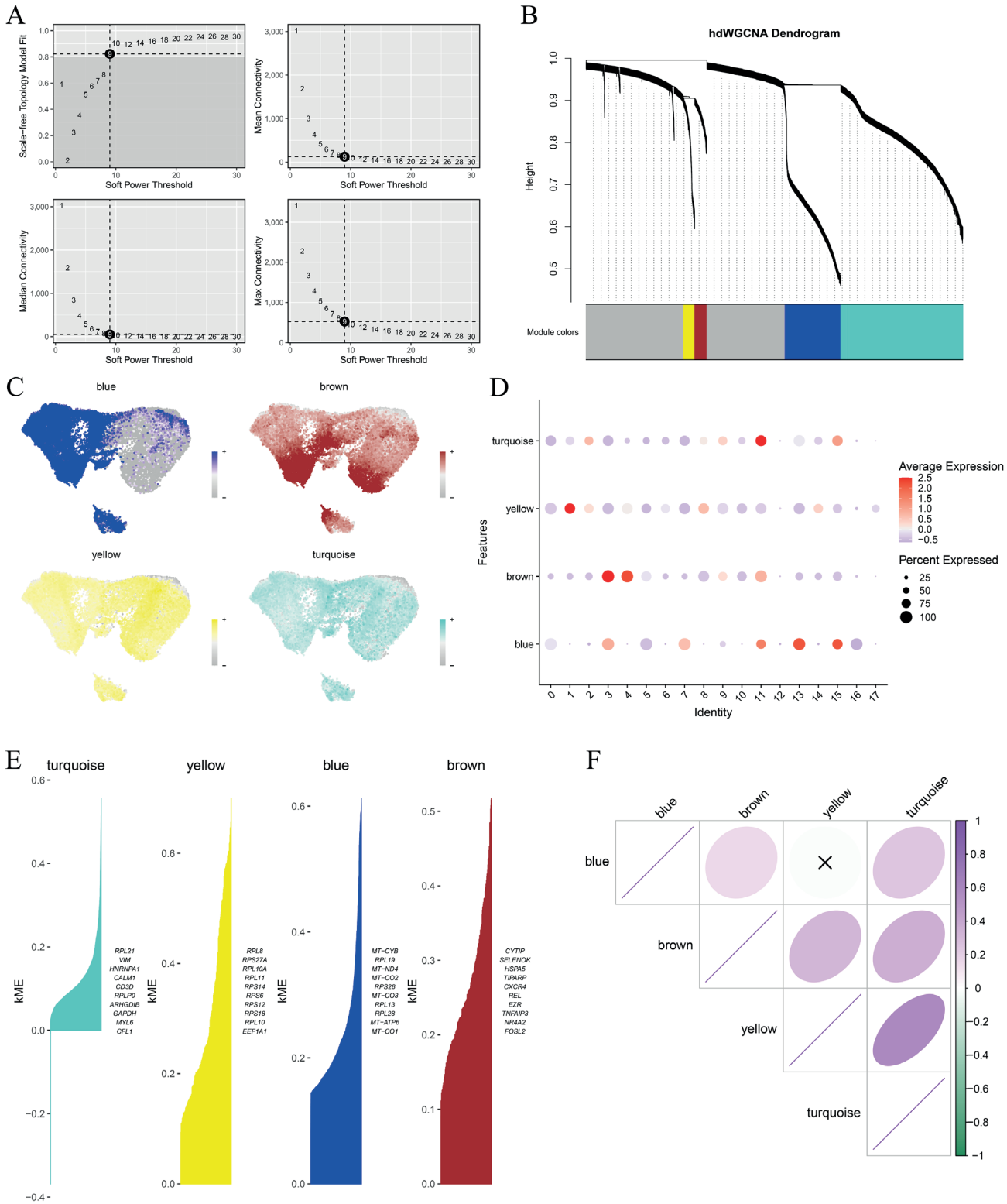


Fig. 2. High-dimensional weighted gene co-expression network analysis (hdWGCNA) of oral lichen planus (OLP)-specific T cells. A. Selection of power value 9 for scale-free network topology; B. Clustering of highly variable genes into 4 modules; C. Feature plots displaying gene expression scores for the blue and brown modules in OLP_T cells; D. Bubble plot showing module scores across T-cell subtypes. E. Hub genes ranked according to eigengene connectivity (kME); F. Correlation matrix showing relationships between modules

OLP_T – OLP-specific T cells.

gene (Fig. 7A). GSE52130 served as the training set, and GSE38616 served as the validation set. After 300 epochs of training (Fig. 7B), the CNN model demonstrated strong

predictive accuracy in both datasets (Fig. 7C,D; training AUC = 1.000, testing AUC = 0.735), suggesting potential for broader applications in OLP diagnosis.

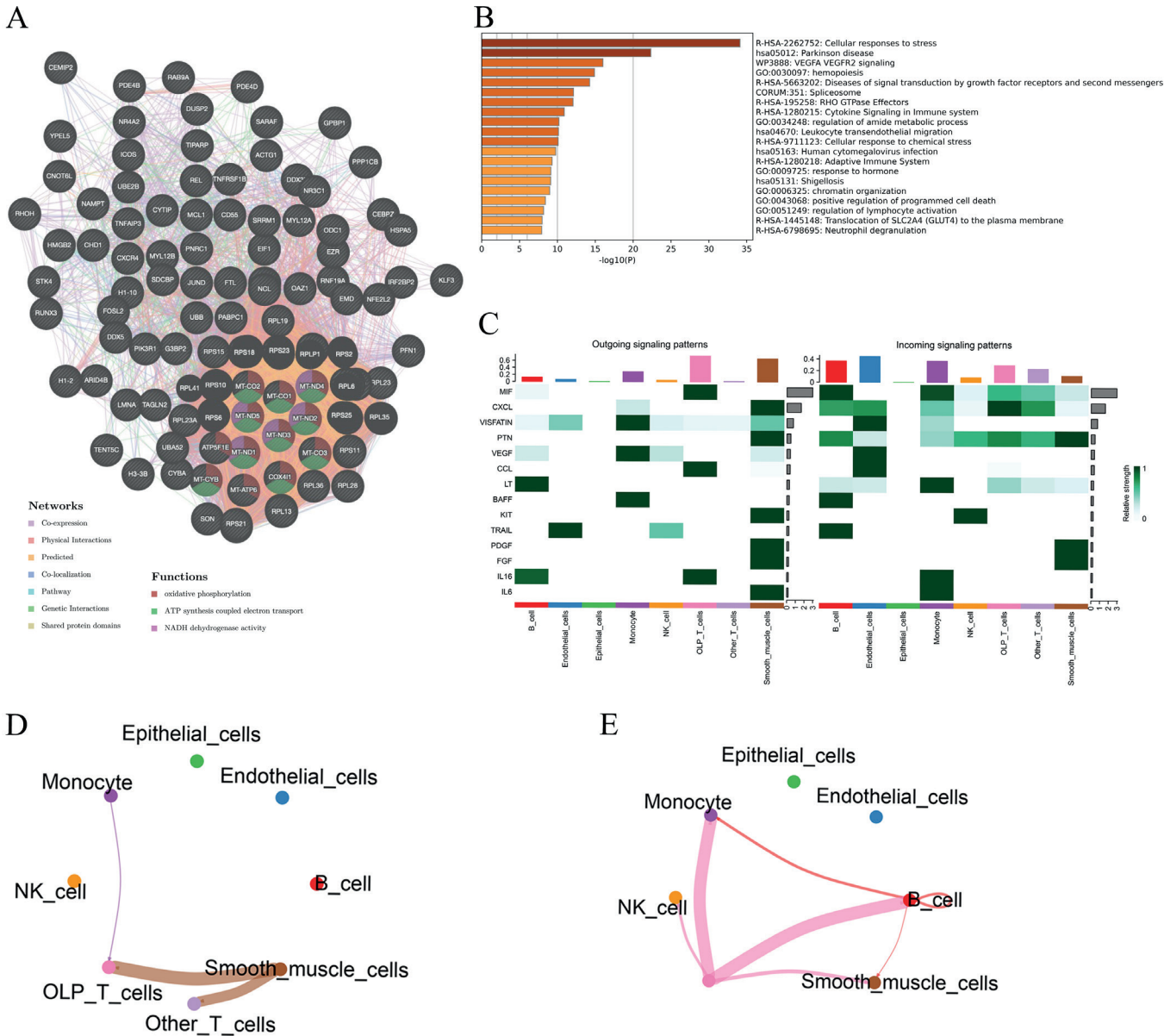


Fig. 3. Functional enrichment and cell interactions of the blue and brown modules. A. Protein–protein interaction network of genes in the blue and brown modules; B. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis linking genes to oxidative stress and cellular responses; C. Heatmap of outgoing and incoming signaling patterns across cell types; D,E. Circle plots showing the number and strength of cell–cell communication between OLP_T cells and other cell types

OLP_T – OLP-specific T cells; NK cells – natural killer cells.

SMR analysis identifies OLP-related gene targets

SMR analysis of GWAS and eQTL data identified regulatory genes causally associated with OLP using significance thresholds of PSMR < 0.05 and PHEIDI > 0.05. A total of 231 genes were found to be pleiotropically linked to OLP (Supplementary Table 1). Among these, the top 10 genes are highlighted because of their significant associations (Table 1). Notably, *CEBPZ* was the only gene that overlapped with the LASSO regression results (Fig. 8A). CCAAT/enhancer-binding proteins (CEBPs), including *CEBPZ*, have been reported to regulate cytokine

production and macrophage function, thereby enhancing inflammatory responses.^{44,45} Our findings showed a correlation between OLP risk and increased *CEBPZ* expression (odds ratio (OR) = 1.057, 95% confidence interval (95% CI): 1.013–1.102, $p = 0.010$; Fig. 8B).

We acknowledge that esophageal mucosa eQTLs served as proxies for oral epithelium because of the lack of adequately powered oral tissue eQTL data. Although both tissues share squamous epithelial characteristics, tissue-specific regulatory mechanisms may differ. Future studies should validate these findings using oral tissue-specific eQTLs. Sensitivity analyses using whole-blood eQTLs or other immune-relevant tissues would strengthen causal inferences.

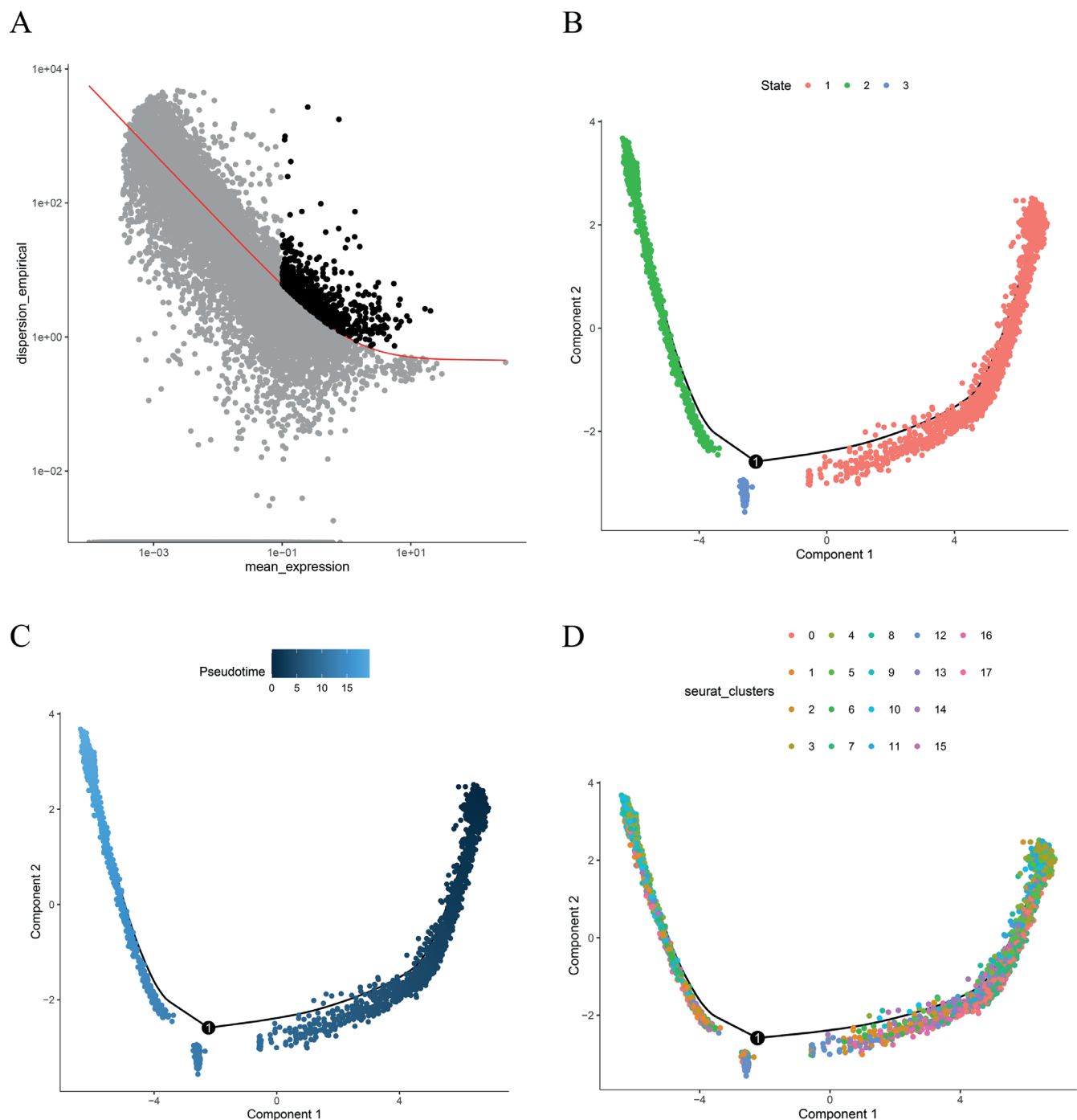


Fig. 4. Pseudotime analysis of T-cell subtypes in oral lichen planus (OLP). A,B. Trajectory plots showing developmental states of OLP_T cell clusters characterized by the blue and brown modules; C. Monocle time-series plot of T-cell development; D. Time-series plot of T-cell subtype differentiation stages OLP_T – OLP-specific T cells.

Expression levels of SMR outcome gene in oral lichen planus

CEBPZ expression was significantly higher in OLP patients than in healthy individuals across all cells and T cells (Fig. 9A,B). The UMAP plot and enrichment analysis demonstrated *CEBPZ* expression across all OLP cell populations (Fig. 9C,D). Expression was increased in T cells, epithelial cells, and smooth muscle cells (Supplementary

Fig. 8A). Unsupervised developmental inference analysis indicated that *CEBPZ* expression increased with cell differentiation (Supplementary Fig. 8B–D).

Given that OLP is a chronic T-cell-mediated inflammatory disease, and consistent with our previous analyses, we focused on *CEBPZ* expression in T cells. We divided T cells into *CEBPZ*⁺ (high-expression) and *CEBPZ*[–] (low-expression) groups according to their expression levels. Using CellChat to calculate receptor–ligand

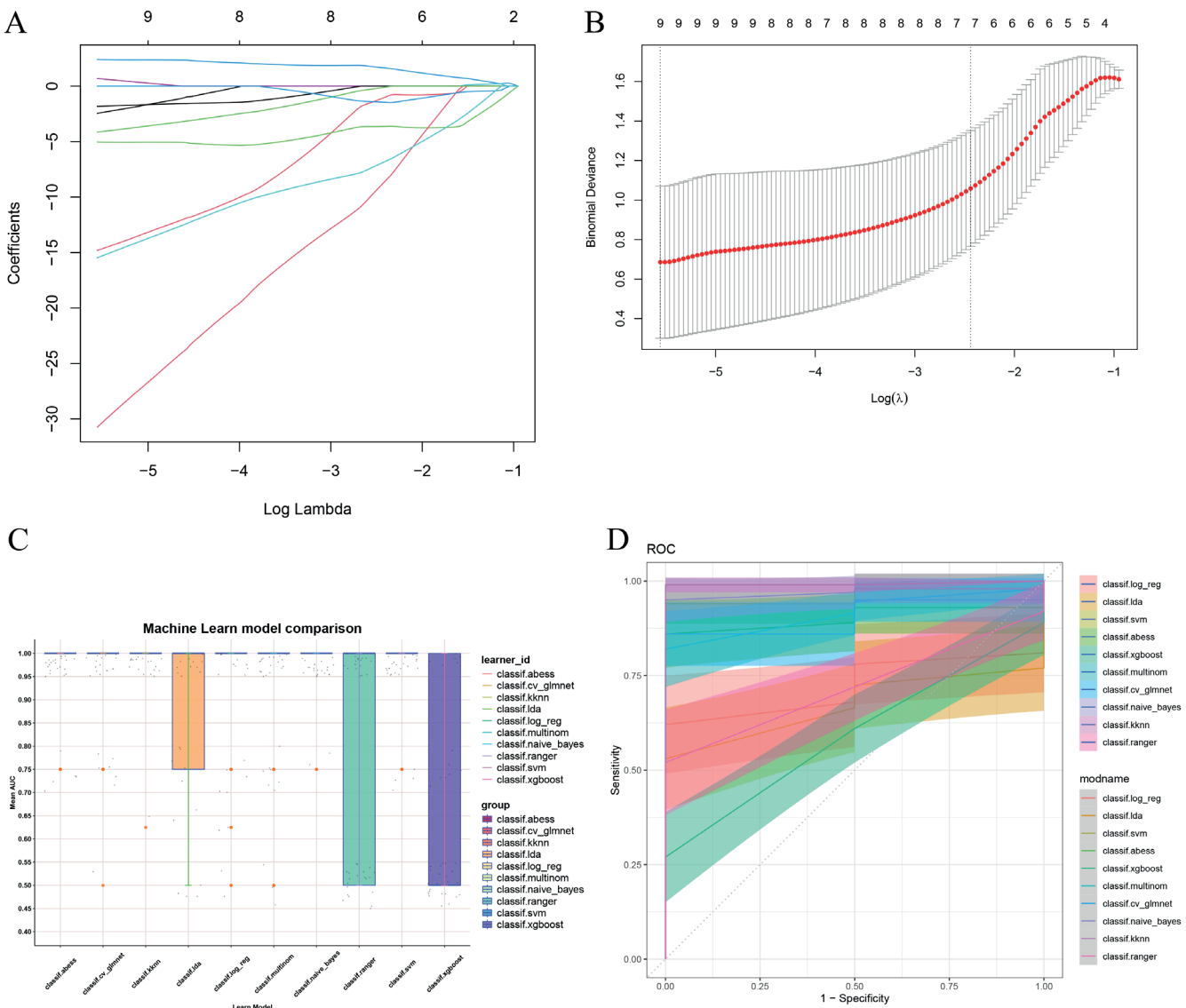


Fig. 5. Machine learning (ML) diagnostic model for oral lichen planus (OLP). A. Least absolute shrinkage and selection operator (LASSO) regression coefficient plot; B. Penalty parameter selection plot; C. Average area under the curve (AUC) from cross-validation for ML models; D. Receiver operating characteristic (ROC) curves with confidence intervals

Table 1. Top genes identified using summary data-based Mendelian randomization (SMR) analysis for oral lichen planus

No.	ProbeID	Gene	P_SMR	P_HEIDI
61	ENSG00000138792	ENPEP	0.0007440228	0.5696891
81	ENSG00000135587	SMPD2	0.000723646	0.4871803
71	ENSG00000183323	CCDC125	0.0009409793	0.5892023
127	ENSG00000121691	CAT	0.0008846088	0.402772
86	ENSG00000010030	ETV7	0.001591347	0.5681294
123	ENSG00000164978	NUDT2	0.001405524	0.4440702
85	ENSG00000204420	C6orf25	0.002674887	0.5592639
217	ENSG00000104852	SNRNP70	0.002695897	0.732081
224	ENSG00000099949	LZTR1	0.003013516	0.9810746
31	ENSG00000115816	CEBPZ*	0.02483984	0.7211582

CEBPZ was the only gene overlapped with least absolute shrinkage and selection operator (LASSO) regression results (see Fig. 9A). P_SMR – p-value from SMR analysis indicating pleiotropic association with OLP; P_HEIDI – p-value from the Heterogeneity in Dependent Instruments (HEIDI) test assessing heterogeneity.

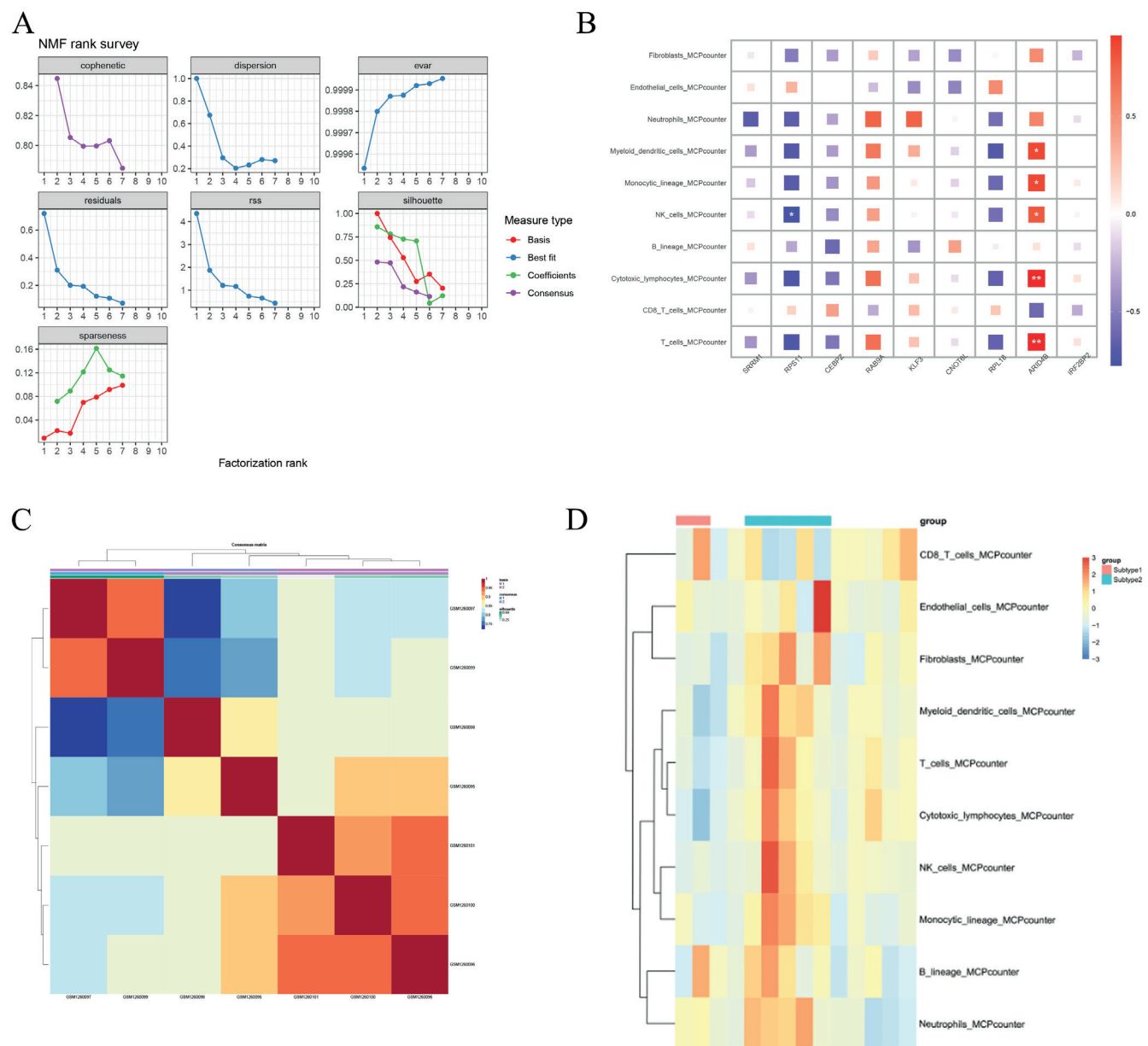


Fig. 6. Oral lichen planus (OLP) patient subtyping and immune infiltration. A. Non-negative matrix factorization (NMF)-based clustering of OLP patients; B. Consensus matrix heatmap (k = 2); C. Correlation heatmap between 9 genes and immune cell types; D. Microenvironment Cell Populations (MCP)-counter comparison of immune cell infiltration between subgroups

interaction strength, we found that signaling pathways between *CEBPZ*+ T cells and monocytes were significantly enhanced compared with those between *CEBPZ*–T cells (Fig. 10A). The enhanced interactions involving “MIF-(CD74+CXCR4)” and “MIF-(CD74+CD44)” were consistent with the enhanced communication observed in OLP_T cells through *MIF*-related pathways (Fig. 10B). According to recent studies, *MIF*, *CD74*, *CXCR4*, and *CD44* are associated with inflammation. *MIF* mediates the chronicity and potential malignant transformation of OLP. A protein–protein interaction (PPI) network generated using the STRING database identified interactions among *CEBPZ*, *MIF*, *CD74*, *CD44*, and *CXCR4* (Fig. 10C).

Discussion

Oral lichen planus is an immune-mediated inflammatory disease that primarily affects the tongue, gingiva, and buccal mucosa. Research indicates that 1.63% of OLP cases develop into OSCC within 7 years, leading the World Health Organization (WHO) to classify OLP as a potentially malignant condition. Early diagnosis and ongoing monitoring are crucial for the management of OLP. However, current diagnostic methods lack precision and are noninvasive.⁴⁶ The development of high-throughput sequencing technology has enabled rapid and precise analysis of gene expression differences between healthy and diseased tissues.⁴⁷ However, using bulk sequencing techniques to determine

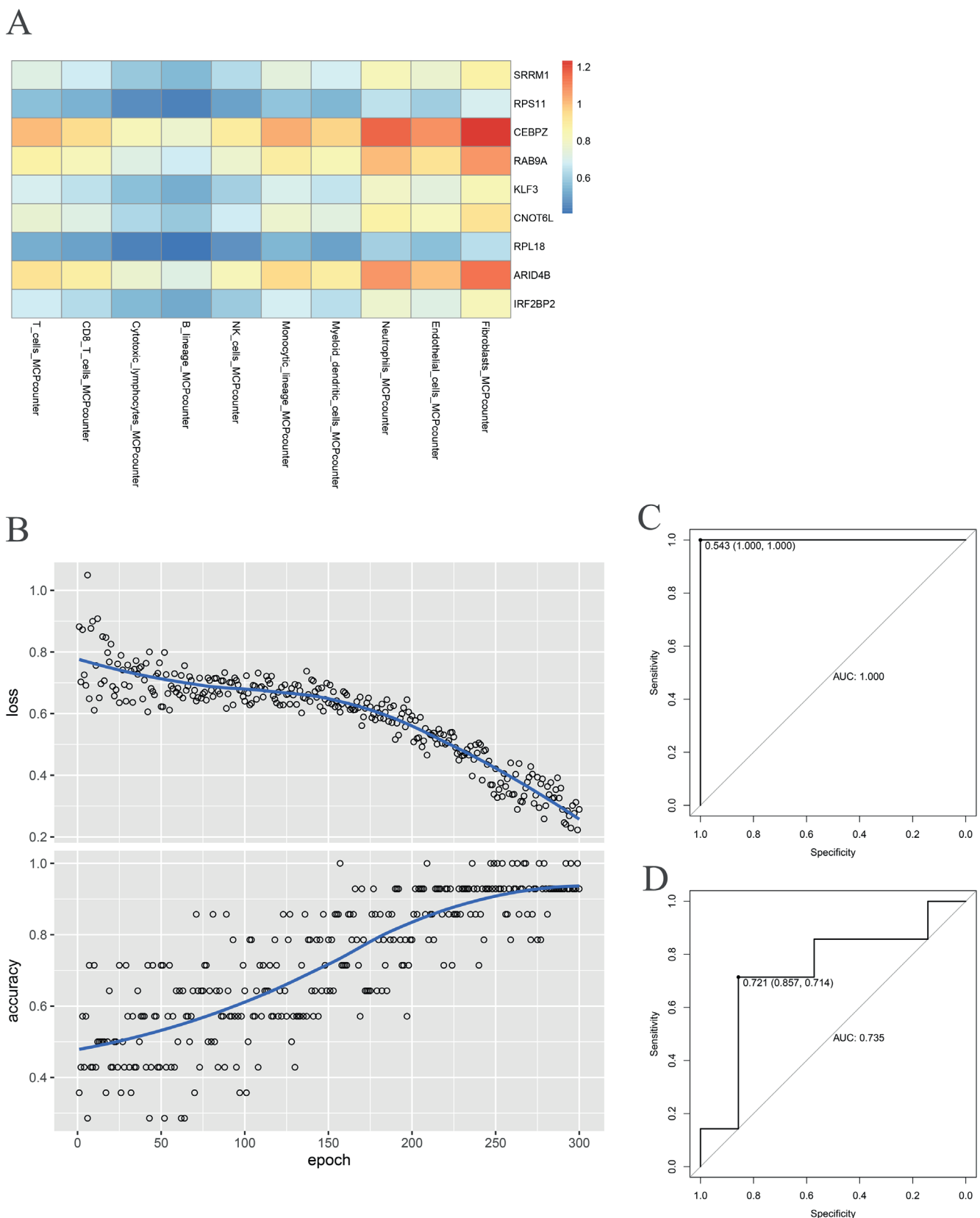


Fig. 7. Gene-immune convolutional neural network (CNN) model. **A.** Schematic representation of the CNN architecture integrating 9 genes and 10 immune cell types; **B.** Training curve over 300 epochs; **C,D.** Model performance in the training (area under the curve (AUC) = 1.000) and validation (AUC = 0.735) datasets

cellular composition and the involvement of specific subpopulations in diseased tissues remains challenging.⁴⁸ Single-cell sequencing technology addresses these limitations

by enabling detailed analysis of cellular heterogeneity and communication within diseased tissues.⁴⁹ SMR analysis helps identify disease-associated genes while minimizing

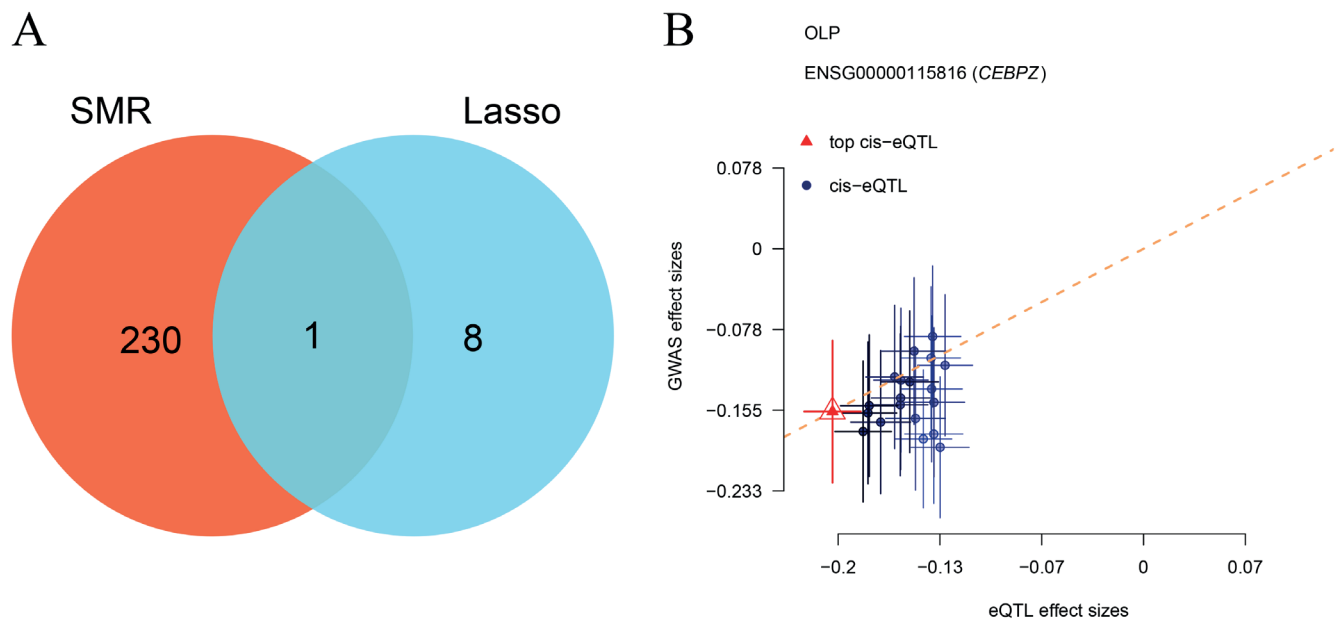


Fig. 8. Summary data-based Mendelian randomization (SMR) analysis of oral lichen planus (OLP)-associated genes. A. Venn diagram showing the overlap between SMR and lasso least absolute shrinkage and selection operator (LASSO) results; B. Mendelian randomization locus plot showing the association between *CEBPZ* expression and OLP risk (odds ratio (OR) = 1.057, 95% confidence interval (95% CI) = 1.013–1.102, $p = 0.010$)

confounding factors and providing important information regarding causality, thereby offering a dynamic view of disease progression.⁵⁰

Studies have indicated that OLP has a major immunological component.¹⁶ The current study found TNF- α expression throughout the subepithelial T-cell infiltrate and TNF receptor 1 (TNFR1) expression in basal and suprabasal epithelial cells in OLP. Oral lichen planus is characterized by a balance between pro-inflammatory (IFN- γ , TNF- α) and immunosuppressive (transforming growth factor beta 1 (TGF- β 1)) cytokines. Insufficient immunosuppression associated with decreased TGF- β 1 activity may promote hyperactive immune responses in OLP. Conversely, high TGF- β 1 activity may impair antitumor immunity and thereby promote OLP carcinogenesis.^{51,52}

Corticosteroids, used as first-line treatment for OLP, primarily suppress the CD4⁺ Th-cell subgroup and down-regulate NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling, thereby reducing IFN- γ levels and exerting immunomodulatory effects.^{53,54} Tacrolimus, a second-line treatment, inhibits cytokine production and T-cell activation, reduces Treg proliferation, inhibits mast cell activation, and affects Langerhans cell activity.^{55–57} Studies based on the immune landscape of OLP have identified potential therapeutic targets, including β -integrins and ICAM-1 inhibitors for controlling adhesion molecule expression, RANTES inhibitors for stabilizing mast cells, Fas cell surface death receptor (FAS) or Fas ligand (FASL) inhibitors, and TNF- α receptor inhibitors for reducing keratinocyte apoptosis.^{58,59} However, further research is required before these approaches can be translated into clinical practice based on the OLP immune microenvironment.

Based on oral scRNA-seq transcriptomic features, we developed a 9-gene classifier that may assist in OLP diagnosis and highlights the potentially important role of specific T-cell subsets characterized by pronounced oxidative stress in OLP pathophysiology. The expression patterns of these 9 genes correlate with oxidative stress, inflammation-related pathways, and treatment-related responses in T cells.^{2,60,61} The application of scRNA-seq has revealed the heterogeneity of immune cells in OLP and their interactions with epithelial cells, which is crucial for understanding disease progression and developing targeted therapies.

CCAAT/enhancer-binding proteins including CEBPA, CEBPB, CEBPD, CEBPE, CEBPG, and CEBPZ, play important roles in physiological and pathological processes.^{62–64} *CEBPZ* functions as either an activator or a suppressor depending on cellular context and is associated with programmed cell death, cell-cycle arrest, and cellular stress.⁶⁵ Strong correlations have been reported between *CEBPZ* expression and methylation in acute myeloid leukemia,⁶⁶ and overexpression has also been observed in esophageal squamous cell carcinoma.⁶² Nearly all 9 LASSO-selected genes were strongly associated with oxidative stress, inflammation, and OLP pathogenesis. The intrinsic OLP score was markedly elevated according to single-sample Gene Set Enrichment Analysis (ssGSEA) based on genes from the blue and brown modules, suggesting worsening immunological dysregulation and inflammation within the oral microenvironment. Strong correlations between the expression patterns of blue- and brown-module genes and immune infiltration suggest that these genes may participate in immune regulation.

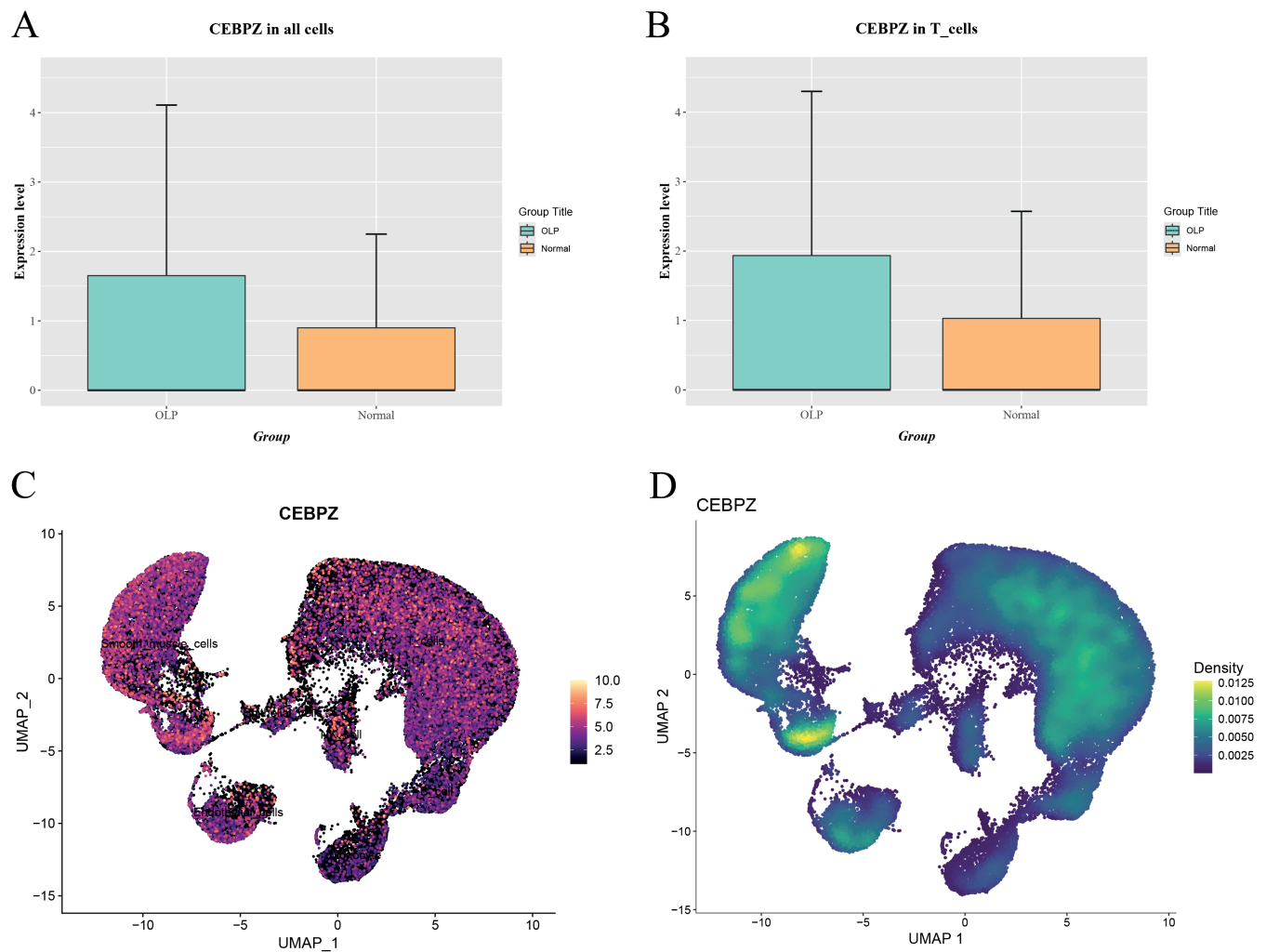


Fig. 9. *CEBPZ* expression in oral lichen planus (OLP). A,B. Box plots of *CEBPZ* expression in all cells and T cells comparing OLP and normal groups; C. Uniform Manifold Approximation and Projection (UMAP) plot showing the distribution of *CEBPZ* expression across all OLP cells; D. Density plot showing *CEBPZ* expression enrichment

By combining SMR and scRNA-seq analyses, we found that high *CEBPZ* expression is associated with activated MIF pathway signaling between T cells and monocytes. *CEBPZ* may influence the expression of *MIF*, *CXCR4*, *CD74*, and *CD44* through the MIF pathway, creating a feed-forward loop that perpetuates inflammation and oxidative stress in the OLP microenvironment. Studies have demonstrated that T-cell-mediated inflammation in OLP is associated with increased *TRIM21* expression.⁶⁷ *TRIM21* links ubiquitinated NF- κ B via K63, thereby activating the NF- κ B signaling pathway and promoting inflammation.⁶⁸ Corticosteroids are first-line therapies that target NF- κ B and have considerable therapeutic significance in OLP treatment. T cells may trigger keratinocyte apoptosis through TNF- α binding to TNF receptor 1 (TNFR1), CD95L binding to CD95, or granzyme B entering through perforin-created membrane pores.^{69–71} This finding highlights the potential significance of *CEBPZ* in immune modulation and provides a novel direction for investigating its potential as a therapeutic target or biomarker. While clinical examination and histological assessment

remain the preferred approaches for evaluating OLP, molecular diagnostic methods such as oral tissue RNA sequencing may significantly enhance diagnostic accuracy.

The clinical implications of *CEBPZ* extend beyond diagnosis. *CEBPZ* expression could potentially guide treatment selection, with high expression indicating patients who may benefit from MIF pathway-targeted therapies or oxidative stress interventions. Integration of *CEBPZ* into ML models could enable risk stratification and identify patients at higher risk of malignant transformation who require more intensive surveillance.

Limitations of the study

This study has several limitations that should be considered when interpreting the results. First, the analyses relied on publicly available datasets from predominantly European populations, which may lack comprehensive clinical metadata, potentially limiting the generalizability of the findings to other ethnic groups. Second, the cross-sectional nature of the data restricts the ability

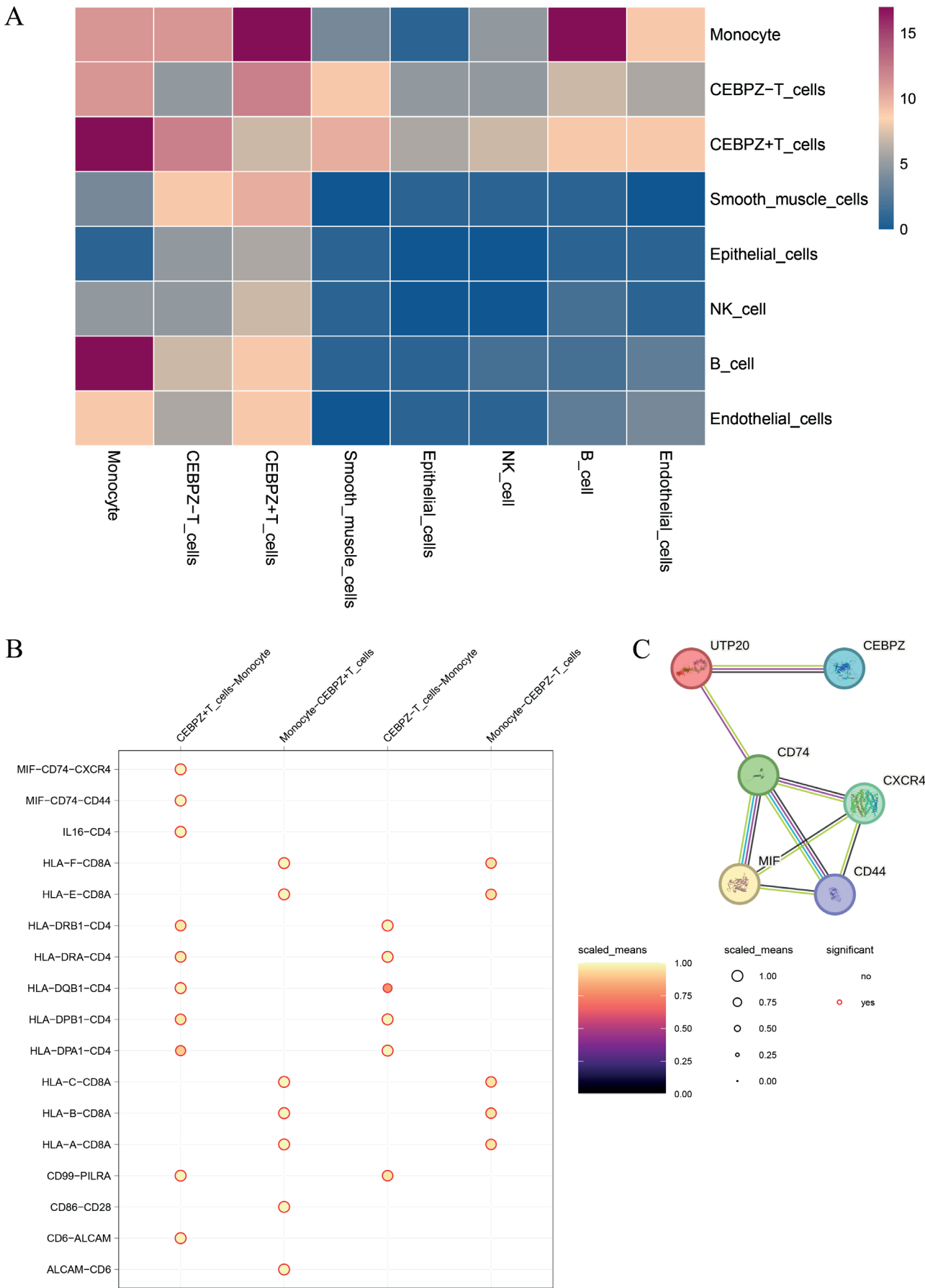


Fig. 10. Role of *CEBPZ* in oral lichen planus (OLP) immune interactions. A. Heatmap of cell-type interaction frequencies; B. Dot plot of receptor–ligand interactions between *CEBPZ*+ and *CEBPZ*– T cells and monocytes; C. Protein–protein interaction (PPI) network generated using the STRING database

to infer temporal changes beyond the genetic associations established through SMR. Third, esophageal mucosa eQTLs served as proxies for oral epithelium; although both tissues share squamous epithelial characteristics, tissue-specific regulatory mechanisms may differ, and future validation using oral tissue-specific eQTLs will be essential. Fourth, experimental validation of identified biomarkers, such as *CEBPZ*, is needed to confirm their functional roles through in vitro and in vivo studies. Fifth, potential batch effects between different datasets, despite computational correction, may have influenced the results. Finally, ML models require prospective validation in independent, clinically characterized cohorts to assess their real-world applicability. Future studies incorporating longitudinal samples, functional assays, and clinical trials will be essential to strengthen the translational impact.

Conclusions

This study pioneers an integrative genomic approach to unravel the immune-mediated mechanisms of OLP by combining scRNA-seq, SMR, hdWGCNA, and ML. We identified a novel T-cell subpopulation in the OLP LP characterized by heightened oxidative stress and enhanced MIF pathway interactions with monocytes and B cells. *CEBPZ* emerged as a pivotal biomarker at the intersection of SMR and LASSO analyses, with elevated expression correlating with immune infiltration and OLP pathogenesis. The developed diagnostic models (AUC: 0.653–0.745) offer promising tools for clinical application. These findings deepen our understanding of the molecular landscape of OLP and highlight *CEBPZ* as a potential therapeutic target, paving the way for precision medicine strategies in OLP.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.18939928>. The package contains the following files:

Supplementary Fig. 1. SMR analysis workflow schematic. Step-by-step diagram illustrating the SMR analysis pipeline, including data acquisition, quality control, instrument selection, SMR/HEIDI analysis, and multi-omic integration.

Supplementary Fig. 2. Quality control of the scRNA-seq dataset. UMAP plots showing the GSE211630 dataset before and after quality-control preprocessing.

Supplementary Fig. 3. Signaling pathways in cell–cell communication. Bubble plots highlighting significant signaling pathways involved in cell–cell communication between T-cell subtypes and other cell types, identified using CellChat.

Supplementary Fig. 4. MIF and CXCL signaling pathway analysis. A. Role of T-cell subtypes in the MIF signaling pathway network; B. Role of T-cell subtypes in the CXCL

signaling pathway network; C. Scatter plot of outgoing vs incoming interaction strengths across cell types.

Supplementary Fig. 5. Expression of LASSO-selected genes. UMAP plots displaying expression patterns of 9 LASSO-selected genes in OLP and healthy tissues.

Supplementary Fig. 6. External validation of ML models. ROC curves for the Logreg, ABESS, and LDA models in the external validation set GSE38616 (AUC = 0.745, 0.694, and 0.653, respectively).

Supplementary Fig. 7. Immune cell proportions between OLP subtypes. Box plot comparing immune cell composition between Subtype 1 and Subtype 2 across 10 MCP-counter cell types.

Supplementary Fig. 8. *CEBPZ* expression across cell types and developmental trajectory. A. Dot plot of *CEBPZ* expression across cell types; B. RNA velocity analysis showing developmental vectors; C. UMAP of T-cell clusters in OLP and normal tissues; D. *CEBPZ* expression distribution across T-cell clusters.

Supplementary Table 1. Complete SMR analysis results. Excel file containing all 231 genes identified through SMR analysis, together with their corresponding statistical values.

Data Availability Statement

The datasets analyzed in this study are publicly available. Single-cell RNA sequencing data (GSE211630, GSE52130, and GSE38616) were obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo>). GWAS summary data for oral lichen planus were obtained from the FinnGen database (release 8; <https://www.finnngen.fi>). Esophageal mucosa eQTL data were retrieved from the GTEx database (version 8; <https://gtexportal.org>). Protein interaction data were obtained from the STRING database (<https://string-db.org>). The complete results of the SMR analysis, including 231 genes, are provided in Supplementary Table 1. All data were processed in accordance with the guidelines and policies of the respective repositories. No new data were generated in this study.

Consent for publication of personal information

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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Pancytopenia and isolated cytopenias as hematological complications of SLE: A narrative review

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Abstract

Pancytopenia is a significant hematological complication in patients with systemic lupus erythematosus (SLE), with an estimated prevalence of 10–40%. It is defined as a simultaneous decrease in red blood cells, white blood cells, and platelets. Although isolated cytopenias are more common, pancytopenia may indicate severe disease activity or secondary complications, such as bone marrow suppression or hemophagocytic syndromes. This review aims to summarize and synthesize current knowledge on the etiology, pathophysiology, diagnostic approach, and treatment strategies for pancytopenia in SLE, as well as its differentiation from other causes of bone marrow failure. The pathogenesis of pancytopenia in SLE is multifactorial and includes drug-induced bone marrow suppression, hypersplenism, myelofibrosis, macrophage activation syndrome (MAS), and autoimmune bone marrow failure. The diagnostic evaluation includes hematologic assessment, bone marrow examination, and exclusion of alternative diagnoses, such as aplastic anemia and paroxysmal nocturnal hemoglobinuria. Therapeutic management depends on the underlying cause. We also summarize published case reports comparing treatment approaches and clinical outcomes. The role of rituximab (RTX) in the management of pancytopenia and isolated cytopenias associated with SLE is discussed in detail. Particular attention is given to its mechanisms of action, safety profile and mixed clinical outcomes, documented in multicenter retrospective cohort studies, meta-analyses, and case series. Early recognition of pancytopenia remains a clinical challenge and requires well-structured diagnostic and therapeutic strategies. A thorough understanding of its underlying mechanisms and clinical manifestations is essential to avoid delays in treatment and prevent complications.

Key words: systemic lupus erythematosus, pancytopenia, rituximab, leukopenia, thrombocytopenia

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Highlights

- Pancytopenia affects 10–40% of patients with systemic lupus erythematosus (SLE) and may indicate severe disease activity or secondary bone marrow pathology.
- The pathogenesis is multifactorial, involving autoimmune bone marrow suppression, drug toxicity, hypersplenism, myelofibrosis, and macrophage activation syndrome.
- Accurate diagnosis requires comprehensive hematologic evaluation, bone marrow examination, and exclusion of differential diagnoses such as aplastic anemia and paroxysmal nocturnal hemoglobinuria.
- Rituximab demonstrates high initial response rates in SLE-related cytopenias, however randomized controlled trials show frequent relapses and difficulties maintaining long-term remissions.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder characterized by the production of autoantibodies against nuclear antigens and the formation of immune complexes that contribute to multiorgan tissue damage. Hematological manifestations are among the most common clinical features of SLE, with pancytopenia – defined as a concurrent reduction in red blood cells, white blood cells, and platelets – representing a particularly important clinical challenge. Its clinical presentation ranges from asymptomatic or mild, incidentally detected cases to severe manifestations associated with, e.g., sepsis.¹

Pancytopenia in the context of SLE may result from several pathogenic mechanisms, including autoimmune destruction of hematopoietic cells, hypersplenism, bone marrow suppression, and adverse effects of immunosuppressive therapy. Coexisting conditions, such as macrophage activation syndrome (MAS) or secondary myelofibrosis, may further complicate the clinical picture and pose significant diagnostic challenges.² Differentiating primary autoimmune cytopenias from other causes often requires a comprehensive diagnostic workup, including bone marrow biopsy, immunologic studies, and infectious disease screening.¹ The search for optimal diagnostic algorithms, supported by multicenter retrospective cohort studies, has led to the adoption of tools such as the Hemophagocytic Syndrome Diagnostic Score (HScore), which facilitates the diagnosis of hemophagocytic lymphohistiocytosis (HLH) and reduces the risk of overlooking the underlying cause of the patient's condition.³

Pancytopenia is considered not only a hematological manifestation of SLE but also a marker of disease activity and severity. Although isolated leukopenia, lymphopenia, and thrombocytopenia are relatively common, their coexistence as pancytopenia warrants a thorough etiological investigation. Effective treatment depends on identifying the underlying cause – whether uncontrolled autoimmunity, iatrogenic effects, or overlapping syndromes – and determining whether the pathology involves peripheral destruction of blood cells, intrinsic bone marrow dysfunction, or both.¹

Moreover, the 2nd part of this review focuses on the role of rituximab (RTX) in the treatment of pancytopenia and isolated cytopenias associated with SLE. Particular emphasis is placed on the mechanisms of action of RTX, with consideration given to the pathophysiological basis of the autoimmune process and its molecular targets. The efficacy of RTX is discussed in the context of evidence from multicenter retrospective cohort studies, meta-analyses, and case series. Its safety profile is also reviewed, with particular attention to patients with immune-mediated cytopenias.

Objectives

This review synthesizes current knowledge on the pathophysiology, differential diagnosis, and treatment of pancytopenia in SLE.⁴ Based on clinical studies, retrospective analyses, and current therapeutic guidelines, it aims to provide a comprehensive overview of this multifaceted complication while emphasizing the importance of an individualized, multidisciplinary approach.

Materials and methods

This review aimed to synthesize current knowledge on pancytopenia and other hematological complications associated with SLE. To achieve this, a comprehensive literature search was conducted using major scientific databases, focusing primarily on studies published within the past 15 years. The search keywords included “systemic lupus erythematosus,” “pancytopenia,” “cytopenia,” “autoimmune myelofibrosis,” “hemophagocytic syndrome,” “rituximab in SLE,” and “iatrogenic cytopenia.” We included studies addressing the pathophysiology, clinical presentation, differential diagnosis, and treatment of pancytopenia, as well as isolated cytopenias in the context of SLE. Eligible publications included original clinical studies, case series, and systematic reviews.

A total of more than 90 articles were screened, of which 71 peer-reviewed publications and case reports were

selected for in-depth analysis. These publications covered topics such as autoimmune mechanisms, drug-induced toxicity (e.g., iberdomide-induced degradation of the transcription factors Ikaros and Aiolos), laboratory biomarkers, differential diagnosis, and various immunosuppressive treatment protocols, including those described in case series as practical references for clinicians. In addition, the role of RTX in the treatment of immune-mediated cytopenias associated with SLE was thoroughly analyzed. Population-based studies were also included, providing broader insights into the true prevalence of hematological complications in this patient population. The data were organized thematically to illustrate the relationship between SLE and pancytopenia, leukopenia – with particular emphasis on neutropenia – and thrombocytopenia, followed by a section on differential diagnosis, including aplastic anemia, and concluding with evidence-based treatment strategies.

Pancytopenia in the context of SLE

Pancytopenia, defined as a reduction in all 3 major blood cell lineages, is a potential hematological complication in patients with SLE. This condition is diagnosed when hemoglobin (Hb) levels are below 10 g/dL, the total white blood cell count is $\leq 4 \times 10^9/\text{L}$, the neutrophil count is $< 1.5 \times 10^9/\text{L}$, and the platelet count is $< 100 \times 10^9/\text{L}$.⁵ Pancytopenia occurs less frequently than isolated cytopenias in patients with SLE. It is estimated to affect 10–40% of patients with SLE, whereas neutropenia and thrombocytopenia are observed more frequently than aplastic anemia.^{5,6}

Pathogenesis of pancytopenia in SLE

Potential causes of pancytopenia in patients with SLE include medications, peripheral blood cell destruction, autoimmune myelofibrosis, and rare conditions such as MAS.⁷ In addition, bone marrow aspiration typically reveals hypocellularity and necrosis, which are attributed to autoimmune mechanisms. Various triggering factors, including immune activation and infections, may lead to excessive macrophage activation in the bone marrow and other tissues.⁸ Moreover, hemophagocytosis is frequently observed in the bone marrow but is neither pathognomonic nor sufficient for establishing the diagnosis.⁹

Autoimmune diseases such as SLE, rheumatoid arthritis, and Felty's syndrome contribute to pancytopenia through hypersplenism, which results in peripheral destruction of blood cells by the reticuloendothelial system. Furthermore, hypersplenism occurs in 9–46% of patients with SLE,¹⁰ particularly during periods of active disease.⁵

Myelofibrosis is another important cause of pancytopenia and is characterized by clonal proliferation of myeloid stem cells and the production of a fibrotic bone marrow

stroma.⁶ In addition, life-threatening conditions such as HLH, disseminated intravascular coagulation (DIC), and paroxysmal nocturnal hemoglobinuria (PNH) should also be considered in the differential diagnosis of pancytopenia in critically ill patients.⁵

Iatrogenic pancytopenia in SLE

Pancytopenia may be induced by various iatrogenic factors, including chemotherapy, radiotherapy, and antiepileptic drugs such as carbamazepine and valproate. Drug interactions, including those between allopurinol and azathioprine (AZA) or methotrexate (MTX) and trimethoprim, may also contribute to the development of pancytopenia. Additional causes include sepsis and certain antibiotics, particularly chloramphenicol, trimethoprim, and linezolid.⁵

In addition, immunosuppressive drugs used in the treatment of SLE, including AZA, MTX, and cyclophosphamide (CYC), may cause bone marrow suppression.⁵ The mechanisms underlying drug-induced cytopenia include immune-mediated reactions. These reactions may impair hematopoiesis within the bone marrow or enhance peripheral destruction of blood cells. They may also be associated with the patient's immune profile or coexisting diseases.⁶ Moreover, pancytopenia in SLE may be induced by interferon alpha (IFN- α) in the context of HLH.¹¹ Cases of pancytopenia developing during sulfasalazine therapy have also been reported. Furthermore, resolution of pancytopenia following discontinuation of sulfasalazine has been described.¹²

Anemia

Studies consistently identify anemia as the most common hematological manifestation in patients with SLE, with a reported prevalence exceeding 50%.⁶ Another review reported rates as high as 98%, reflecting the diverse etiologies of anemia in SLE, including anemia of chronic disease, autoimmune hemolytic anemia, and microangiopathic hemolytic anemia. Anemia of chronic disease was the predominant subtype, underscoring the impact of chronic inflammation and hematologic dysregulation associated with SLE.¹³

Anemia of chronic disease is considered the most common type of anemia in SLE, accounting for approx. 33% of cases. In asymptomatic patients, treatment beyond adequate control of the underlying disease is usually unnecessary; however, in severe cases, erythropoiesis-stimulating agents may be considered.⁶

Autoimmune hemolytic anemia (AIHA) occurs in approx. 10% of patients with SLE, may precede the diagnosis of SLE by several years, and may represent the predominant clinical manifestation. Warm AIHA is the most common subtype, and its treatment is based primarily

on glucocorticosteroids (GCs). However, increasing attention has been paid to the use of RTX in patients with SLE-associated AIHA, as it may avoid the need for splenectomy and its associated complications.⁶ Aplastic anemia may also occur in patients with SLE, and careful monitoring while awaiting the response to treatment is essential because blood transfusions may be required.⁶

Microangiopathic hemolytic anemia leading to thrombotic microangiopathy (TMA) occurs in approx. 0.5–10% of patients with SLE and is associated with a poor clinical prognosis. Although this is a rare complication, its coexistence with SLE is associated with high mortality, ranging from 33.9% to 62.5%, despite the use of plasmapheresis, which remains the cornerstone of treatment in this setting.⁶

Regardless of the underlying etiology of anemia in SLE, it is essential to determine whether the patient requires urgent intervention, including blood transfusion. It should be emphasized that transfusion is a supportive measure aimed at stabilizing the patient's condition until immunosuppressive or hematological treatment becomes effective.

Transfusion is generally considered when the Hb concentration is <7 g/dL in hemodynamically stable patients, or <9 g/dL in patients with cardiovascular disease (CVD), impaired tissue perfusion, acute hemolytic anemia, or active AIHA. Transfusion is also indicated in symptomatic AIHA accompanied by signs of circulatory failure and in anemia resulting from aplastic anemia or myelofibrosis when causal treatment is ineffective.¹

In patients with SLE, the use of leukoreduced blood components is recommended because it reduces the risk of febrile nonhemolytic transfusion reactions, alloimmunization, and transmission of leukocyte-associated viruses. In addition, irradiated blood components are recommended for patients with profound immunosuppression, particularly those receiving RTX, CYC, mycophenolate mofetil, or those who have undergone hematopoietic stem cell transplantation (HSCT). This approach aims to prevent transfusion-associated graft-versus-host disease (TA-GVHD), a potentially life-threatening complication in this patient population.¹

Leukopenia

Leukopenia, defined as a reduced white blood cell count, is a common hematological manifestation of SLE and often correlates with disease activity. Proposed mechanisms include increased peripheral destruction of granulocytes, alterations in the marginal granulocyte pool and spleen, and reduced bone marrow production.⁶ The most common form of lymphopenia in SLE is T-cell lymphopenia, which is strongly associated with disease activity, particularly when accompanied by thrombocytopenia. Notably, both the American College of Rheumatology (ACR) and

the Systemic Lupus International Collaborating Clinics (SLICC) recognize leukopenia as an important classification criterion for SLE.⁶

In addition, lymphopenia has been reported to be significantly associated with overall SLE disease activity, erythrocyte sedimentation rate (ESR), serological findings, and the use of various medications, including prednisolone, AZA, MTX, CYC, and RTX.¹⁴ In contrast, MTX and cyclosporine have been reported to be associated with a lower risk of neutropenia. Lower SLE disease activity has likewise been associated with a lower prevalence of both lymphopenia and neutropenia.¹⁵

Neutropenia

Neutropenia is the most common neutrophil abnormality in SLE, occurring in 20–47% of patients. It is classified as mild when the neutrophil count is $1.0\text{--}1.5 \times 10^9/\text{L}$, moderate at $0.5\text{--}1.0 \times 10^9/\text{L}$, and severe at $<0.5 \times 10^9/\text{L}$.¹⁶ Neutrophils have long been recognized as a potential source of autoantigens and inflammatory cytokines in SLE.¹⁷ Neutrophil populations in patients with SLE differ from those in healthy individuals, with impaired phagocytic function, altered metabolism, and increased apoptosis representing the principal abnormalities.¹⁸ Consequently, these neutrophils exhibit an increased capacity to form neutrophil extracellular traps (NETs) and contain autoantigens as well as elevated levels of other immunostimulatory molecules.¹⁹ Excessive NET formation may contribute to disease progression by stimulating type I interferon production by plasmacytoid dendritic cells and promoting endothelial dysfunction and prothrombotic changes.¹⁷

In addition to reduced bone marrow production and immune-mediated destruction, transient reductions in circulating neutrophil counts in SLE may also result from increased margination of neutrophils along the vascular endothelium.²⁰ Margination reflects the redistribution of neutrophils from the freely circulating pool into marginated intravascular reservoirs (e.g., the spleen and the pulmonary and hepatic microvasculature) and may result in apparent peripheral neutropenia without a true reduction in neutrophil mass.²¹ Moreover, iberdomide, a novel therapeutic agent under investigation for SLE, promotes degradation of the transcription factors Ikaros and Aiolos, thereby influencing leukocyte development and autoimmune processes. However, its use is associated with adverse effects, including neutropenia.²²

A study comparing 208 patients with neutropenia and 779 patients with SLE without neutropenia found that neutropenia was significantly associated with thrombocytopenia, lymphopenia, and reduced complement C3 levels.¹⁷ Chronic neutropenia was also associated with the presence of anti-Ro/SSA antibodies. Kurien et al. proposed that anti-Ro/SSA antibodies may cross-react with neutrophil surface

proteins, triggering complement activation and subsequent cell damage.²³ Furthermore, Beyan et al.²⁴ reported a 20% prevalence of neutropenia among 115 patients with SLE ($<1.8 \times 10^9/L$), whereas Dias et al.²⁵ documented a prevalence of 40.3% among 124 patients with SLE, with severe and persistent neutropenia occurring in 0.8% and 4.8% of patients respectively.

Thrombocytopenia

Mild thrombocytopenia (platelet count: $100\text{--}150 \times 10^9/L$) is observed in 25–50% of patients, whereas severe thrombocytopenia (platelet count $<50 \times 10^9/L$) occurs in approx. 10%.²⁶ Immune thrombocytopenia (ITP), the primary cause of SLE-associated thrombocytopenia,⁶ is defined as a platelet count $<100 \times 10^9/L$ and affects 10–40% of patients with SLE.²⁷ Immune thrombocytopenia may precede the clinical manifestations of SLE in 5–16% of patients.²⁸ Moreover, severe thrombocytopenia is associated with a poor prognosis and increased mortality.²⁹ The underlying mechanisms include impaired platelet production by megakaryocytes and increased peripheral platelet destruction mediated by autoimmune processes.⁷

Treatment of SLE-associated immune thrombocytopenia

Currently, the treatment of SLE-associated ITP (SLE-ITP) is based on immunosuppressive therapy, similarly to the treatment of primary ITP. Approximately 75% of patients with SLE-ITP respond to GCs and antimalarial drugs.²⁹ In cases in which ITP occurs secondary to SLE, GCs play a central role in treatment, resulting in clinical improvement in approx. 2/3 of patients. Moreover, GC monotherapy achieves remission in nearly 20% of patients. Dexamethasone is the preferred GC because of its more rapid onset of action and lower risk of toxicity compared with prolonged prednisolone therapy.³⁰ Furthermore, intravenous immunoglobulin (IVIG) can temporarily increase platelet counts; however, it is used primarily in patients who are intolerant of corticosteroids. Immunoglobulin G (IgG) is administered intravenously at a dose of 1 g/kg/day for 1 or 2 days.⁶

The latest therapeutic options include thrombopoietin receptor agonists (TPO-RAs), platelet desialylation inhibitors (e.g., oseltamivir),³¹ neonatal Fc receptor (FcRn) inhibitors, spleen tyrosine kinase (Syk) inhibitors, Bruton's tyrosine kinase (BTK) inhibitors,³² and B-cell-targeted therapies (e.g., RTX and belimumab).³³

The introduction of RTX into the treatment of ITP has resulted in response rates of up to 60%, providing an alternative to splenectomy. However, observational studies indicate that only 20–30% of patients maintain long-term remission, with treatment being more effective in younger women before the disease enters the chronic phase.³⁴

Differentiation of pancytopenia in SLE from aplastic anemia and other types of anemia

The diagnosis of aplastic anemia (AA), a rare complication in patients with SLE, is often delayed because of the presence of other potential causes of pancytopenia.³⁵ In addition, several other types of anemia may occur in patients with SLE, including microangiopathic hemolytic anemia, iron deficiency anemia, autoimmune hemolytic anemia (with a positive Coombs test), pure red cell aplasia, and anemia secondary to chronic kidney disease (CKD). Autoimmune cytopenias in SLE are associated with the presence of autoantibodies directed against blood cell antigens, leading to immune-mediated destruction of hematopoietic cells.⁴

Differential diagnosis of pancytopenia in SLE

Once pancytopenia has been confirmed based on the initial complete blood count, preliminary noninvasive investigations are recommended, including reticulocyte count and peripheral blood smear analysis. The reticulocyte count serves as a reliable indicator of bone marrow function.¹⁴

In recent years, several biomarkers of primary myelofibrosis have been identified, including circulating YKL-40 and GATA-1; however, they are not useful for diagnosing myelofibrosis associated with SLE.³⁶ Further hematological evaluation is typically recommended, including bone marrow aspiration and trephine biopsy, which may reveal a hypercellular bone marrow. Trephine biopsy is also used to confirm hypocellularity or bone marrow necrosis⁵ (Fig. 1).

Chronic SLE and long-term immunosuppressive therapy increase the risk of lymphoid malignancies. Therefore, in patients with persistent cytopenia or new clinical features, such as progressive lymphadenopathy, fever, night sweats, or weight loss, lymphoma should be considered in the differential diagnosis.⁵

In the differential diagnosis, it is important to distinguish between bone marrow suppression caused by medications or active autoimmune disease and malignant bone marrow infiltration. The final diagnosis is based on bone marrow aspiration and trephine biopsy with histopathological evaluation, flow cytometry, and cytogenetic and molecular analyses for monoclonal lymphocyte populations, allowing lymphoma to be confirmed or excluded as the cause of pancytopenia. In diagnostically challenging cases, additional molecular and immunohistochemical analyses, including GATA-1 assessment, may be considered to help distinguish myeloproliferative neoplasms from lymphoproliferative disorders.⁵

Moreover, infectious causes should also be considered in the differential diagnosis of pancytopenia in patients

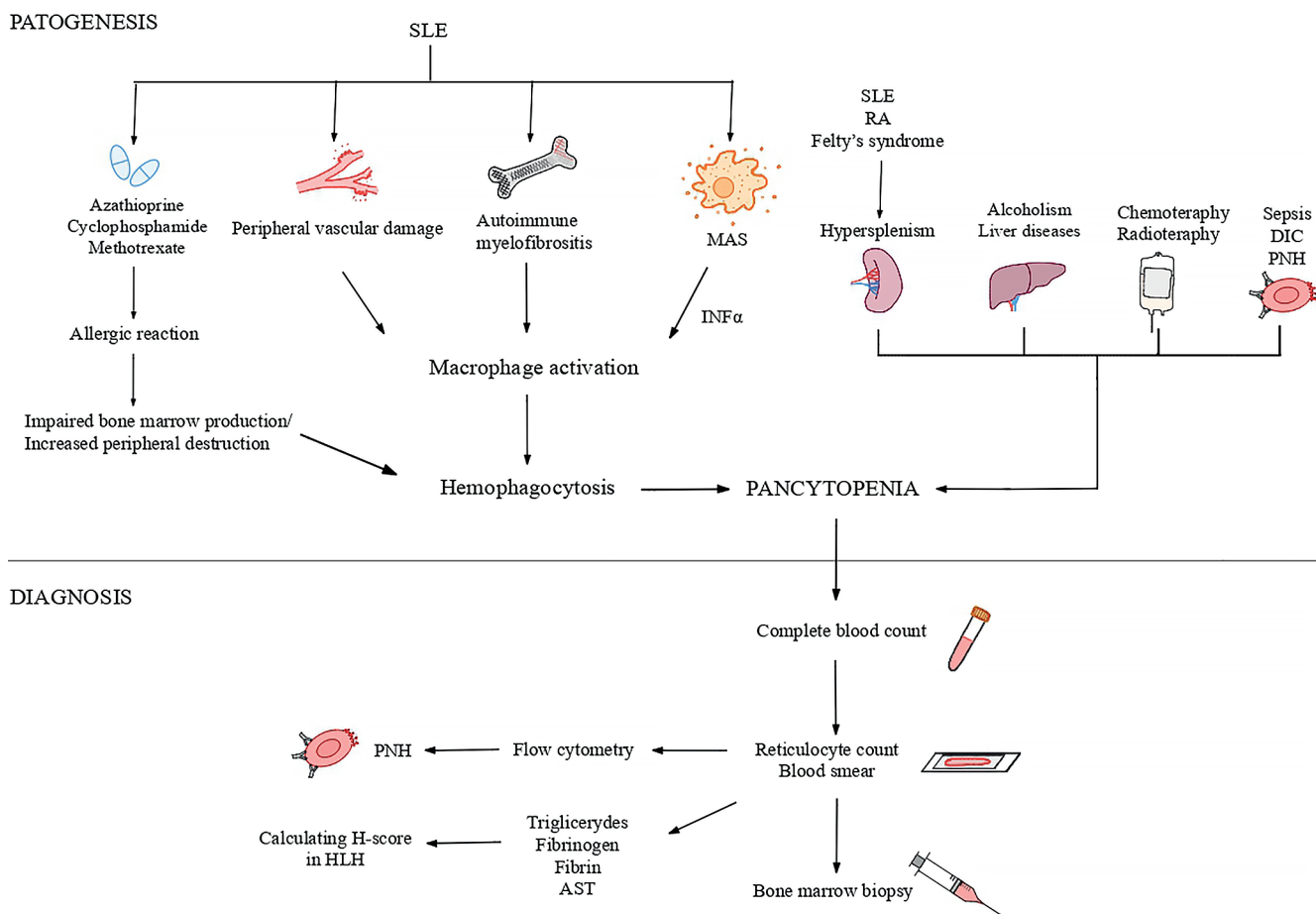


Fig. 1. Pathogenesis and differential diagnosis of pancytopenia in systemic lupus erythematosus (SLE)

RA – rheumatoid arthritis; MAS – macrophage activation syndrome; DIC – disseminated intravascular coagulation; PNH – paroxysmal nocturnal hemoglobinuria; HLH – hemophagocytic lymphohistiocytosis; AST – aspartate aminotransferase; $\text{TNF-}\alpha$ – tumor necrosis factor alpha.

with SLE. Viral infections, including parvovirus B19, HIV, Epstein–Barr virus (EBV), cytomegalovirus (CMV), and hepatitis viruses (HAV, HBV, and HCV), may impair bone marrow hematopoiesis because of their tropism for hematopoietic progenitor cells and their ability to induce aplastic anemia.⁵ In endemic regions, visceral leishmaniasis should also be considered because it may closely mimic SLE flares by presenting with fever, pancytopenia, splenomegaly, hypergammaglobulinemia, and autoantibody production.³⁷

Furthermore, additional investigations, including flow cytometry for PNH and measurements of aspartate aminotransferase (AST), ferritin, triglycerides, and fibrinogen, are important for calculating the HScore in patients with suspected HLH.⁵

Treatment of pancytopenia is primarily directed at identifying and eliminating the underlying cause (Table 1). In addition, antibiotics are indicated in cases of severe neutropenia, and blood transfusions may be administered when clinically indicated to manage severe anemia.⁵ Prompt recognition and comprehensive diagnostic evaluation are essential to distinguish SLE-related pancytopenia from other potentially life-threatening conditions.

A thorough understanding of the pathophysiology and clinical implications of pancytopenia in SLE is crucial for guiding appropriate therapeutic strategies and improving patient outcomes. Further research is warranted to clarify the underlying mechanisms and optimize management strategies for this complex and heterogeneous patient population.

The role of rituximab in the treatment of cytopenias associated with SLE

Given the central role of B lymphocytes in the pathogenesis of SLE, RTX, a chimeric mouse/human monoclonal antibody targeting the CD20 antigen, has been evaluated in numerous randomized controlled trials for the treatment of SLE, including studies assessing its B-cell specificity and efficacy in SLE as well as in other rheumatic diseases.⁴⁸ Rituximab induces depletion of mature B lymphocytes through complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC),

Table 1. Summary of case reports on systemic lupus erythematosus (SLE)-associated pancytopenia and isolated cytopenias

Authors and year of publication	Case report	Treatment	Clinical outcome
Serin et al., 2021 ³⁸	30-year-old female patient with SLE, suspected aplastic anemia/myelodysplastic syndrome (MDS) with a positive fluorescein-labeled aerolysin (FLAER) test.	Pulse methylprednisolone 1 g/day for 2 days; prednisone 20 mg; cyclosporine 2 × 50 mg.	Good response; transfusion no longer needed; patient transferred to outpatient care.
Frikha et al., 2022 ³⁹	45-year-old woman with rheumatoid arthritis, agranulocytosis in SLE.	Methylprednisolone pulses (1 g/day × 3 days); hydroxychloroquine 400 mg/day; adjuvant iron and folic acid; high-dose corticosteroids (1 mg/kg/day) × 8 weeks.	Resolution of epigastralgia, fever, diarrhea, leukopenia, and thrombocytopenia.
Sahbani et al., 2024 ⁴⁰	16-year-old boy with hemorrhagic syndrome (atypical presentation of SLE).	Dexamethasone 40 mg/day × 3 days; intravenous immunoglobulin (IVIG) 0.5 g/kg × 4 days; platelet transfusion; later hydroxychloroquine and corticosteroids; maintenance with azathioprine (AZA) and corticosteroids.	Stabilization of thrombocytopenia; long-term stability on AZA and corticosteroids.
Horino et al., 2025 ⁴¹	67-year-old woman with pancytopenia and schizophrenia (neuropsychiatric SLE in a patient with pancytopenia and chronic schizophrenia).	Oral prednisolone 40 mg/day, tapered to 5 mg/day over 2 years.	Improvement in pancytopenia, pleural effusion, and ascites; no recurrence during 2-year follow-up.
Haidar et al., 2024 ⁴²	63-year-old female patient with pancytopenia as a first presentation of late-onset SLE.	Prednisone 60 mg/day; hydroxychloroquine 200 mg/day; prednisolone taper 5 mg/week.	Improved fatigue, no fever; normalization of white blood cells, hemoglobin, and platelets; decreased erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP); 2 kg weight gain in 4 weeks.
Brar et al., 2022 ⁴³	73-year-old male patient, hydralazine-induced lupus presenting with pancytopenia.	Discontinuation of hydralazine.	Marked symptom improvement; complete resolution of night sweats, fevers, and weight loss within 2 months.
Ungprasert et al., 2016 ⁴⁴	33-year-old female patient with autoimmune myelofibrosis with pancytopenia as a presenting manifestation of SLE.	Glucocorticoids; mycophenolate mofetil.	Good response to therapy.
Guevara et al., 2023 ⁴⁵	34-year-old female patient with pancytopenia and autoimmune history.	Steroids; hydroxychloroquine.	Complete resolution of symptoms; pancytopenia returned to baseline with mild persistent anemia.
Abu Jheasha et al., 2024 ⁴⁶	22-year-old female patient with protein-losing enteropathy (rare SLE manifestation).	Pulse methylprednisolone 1 g/day; hydroxychloroquine 200 mg/day.	Clinical and laboratory improvement; stable vitals; discharged with regular rheumatology follow-up.
Ameer et al., 2024 ⁴⁷	30-year-old female patient with pancytopenia and SLE.	Mycophenolate 1,500 mg twice daily; lisinopril 30 mg/day; prednisone 10 mg/day; ongoing acute promyelocytic leukemia (APML) therapy with all-trans-retinoic acid/arsenic trioxide (ATRA/ATO).	Continuation of therapy until remission.

and induction of apoptosis, providing a biological rationale for its use in autoimmune cytopenias associated with SLE.⁴⁹

In many countries, RTX is used off-label for the treatment of primary immune cytopenias in adults. Prospective randomized studies have demonstrated response rates of approx. 50–60% in ITP⁵⁰ and up to 75% in warm AIHA.⁵¹ However, studies evaluating RTX in SLE-related cytopenias have not demonstrated a significant clinical benefit; therefore, its use in this setting remains off-label.⁴⁸

Mechanisms of action of RTX

Direct depletion of CD20-positive B lymphocytes through CDC and ADCC prevents the generation of autoantibody-producing plasma cells directed against blood

cell antigens (e.g., antiplatelet antibodies in ITP and antierythrocyte antibodies in AIHA), thereby reducing opsonization and peripheral destruction of blood cells.⁴ In cases of severe thrombocytopenia, RTX treatment stabilized platelet counts above $100 \times 10^9/\mu\text{L}$ for more than 6 months and was accompanied by the disappearance of anti-DNA antibodies⁴ (Fig. 2).

Importantly, RTX targets both immature pre-B cells in the bone marrow and mature circulating B lymphocytes. As B cells differentiate into immunoglobulin-producing plasma cells, they lose CD20 expression. Consequently, plasma cells are generally not depleted during RTX therapy, and serum immunoglobulin levels usually remain within the normal range. In addition, RTX does not affect hematopoietic stem cells (HSCs).⁵²

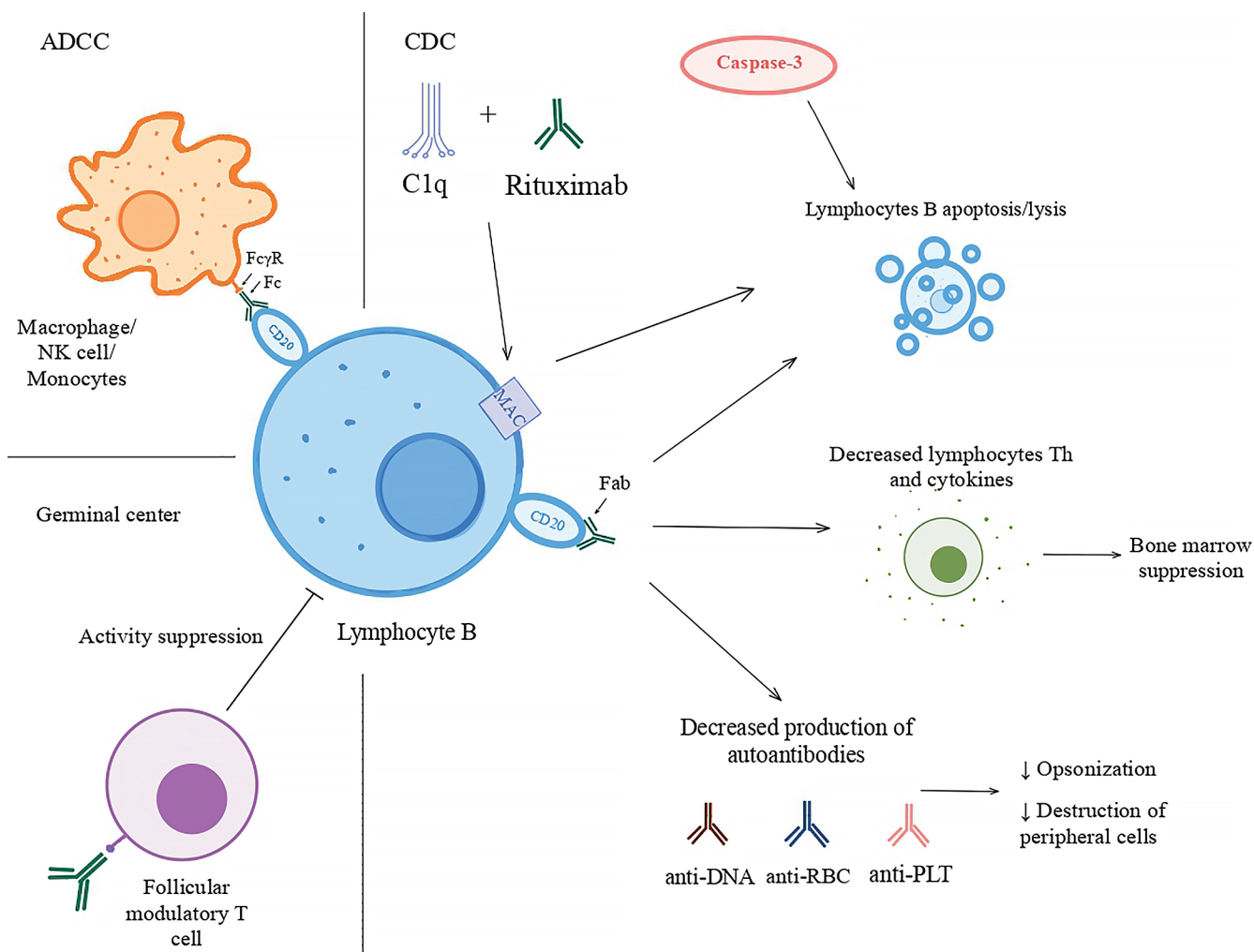


Fig. 2. Mechanism of action of rituximab (RTX)

CDC – complement-dependent cytotoxicity; ADCC – antibody-dependent cellular cytotoxicity; FcγR – Fc gamma receptor; C1q – complement component 1q; RBC – red blood cell; PLT – platelet; Tfh – T follicular helper cell; Tfr – T follicular regulatory cell.

B-cell depletion also influences the number and function of helper T lymphocytes, including T follicular helper (Tfh) cells, as well as cytokine production, which is relevant in immune-mediated bone marrow suppression.³³ Furthermore, RTX exerts complement-dependent cytotoxicity by binding C1q, leading to the formation of the membrane attack complex and subsequent lysis of CD20-positive B cells. In addition, RTX induces apoptosis through activation of the caspase-3 signaling pathway and via Fab-dependent mechanisms.⁵³ Antibody-dependent cellular cytotoxicity represents another important mechanism of RTX action. This process involves binding of Fc receptors expressed on monocytes, macrophages, and natural killer (NK) cells to the Fc region of RTX, resulting in activation of effector cells and lysis of B lymphocytes.⁵⁴ Rituximab may also modulate germinal center responses and the activity of follicular regulatory T (Tfr) cells, which suppress B-cell activation within germinal centers.⁵³

In cases associated with MAS or HLH, the therapeutic effect of RTX may result from disruption of the pathological feedback loop between autoreactive B cells and macrophage

overactivation through modulation of the cytokine network. Rituximab may be used both as monotherapy and as an adjunct to the HLH-2004 treatment protocol. Compared with etoposide, RTX has a more favorable safety profile and is generally better tolerated.⁵⁵

Efficacy of RTX in the treatment of hematological manifestations of SLE

Evidence supporting the efficacy of RTX in the treatment of hematological manifestations of SLE is derived primarily from case series, large observational cohorts, and meta-analyses. In a multicenter retrospective study by Serris et al., RTX produced a high rate of rapid responses in immune cytopenias associated with SLE. The overall initial response rate was 82%, including a complete response (CR) rate of 59%. The initial response rates were 87% for ITP, 86% for AIHA, and 50% for Evans syndrome. Although relapse occurred in 41% of patients despite an initial response, re-treatment with RTX was effective in 94% of these cases.⁵⁶

In the study by Sans-Pola et al., RTX was administered to 33 patients with extrarenal SLE, resulting in partial or complete responses. The median SLEDAI-2K score decreased from 9 to 1.5, and patients with thrombocytopenia experienced significant increases in platelet counts. Anti-double-stranded DNA (anti-dsDNA) antibody levels also declined, from a median of 64.3 to 32.7. However, the median time to relapse after the first treatment cycle was 1.6 years, and all patients required ongoing therapy to maintain remission.⁵⁷

In a multicenter retrospective cohort study including 71 adults with SLE-associated cytopenias, 86% of patients responded initially to RTX (91% with ITP, 87.5% with AIHA, and 60% with Evans syndrome), and 60.5% achieved complete remission. No cases of RTX-induced neutropenia were reported, and only 3 patients developed severe infections. Among the 24 patients (39.3%) who relapsed after an initial response, 18 underwent re-treatment with RTX, which was successful in 88.8% of cases.⁴⁹

Pediatric case reports have also described severe, treatment-refractory pancytopenia that achieved remission following RTX therapy without significant adverse events.⁵⁸

Although the placebo-controlled randomized EXPLORER (extrarenal lupus) and LUNAR (lupus nephritis) trials did not demonstrate statistically significant superiority of RTX combined with standard therapy, observational evidence suggests that carefully selected patients with hematological manifestations of SLE, including ITP, AIHA, and pancytopenia, may benefit from RTX treatment.⁵⁹

In an interventional study by Zhang et al., 8 patients with treatment-refractory SLE-associated ITP received low-dose RTX (2 infusions of 200 mg administered at 2-week intervals) together with tapering corticosteroid therapy. Overall response rates (ORRs) at 1, 3, 6, and 12 months were 25.0%, 87.5%, 75.0%, and 75.0%, respectively, suggesting that this regimen may represent a promising therapeutic approach.⁶⁰

In a retrospective cohort study by Harris et al., the ORR to RTX (56%) did not differ significantly between children (58%, $n = 24$) and adults (55%, $n = 40$). Response rates were also similar in primary and secondary ITP (53% and 62%, respectively). Evans syndrome was more common in children (42%) than in adults (18%) treated with RTX.⁶¹

Safety profile of RTX, with emphasis on patients with immune cytopenias

Rituximab is not without adverse effects. It may induce secondary hypogammaglobulinemia, thereby increasing the risk of severe infections, particularly in patients with low IgG levels before treatment or after repeated treatment courses. This complication may require IVIG replacement therapy. The risk is particularly relevant in patients with baseline pancytopenia, as this condition itself predisposes them to infections and necessitates regular monitoring of serum immunoglobulin levels.⁶²

In addition, late-onset neutropenia (LON) may occur weeks to months after RTX administration, although it is usually transient. Concomitant use of other immunosuppressive agents or previous treatment courses may increase this risk.⁶³

Infusion-related reactions may also occur; however, they are generally mild to moderate.⁵³ There are also isolated reports suggesting that RTX may exacerbate bone marrow suppression.⁶⁴

Other treatment methods in SLE patients with cytopenias

Clinical studies have demonstrated that mesenchymal stem cell (MSC) transplantation can improve outcomes in patients with SLE by enhancing regulatory T-cell (Treg) and T-helper 2 (Th2) responses while suppressing pro-inflammatory Th1, Th17, and B-cell responses, with an overall favorable safety profile. However, therapeutic efficacy remains variable, which may reflect differences in donor cell quality and recipient immune characteristics.⁶⁵

An alternative treatment for severe SLE is hematopoietic stem cell transplantation (HSCT), which may induce long-term remission but is associated with risks such as infections and conditioning-related toxicity.⁶⁶

Belimumab, a B-lymphocyte stimulator (BLyS/BAFF) inhibitor, has beneficial effects on the course of SLE, including its hematological manifestations, by suppressing excessive activation of autoreactive B lymphocytes.²⁹ Analyses of published case reports suggest that belimumab may improve platelet counts and Hb concentrations in patients with SLE-ITP and autoimmune hemolytic anemia (AIHA).⁶⁷ In addition, belimumab therapy may allow reduction of GC doses.⁶⁸ Its safety profile has been shown to be favorable across different clinical phenotypes of SLE. However, it should be noted that patients with severe, life-threatening cytopenias were excluded from the pivotal registration trials. Therefore, belimumab may be considered an adjunctive treatment option for chronic, refractory cytopenias associated with SLE, although the decision to initiate therapy should be individualized.⁶⁹

Another biologic agent that may be useful in the treatment of cytopenias in patients with SLE is anifrolumab. It is a monoclonal antibody that blocks the type I interferon receptor (IFNAR1), thereby targeting a key pathogenic pathway in SLE and reducing disease activity, including improvement of hematological parameters in some patients.⁷⁰ Post hoc analyses of clinical trials have shown that inhibition of type I interferon signaling is associated with improvements across multiple domains of disease activity, including hematological manifestations. However, no clinical studies have specifically evaluated anifrolumab for the treatment of severe cytopenias in patients with SLE.⁷¹

Limitations of the study

This review has several limitations, primarily related to the heterogeneity and limited availability of large, high-quality studies investigating pancytopenia in SLE. Much of the available evidence is derived from small case series, introducing the potential for selection and reporting bias. In addition, the retrospective design of many studies and the overlap between iatrogenic causes and disease manifestations limit causal inference. There is a clear need for prospective cohort studies and randomized clinical trials to support the development of robust diagnostic and therapeutic guidelines for the management of hematological disorders in patients with SLE.

Conclusions

Pancytopenia in SLE is a multifactorial complication with a diverse etiology. It may reflect primary autoimmune activity or arise secondary to infections, drug toxicity, bone marrow suppression, or concomitant hematological disorders such as MAS. Early recognition and a systematic diagnostic evaluation are essential for identifying the underlying cause and selecting appropriate treatment. Bone marrow examination, serological testing, and immunologic profiling are key components of the diagnostic workup and help exclude conditions that may mimic SLE-associated pancytopenia, such as hematological malignancies or aplastic anemia.

Therapeutic interventions should be directed at the underlying cause. In autoimmune-mediated pancytopenia, immunosuppressive therapy – including GCs, IVIG, and biologic agents such as RTX – may be effective. In cases related to drug toxicity or infection, supportive care and modification of the immunosuppressive regimen are required. Novel therapeutic approaches, including neonatal Fc receptor (FcRn) inhibitors and immunomodulatory agents such as iberdomide, are currently under clinical investigation and may offer promising treatment options for refractory cases.

Importantly, pancytopenia should not be regarded merely as a laboratory abnormality; it often reflects increased disease activity and an elevated risk of systemic complications. Its presence should prompt comprehensive clinical reassessment and multidisciplinary collaboration involving rheumatologists, hematologists, and infectious disease specialists.

Further research is needed to clarify the immunopathogenesis of pancytopenia in SLE and to optimize evidence-based treatment algorithms. Given its potential for rapid progression and its prognostic significance, pancytopenia should be recognized as a serious clinical manifestation requiring prompt evaluation and appropriate management within the broader context of systemic autoimmunity.

Use of AI and AI-assisted technologies

Not applicable.

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Situational burden and challenges faced by informal caregivers of post-stroke patients: A narrative review

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Abstract

Stroke is a leading cause of long-term functional disability and often requires caregiver support during recovery. Informal caregivers of post-stroke patients face multiple challenges related to medical and nursing competence, workload, and access to information. These challenges remain insufficiently explored and inadequately addressed in healthcare systems. Using predefined criteria, we searched PubMed and Google Scholar for English-language studies involving human participants and published between January 2015 and August 2025 on issues related to informal caregiving. After initial abstract screening, we selected 60 relevant publications from each database for discussion in this narrative review, using a PRISMA-based approach. The findings reveal a multidimensional caregiving burden, with nearly 90% of caregivers reporting shortages in practical knowledge and skills training. They also often face psycho-emotional and economic strain, compounded by a lack of institutional support and social isolation. Caregivers experience chronic frustration and exhaustion, leading to depressive and somatic symptoms, while also lacking adequate medical attention. Their needs are largely unsupported and unmet by available institutional support. There is a lack of systemic solutions to integrate caregiving responsibilities with personal activities and preventive healthcare. These issues necessitate urgent research and the implementation of effective supportive interventions to enhance the quality of life of post-stroke caregivers. Key recommendations include developing standardized educational programs implemented at hospital discharge, ensuring access to professional medical support, and establishing flexible respite care services to prevent caregiver burnout. Caregivers' psychosocial and somatic wellbeing is essential for the successful rehabilitation and recovery of post-stroke patients.

Key words: caregivers, quality of life, disability, stroke patients, psycho-emotional burnout

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Highlights

- Post-stroke informal caregivers experience a substantial multidimensional burden, with nearly 90% reporting inadequate training in medical and nursing care after hospital discharge.
- Caregiving is associated with significant psycho-emotional, physical, and economic strain, including chronic stress, exhaustion, depressive symptoms, and limited access to professional healthcare support.
- Current healthcare systems fail to adequately address caregivers' unmet needs, with insufficient institutional support, poor access to information, and lack of integrated preventive care solutions.
- Standardized caregiver education, professional follow-up, and flexible respite care are urgently needed to prevent caregiver burnout and improve rehabilitation outcomes for stroke survivors.

Introduction

Stroke is one of the most devastating human pathologies and a leading cause of long-term disability worldwide. Recovery outcomes are difficult to achieve and depend on variable loss of brain plasticity due to neuronal mitochondrial dysfunction following ischemia/reperfusion (I/R) episodes associated with oxidative stress and the resulting dysfunctional sensorimotor performance.¹ Maintaining an acceptable standard of living for stroke survivors is extremely difficult. Most studies show that approx. 20–30% of stroke survivors experience limitations in activities of daily living, and approx. 30% have difficulties with instrumental activities of daily living. The informal caregiving burden remains high for up to 1 year after stroke, with functional dependency on caregivers.^{2–4} Stroke survivors discharged to an inpatient rehabilitation facility have more than twice the 1-year survival rate of those discharged to nursing facilities.⁵ Proper institutional care is important, as only a fraction of low-functioning post-stroke patients survive when deprived of care.

Care supports recovery from the acute physical and emotional consequences of stroke and is essential for the long-term management of sustainable recovery. Caregivers' work is also essential for reducing the high socioeconomic burden of stroke on public healthcare systems. Nonetheless, informal caregivers face numerous problems, including insufficient knowledge of caregiving principles and limited psychological and medical support, despite demanding and exhausting working conditions and a lack of formal support from healthcare policymakers.⁶ The situation of informal caregivers of post-stroke patients is an important but under-researched area.

Objectives

Therefore, in this study, we focused on the issues that make caregivers' work difficult and hinder their efforts to support patient rehabilitation. To this end, we investigated the most recent literature on the subject in major medical databases. We aimed to provide a concise analysis

of caregivers' status, the drawbacks and deficiencies limiting their work, and the educational shortcomings they perceive. We also offer a viewpoint on preventive measures to avoid a decline in caregivers' work effectiveness and psychophysical resilience, which may adversely affect rehabilitation processes in post-stroke patients.

Materials and methods

This article is a desk review of the most relevant publications on the work-related problems encountered by caregivers of post-stroke patients and possible ways of addressing these problems. The literature was assessed by both authors of this article. We retrieved English-language articles on studies conducted in human participants aged 18 years or older and published in PubMed and Google Scholar between January 2015 and August 2025. Our search strategy in PubMed, a curated source of biomedical literature, used the Medical Subject Headings (MeSH) vocabulary. We designed the following complex search string, emphasizing the role of post-stroke caregivers: ("Disability Evaluation" AND "Stroke" AND "Caregivers") OR ("Patient Compliance" AND "Rehabilitation" AND "Stroke" AND "Caregivers") OR ("Social Support" AND "Caregivers" AND "Stroke"). We paid particular attention to publication originality, relevance to the main topic of this review, proper formulation of aims and outcomes, evidence-based findings, fact-checked data, and neutral presentation by the source authors. The screening included:

- original reports of randomized controlled trials (RCTs), meta-analyses, systematic reviews, and psychosocial studies;
- reports on work-related and health problems experienced by caregivers of post-stroke patients;
- issues hindering caregivers' efforts to care for and rehabilitate patients;
- reports on the association between treatment outcomes and complications and patient caregiving;
- caregivers' perceptions of limitations in accessing preventive healthcare measures and patient care-oriented educational resources.

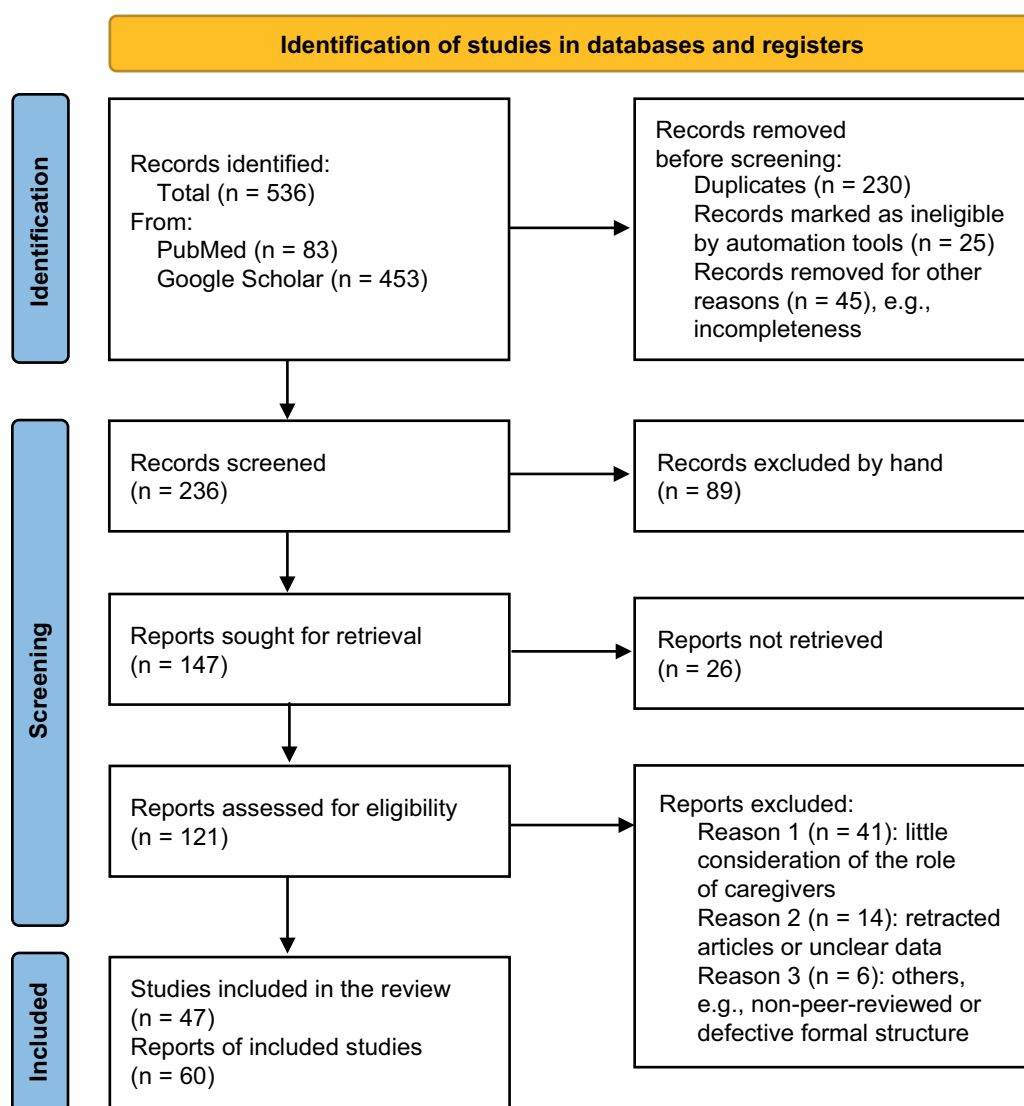


Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram showing the identification of studies from databases

Published conference proceedings, case reports, commentaries, short communications, redundant articles, promotional materials, and publications lacking controlled research or otherwise failing to meet academic standards were excluded.

We employed a multistage screening process to retrieve articles from the databases, in line with the standard sequential Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for review reporting, as shown in Fig. 1. The database search yielded a total of 536 articles: 83 in PubMed and 453 in Google Scholar. The articles were further screened for thematic relevance to the work of informal caregivers of post-stroke patients according to the pre-established criteria outlined above.

Briefly, after abstract screening, we removed duplicate records and records marked as ineligible by automated tools or for other reasons. The remaining 236 records were evaluated based on how well their titles and abstracts met the predefined substantive criteria, which led to the exclusion of 89 manually selected reports. Of the remaining

147 records, 121 were successfully retrieved for detailed eligibility assessment. Only full-text files in accessible document formats, including PDF, DOC, DOCX, ODT, and RTF, were retrieved; the remaining 26 records, which were available only as executable files or archives, such as EXE, JS, and ZIP files, were automatically discarded. The assessment selected 60 papers, covering 47 studies, for further discussion in this review.

Results and discussion

Knowledge, information, and education about stroke recovery and care

Caregivers' insufficient medical competence appears to be a significant issue. Empirical studies indicate that the vast majority of caregivers (86.7%) report a need for training in key skills, including self-care, safety supervision, and functional rehabilitation, with 62.7% identifying multiple needs.⁷ Another study showed that 87.3%

of caregivers had poor knowledge of patient positioning, which may increase the incidence of contractures and secondary complications.⁸ With regard to nutrition, 28% of caregivers did not modify food consistency for patients with dysphagia, thereby increasing the risk of aspiration and respiratory infections.^{9–11}

Pressure ulcer prevention in post-stroke patients reveals a critical vulnerability, particularly after hospital discharge, as demonstrated by the marked increase in pressure injury prevalence from 3.06% in clinical settings to 17.25% in the home environment. This significant increase is likely attributable to substantial deficits in caregiver preparedness, as 67.5% of caregivers had never received formal training in pressure ulcer prevention, and 50% had poor knowledge of appropriate management techniques.^{12–15}

Likewise, 33.8% of caregivers were not fully competent in supporting post-stroke physical activity, which may reduce the effectiveness of rehabilitation.^{16–18} They did not receive adequate training in the use of specialized aids for musculoskeletal exercises, despite their crucial role in restoring motor function.¹⁹ In addition, 87% of caregivers had poor knowledge of post-stroke patient positioning and other specific aspects of post-stroke care.^{8,16} A lack of skills in ergonomic patient transfer techniques increases the risk of musculoskeletal injuries affecting both caregivers and patients.^{20,21} Only slightly more than 20% of caregivers receive adequate institutional support, which may reduce care efficiency and quality of life for all those involved.^{22,23}

In response to these deficits, 80% of caregivers expressed a need for systematic training, including recognition of the signs and symptoms of clinical deterioration and specialized long-term care techniques.²⁴ The paucity of hands-on instruction among caregivers of post-stroke patients represents a significant therapeutic barrier in fundamental domains of long-term care. Studies show that educational instruction could increase compliance with clinical guidelines from the current 58% to more than 81%.¹⁰

Caregivers' psychosocial health problems

The overall wellbeing of informal caregivers, understood as a dynamic interplay between individuals' psycho-emotional state and their social environment, particularly the work-related environment, is profoundly affected. Empirical studies show that only a small proportion of caregivers receive systematic psychological support, even though as many as 67% of this population experience symptoms of chronic exhaustion associated with long-term caregiving responsibilities.^{25,26} Deficits in psychological interventions may increase the emotional burden, particularly when high-intensity psycho-emotional needs remain unmet.

The coordination of interdisciplinary non-hospital care also shows systemic dysfunction, with only a small proportion of caregivers receiving precise instructions on how to collaborate with community nursing or physiotherapy

staff.²⁷ As a result, the organization of necessary services is delayed, increasing the risk of both insufficient patient care and psycho-emotional complications among caregivers.^{28,29} Despite these alarming indicators, the availability of psychotherapeutic interventions remains insufficient, meaning that caregivers are often left to deal with psychological challenges on their own.

Unmet emotional needs among caregivers of post-stroke patients represent an important but underexplored clinical problem. Studies show that caregivers experience frustration in interactions with care recipients, which may manifest as communication difficulties, feelings of helplessness, and conflicting caregiving roles. Caregiving involves sacrifices in daily responsibilities and social engagement, resulting in strain, aggravation, anxiety, sleep disturbances, and loss of control.^{30,31} Moreover, the physical disability of stroke survivors takes a psycho-emotional toll on primary family caregivers.

Reciprocal changes in attitudes, reported by 53.6% of caregivers, include the emergence of affective lability with heightened emotional tension, leading to frustration that manifests as dysfunctional relationships within caregiver–patient dyads.^{32,33} Studies show that about 40% of caregivers experience anxiety or clinically evident depression.^{23,34} About 1/3 of caregivers report the regular use of psychotropic agents as a consequence of chronic exposure to stress and psycho-emotional overload, which may further increase the risk of depressive disorders and dyssomnia.^{35–37} Limited access to psychotherapeutic interventions often leads to self-medication, generating a risk of iatrogenic effects, including cognitive dysfunction.³⁸

Reduced social interaction is a significant functional problem among caregivers of post-stroke patients, affecting 60% of this population and occurring particularly among women who provide independent care.^{39,40} Impaired interpersonal functioning may manifest as withdrawal from family gatherings, cultural events, and recreational activities.³¹ Among caregivers of post-stroke patients, sex-based differences in social isolation show significant variation, with women affected more often than men, which is associated with disparities in the distribution of caregiving responsibilities and differing sociocultural expectations.^{40,41} Women are more likely to reduce or discontinue their professional activity, which intensifies social marginalization. The psychological consequence of this chronic isolation has been shown to correlate with an increased likelihood of developing depressive disorders.^{33,42} Deficits in emotional support and limitations in interpersonal relationships may induce chronic stress and lead to deterioration in quality of life.

Caregivers' health barriers

The lack of systematic assessment of caregivers' health stems partly from neglect of their self-care and respite needs and partly from limited access to preventive

healthcare and specialist resources. Half of caregivers report an increased frequency of respiratory infections, gastrointestinal dysfunction, and exacerbations of chronic conditions during the first months of caregiving. Worsening health status correlates with the accumulation of caregiving burden.^{18,43} On average, caregivers spend at least 5–8 h per day on caregiving tasks for older and ill individuals, including feeding, patient transfer, and monitoring of vital signs, which reduces their ability to meet their own biological needs.^{44,45}

Chronic physical and psychological burden intensifies health dysfunction, as long-term exposure to caregiving stress correlates with increased cortisol levels, thereby increasing the likelihood of developing hypertension.^{46,47} Despite clear somatic manifestations of exhaustion, only a small percentage of caregivers undergo systematic diagnostic and preventive procedures.^{48,49} Caregivers' neglect of preventive checkups contributes to health deterioration and decreases the likelihood of implementing effective care, thereby intensifying the burden on both caregivers and their patients.^{6,50}

Deficits in material support

Lack of material support represents a significant challenge for caregivers of post-stroke patients. Only a small percentage of caregivers gain access to in-kind support in the form of necessary medical devices, such as pressure-relieving mattresses and hygiene products, which may reduce the effectiveness of nursing procedures.^{51–53} Likewise, only a small percentage receive financial support to offset expenses related to pharmacotherapy, rehabilitation, or specialized nutritional protocols.

Deficits in material support are directly correlated with the deterioration of socioeconomic conditions. Caregivers report a reduction in household income resulting from changes in employment status or increased caregiving expenses.^{2,54} Limited access to essential equipment and financial resources intensifies the psycho-emotional burden on caregivers and increases the risk of clinical complications in patients, such as pressure ulcers and secondary infections.

Systemic problems and barriers in the healthcare system

With regard to formal support, most caregivers report a lack of understanding of administrative procedures for formal documentation of disability and subsidization of medical equipment, which may prevent them from enforcing their entitlements.⁵⁵ Only half of caregivers receive information about possible financial benefits that are essential for covering the costs of pharmacotherapy and medical and rehabilitation aids.^{22,56}

The coordination of interdisciplinary out-of-hospital care shows significant dysfunction, as only a small proportion of caregivers receive precise instructions on how

to cooperate with community care staff, which delays the implementation of necessary services during patients' transition from hospital to home care. The lack of clear procedures and communication often leads to ineffective care coordination.⁵⁷ Moreover, rest breaks and respite care are available to only about 15% of caregivers, despite 73% reporting symptoms of extreme overload.⁵⁸

Systemic dysfunction in information flow among post-stroke caregivers results in a lack of consistent guidelines for support programs. This facilitates emotional strain and the development of affective and anxiety disorders, which may, in turn, reduce the quality of care provided to post-stroke patients.⁵⁹

Limitations of the study

This study has limitations. The relevance of publications retrieved from a database is subject to inherent selection bias related to the assessors' interests, experience, and perspectives. A different perspective may lead to different interpretations of the same issue or event. To mitigate perceptual bias and improve review reporting, we detailed the selection process using the internationally recognized PRISMA flow diagram. Another limitation may be the non-exhaustive nature of the search, as it was confined to PubMed's MeSH-controlled syntax and Google Scholar, thereby omitting literature indexed in PubMed Central (PMC) and other, albeit smaller, life science repositories.

Although PubMed and Google Scholar are among the most widely used and comprehensive sources of academic literature, the scope of the literature search imposes certain constraints. The search was restricted to English-language articles, which may have introduced geographical or cultural bias and potentially omitted relevant publications indexed elsewhere. Moreover, the exclusion of grey literature, including governmental reports, clinical guidelines, and unpublished dissertations, together with the defined temporal scope of the search, may have resulted in the omission of both contemporary practical data and older foundational works in the field. We also did not conduct a formal methodological quality appraisal of the included studies, meaning that the conclusions were based on evidence of varying scientific rigor.

These limitations underscore the need for future comprehensive systematic reviews and meta-analyses to assess the prevalence and severity of caregiver burden. This requires a more inclusive search strategy encompassing multiple databases, multilingual publications, grey literature, and critical methodological scrutiny of the included research. There is a pressing need for studies that elucidate both universal and culture-specific barriers among caregivers, which is essential for developing tailored support systems. Subsequent analyses should also evaluate the efficacy of specific interventions, such as psychotherapy, respite care, and online training, and investigate the long-term health and economic consequences of post-stroke caregiving for the family unit.

Conclusions

Despite the limitations outlined above, we believe that we have demonstrated that informal caregivers of post-stroke patients are not exempt from professional burnout. Caregiver burden remains an underexplored research area. Caregivers experience high levels of psycho-emotional and physical stress, leading to depression and anxiety. They also experience social isolation and marginalization, which are exacerbated by limited access to respite care, preventive healthcare measures, and social and educational support in caregiving tasks. Understanding the factors underlying family caregivers' perceptions of sacrifice and psychosomatic strain is essential for implementing a holistic care plan.

From a practical perspective, caregivers experience knowledge gaps in specific caregiving skills, such as patient positioning to prevent and manage contractures and pressure ulcers, dietary modifications to prevent dysphagia-related complications, and safe patient transfer techniques. They also experience a significant lack of institutional and administrative support and often neglect their own health needs, with only a fraction having access to respite care. All these factors may adversely affect the quality of care and the psychosomatic health of both caregivers and patients.

Quantitative rather than merely descriptive findings are needed to precisely assess the true magnitude of caregivers' inadequate work-life balance, which often causes them to discontinue caregiving. Our study reveals gaps in research on routine challenges faced by family caregivers in their daily practice. It also highlights the need for longitudinal research focusing on caregivers' health. There is an urgent need to implement systemic solutions that integrate caregiving responsibilities with caregivers' personal activities to counteract burnout and improve wellbeing.

Use of AI and AI-assisted technologies

Not applicable.

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Postoperative nutritional interventions and recovery: Molecular mechanisms, clinical outcomes, and ERAS-based practice

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Abstract

Nutritional interventions play a key role in enhancing postoperative recovery through anti-inflammatory, immunoregulatory, and metabolic effects. However, evidence across different nutrients and surgical settings remains heterogeneous. This review aimed to summarize both the molecular mechanisms and clinical outcomes of nutritional strategies supporting recovery after surgery. Relevant studies were identified through systematic searches of the PubMed, Web of Science, and Scopus databases from January 2000 to February 2025, including randomized controlled trials (RCTs), systematic reviews, and meta-analyses focusing on enteral nutrition, parenteral nutrition, and immunonutrition. Evidence from more than 50 clinical studies indicates that enteral immunonutrition (EIN) reduces postoperative infectious complications by approx. 30% and shortens hospital stay by 2–3 days. Early enteral feeding within 24–48 h improves gastrointestinal recovery and lowers overall morbidity. Omega-3-enriched nutrition modulates inflammatory and immune responses without increasing the risk of adverse events. Emerging technologies, such as nutrigenomics and digital monitoring, further enable individualized nutritional management. Nutritional interventions represent a promising nonpharmacological approach to optimizing postoperative recovery by integrating molecular insights with clinical practice. Standardization of protocols and long-term evaluation remain priorities for advancing evidence-based, personalized nutrition within Enhanced Recovery After Surgery (ERAS)-aligned surgical care.

Key words: postoperative care, nutritional support, Enhanced Recovery After Surgery (ERAS), immunonutrition, enteral nutrition

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Highlights

- Postoperative nutrition significantly improves surgical recovery, with enteral immunonutrition (EIN) reducing surgical site infections by approx. 30% and shortening hospital stay by 2–3 days.
- Early enteral nutrition initiated within 24–48 h enhances gastrointestinal recovery, reduces postoperative morbidity, and supports implementation of Enhanced Recovery After Surgery (ERAS) protocols.
- Omega-3 fatty acid–enriched postoperative nutrition modulates inflammation and immune function without increasing adverse events, supporting safe postoperative immune regulation.
- Personalized postoperative nutrition, supported by nutrigenomics and digital nutritional monitoring, enables evidence-based nutritional management to optimize surgical recovery.

Introduction

Postoperative recovery is a critical area in modern medicine that significantly impacts patient outcomes and quality of life (QoL). However, surgery induces a series of complex physiological and metabolic changes, including an acute inflammatory response, immunosuppression, delayed tissue repair, and disorders of energy metabolism.^{1,2} These factors collectively increase the risk of postoperative complications, such as infections, poor wound healing, and muscle atrophy, thereby delaying the recovery process.^{3–5} Therefore, identifying effective interventions to promote postoperative recovery has become a key focus of clinical research. In recent years, nutritional interventions have gained attention as an area of active research due to their noninvasive nature, cost-effectiveness, and multi-target effects.^{6–8}

Studies have demonstrated that specific nutrients, such as ω -3 fatty acids, glutamine, and vitamin D, play significant roles in suppressing postoperative inflammation, enhancing immune function, and promoting tissue repair.⁹ For instance, ω -3 fatty acids alleviate inflammatory responses by regulating the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway,^{10,11} while glutamine has been shown to enhance T-cell activity and mitigate postoperative immunosuppression.¹² These molecular mechanisms provide a biological rationale for integrating nutritional strategies into perioperative management frameworks and guidelines, such as Enhanced Recovery After Surgery (ERAS) protocols and the European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines. Moreover, an optimized combination of enteral nutrition (EN) and parenteral nutrition (PN) has been shown to significantly reduce postoperative complications.¹³ Despite the increasing number of related studies, notable gaps remain in our understanding of the specific molecular mechanisms, individual nutritional requirements, and standardized clinical intervention protocols. In clinical practice, nutritional interventions have been increasingly integrated into perioperative management for various surgical populations, including patients undergoing gastrointestinal, hepatobiliary, and

orthopedic procedures. The use of nutritional support within ERAS and ESPEN guideline frameworks has demonstrated measurable benefits in reducing complications, shortening hospital stays, and improving functional recovery. These frameworks emphasize early enteral feeding, immunonutrition, and metabolic optimization as essential components of postoperative care. Incorporating these clinical perspectives provides a practical foundation for understanding how molecular mechanisms translate into real-world recovery outcomes.

At present, the role of nutritional interventions in postoperative recovery lacks a comprehensive theoretical framework. On the one hand, the influence of different types of surgery and individual patient variability on nutritional needs requires further exploration. On the other hand, the precise pathways through which nutritional interventions regulate inflammation, immunity, and tissue repair at the molecular level remain inadequately understood. Additionally, many clinical studies lack long-term follow-up data, making it difficult to evaluate the long-term effects of nutritional interventions on recovery and QoL. Therefore, there is an urgent need to systematically integrate existing research findings to clarify the specific mechanisms and optimize nutritional intervention strategies.

Objectives

This work is a comprehensive narrative review that integrates molecular and clinical evidence on perioperative nutritional interventions, with the aim of informing clinical decision-making regarding the timing of EN and PN, the role of immunonutrition, and the use of ω -3-enriched formulas. First, it explores the molecular mechanisms by which nutritional interventions modulate inflammation, immune function, and tissue repair. Second, it reviews the clinical applications of nutritional interventions across various postoperative recovery contexts. Finally, it analyzes research challenges and future directions in this field, providing a scientific basis for optimizing postoperative recovery strategies.

Materials and methods

This review was conducted as a comprehensive narrative literature review. Relevant studies were identified through systematic searches of the PubMed, Web of Science, and Scopus databases for articles published between January 2000 and February 2025. The search strategy included combinations of the following terms: “postoperative recovery,” “nutritional intervention,” “enteral nutrition,” “parenteral nutrition,” “immunonutrition,” “ ω -3 fatty acids,” and “Enhanced Recovery After Surgery.”

Original research articles, randomized controlled trials (RCTs), systematic reviews, and meta-analyses focusing on the effects of perioperative nutritional interventions on postoperative outcomes were included. Non-English-language articles, animal studies, case reports, and conference abstracts were excluded. Each study was evaluated for methodological quality and relevance to clinical practice, and the findings were synthesized narratively according to the phase of care: preoperative, postoperative, and recovery.

Molecular mechanisms of nutritional interventions in postoperative recovery

Regulation of postoperative inflammatory responses by nutritional interventions

Control of acute postoperative inflammation

Postoperative inflammation is both a physiological response to surgical trauma and a critical phase of tissue repair.¹⁴ However, excessive or prolonged inflammation may cause secondary tissue damage, delay functional recovery, and contribute to postoperative complications. Research suggests that nutritional interventions can regulate

postoperative inflammation at the molecular level, thereby mitigating its adverse effects.¹⁵ Among these interventions, ω -3 fatty acids, a group of polyunsaturated fatty acids with anti-inflammatory properties, inhibit the production of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α), by modulating the NF- κ B signaling pathway, thereby alleviating the inflammatory response. Additionally, ω -3 fatty acids can be converted into specialized lipid mediators, such as resolvins and protectins, which promote the resolution of inflammation and reduce inflammation-induced tissue damage.^{16,17} These mechanisms provide a theoretical basis for the nonpharmacological management of postoperative inflammation.

Alleviation of oxidative stress

Surgical trauma is often accompanied by increased oxidative stress, characterized by a marked increase in reactive oxygen species (ROS) production.¹⁸ Excessive ROS can compromise the integrity of cell membranes and cause oxidative damage to proteins and DNA, thereby exacerbating postoperative tissue injury and functional impairment.¹⁹ Therefore, controlling the level of oxidative stress is a critical step in promoting postoperative recovery. Antioxidants have been shown to exert significant protective effects by reducing oxidative stress. For example, vitamin C, a water-soluble antioxidant, directly scavenges ROS, prevents the propagation of free-radical chain reactions, and protects cells from oxidative damage.²⁰ Vitamin E, a fat-soluble antioxidant, is incorporated into cell membranes and stabilizes the lipid bilayer, thereby preventing lipid peroxidation.²¹ Furthermore, the synergistic action of these 2 vitamins can enhance antioxidant capacity and stabilize the postoperative tissue microenvironment. A recent study by Castillo et al.²² highlighted that the combined use of antioxidants may have a more pronounced effect on alleviating postoperative oxidative stress, offering new avenues for developing refined nutritional intervention strategies (Fig. 1).

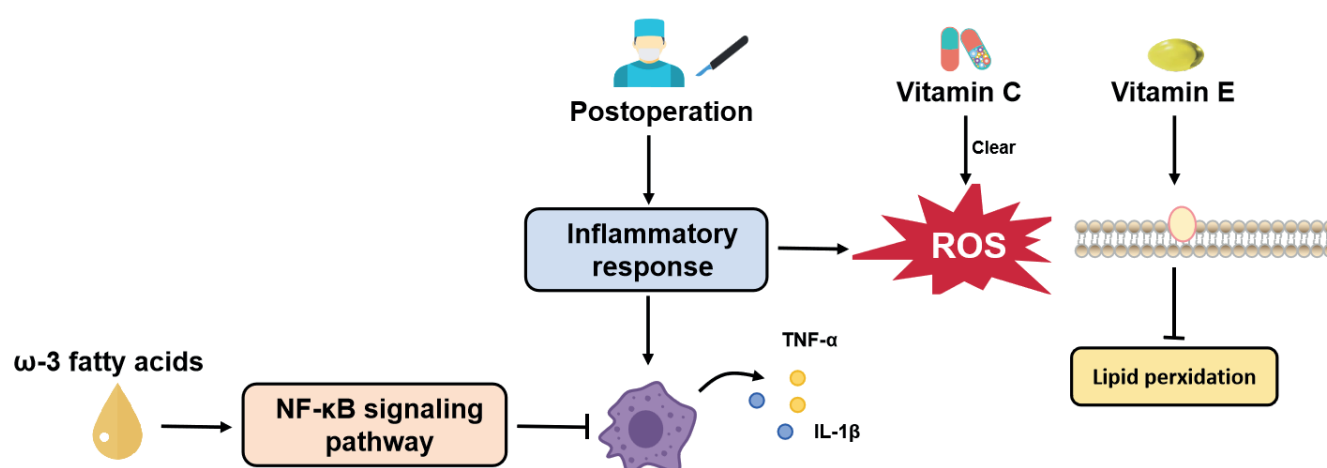


Fig. 1. Antioxidant mechanisms of nutritional interventions in postoperative recovery

Modulation of postoperative immune function by nutritional interventions improvement of postoperative immunosuppression

The physiological stress response triggered by surgery often leads to an immunosuppressive state, particularly in the early postoperative phase. This immunosuppression not only reduces the body's ability to combat infection but also increases the risk of postoperative complications, such as sepsis and wound infections. Nutritional intervention is regarded as a potentially effective strategy for addressing this issue. Glutamine, the most abundant free amino acid in the human body, plays a crucial role in maintaining normal immune function.^{23,24} Studies have shown that postoperative glutamine supplementation significantly enhances T-cell proliferation and increases the activity of natural killer (NK) cells.²⁵ These effects are particularly important during postoperative recovery, as T cells and NK cells are essential for defense against pathogenic infections. Additionally, glutamine promotes repair of the intestinal mucosal barrier, indirectly improving systemic immune function and reducing the incidence of postoperative bacterial translocation.^{26,27} These findings suggest that glutamine is a multifunctional immunonutrient with substantial benefits for postoperative recovery.

Gut microbiota regulation and immune recovery

Surgical stress and postoperative antibiotic use often disrupt the balance of the gut microbiota, resulting in a reduction in beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, and overgrowth of opportunistic pathogens.²⁸ Dysbiosis of the gut microbiota may further suppress host immune function and increase the risk of postoperative infections. Therefore, restoring gut microbiota balance through nutritional interventions is a critical strategy for promoting immune recovery after surgery. Probiotics and dietary fiber have been widely studied and used to modulate the gut microbiota. Probiotics restore immune homeostasis by competitively excluding pathogenic bacteria and modulating gut immune responses.^{29,30} Moreover, dietary fiber is fermented in the gut to produce short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate.^{31,32} Short-chain fatty acids serve as the primary energy source for intestinal epithelial cells and activate immune-related pathways, including those mediated by G-protein-coupled receptors 41 and 43 (GPR41/43), thereby promoting anti-inflammatory responses and enhancing systemic immune function.³³ For instance, butyrate enhances the function of regulatory T cells (Tregs), alleviating postoperative inflammation and ameliorating immunosuppression.³⁴ Recently, Trone et al. demonstrated

that the combined use of probiotics and dietary fiber may have a synergistic effect, thereby optimizing postoperative immune regulation strategies.³⁵ This finding provides new avenues for developing precise nutritional intervention plans for postoperative recovery.

The role of nutrition in supporting postoperative tissue repair and regeneration

Effects of proteins and amino acids on postoperative wound healing

Tissue repair after surgery is a critical component of the recovery process and is highly dependent on an adequate supply of protein and amino acids. Surgical trauma induces a hypermetabolic state characterized by increased protein degradation and a heightened demand for protein synthesis. Adequate protein intake provides essential substrates for new tissue formation, supports collagen synthesis, and promotes cell proliferation. Leucine, an essential amino acid, is particularly important for postoperative recovery. According to Holowaty et al., leucine accelerates muscle and wound repair by activating the mTOR signaling pathway, thereby promoting protein synthesis.³⁶ Additionally, leucine stimulates fibroblast activity and enhances collagen matrix production, which is vital for wound healing.³⁷ Other branched-chain amino acids (BCAAs), such as valine and isoleucine, also play supportive roles in cell repair and regulation of inflammation. Postoperative patients often experience impaired protein synthesis or excessive protein catabolism; therefore, supplementation with high-protein diets or specific amino acid formulations can effectively promote wound healing, shorten recovery time, and reduce the incidence of postoperative complications.

Promotion of angiogenesis and tissue regeneration by micronutrients

Micronutrients play a crucial role in tissue repair after surgery. Zinc and copper are essential cofactors for numerous enzymes involved in collagen cross-linking and metabolic regulation. Zinc enhances extracellular matrix (ECM) remodeling by activating metalloproteinases and promotes fibroblast and keratinocyte activity, thereby expediting wound closure.³⁸ Copper plays a unique role in angiogenesis, acting as a critical regulator of vascular endothelial growth factor (VEGF) signaling pathways, thereby improving blood supply to the wound, facilitating nutrient and oxygen delivery, and creating optimal conditions for tissue regeneration.³⁹ Vitamin C, an essential cofactor in collagen synthesis, aids in converting procollagen into mature collagen, enhancing the strength

and elasticity of wound tissue. Vitamin A regulates epithelial cell proliferation and differentiation and plays a key role in wound epithelialization. The synergistic effects of multiple micronutrients provide comprehensive support for tissue repair and angiogenesis. Clinical studies have demonstrated that postoperative supplementation with a combination of micronutrients significantly improves the quality of wound healing and reduces the risk of infection.⁴⁰ These findings provide a solid basis for developing micronutrient supplementation protocols tailored to postoperative patients. According to the ESPEN micronutrient guideline,⁴¹ the recommended supplementation ranges are as follows: zinc, 3 mg/day intravenously (i.v.; up to 12 mg/day in loss states and 30 mg/day in major burns); copper, 0.3 mg/day in PN (1 mg/day in EN; 4 mg/day i.v. in severe deficiency); vitamin C, 100 mg/day (rising to 200 mg/day in chronic oxidative stress and 2 g/day i.v. during critical illness); and vitamin E, 100 mg/day for deficiency repletion. These ranges reflect the evidence-based safety levels summarized by ESPEN and should be adjusted according to individual clinical conditions.

The role of nutritional interventions in postoperative metabolic balance

Regulation of postoperative metabolic disorders

Surgical trauma triggers a series of metabolic responses, placing patients in a stress state characterized by an increased metabolic rate, catabolism, and insulin resistance. Without timely intervention, this state can exacerbate protein degradation, muscle loss, immune dysfunction, and delayed tissue repair, thereby prolonging postoperative recovery. Nutritional interventions play a pivotal role in managing postoperative metabolic disturbances. Certain nutrients have shown promising effects on metabolic regulation after surgery. For instance, BCAAs, such as leucine, valine, and isoleucine, not only serve as direct substrates for protein synthesis but also activate the mTOR signaling pathway to reduce protein breakdown, thereby facilitating muscle and tissue repair. Furthermore, BCAAs improve insulin sensitivity, stabilize blood glucose levels, and mitigate the hypercatabolic state associated with postoperative stress. Lipid emulsions, a key component of PN, provide an alternative energy source and reduce reliance on glucose metabolism, thereby significantly improving postoperative metabolic disturbances. A study by Klek indicates that lipid emulsions containing ω -3 fatty acids exhibit anti-inflammatory properties, further promoting metabolic stability.⁴²

Optimization of postoperative energy metabolism

During postoperative recovery, patients often have increased energy requirements. However, traditional high-carbohydrate diets may lead to hyperglycemia, thereby increasing the risk of infections and other complications. Identifying efficient and metabolically favorable energy sources has become a focus of nutritional interventions. Medium-chain triglycerides (MCTs) have attracted attention because of their unique metabolic properties. Unlike long-chain fatty acids, MCTs are rapidly absorbed and metabolized directly in the liver to generate ketone bodies, thereby providing an efficient energy source for patients. Ketone bodies serve as an alternative energy substrate to glucose, offering anti-inflammatory and antioxidant benefits that help alleviate postoperative inflammation and improve cellular function. Additionally, ketone metabolism does not significantly elevate blood glucose levels, thereby helping to avoid complications associated with high-carbohydrate diets, such as hyperglycemia. Studies suggest that incorporating MCTs into postoperative nutritional regimens or using ketogenic diets to modulate energy metabolism can significantly accelerate recovery, reduce postoperative fatigue, and improve overall QoL.⁴³ These findings provide important support for the precise management of energy metabolism during postoperative recovery.

Clinical applications of nutritional interventions in postoperative practice assessment of nutritional needs

Evaluation of preoperative nutritional status

Preoperative nutritional status is a critical factor influencing postoperative recovery outcomes. Malnourished patients are at higher risk of postoperative complications, including delayed wound healing, increased infection rates, and prolonged hospital stays. Therefore, a comprehensive assessment of nutritional status before surgery is essential for designing effective nutritional intervention plans. Common parameters used to evaluate nutritional risk include body mass index (BMI), serum albumin concentration, and lymphocyte count. Patients with a BMI below 18.5 kg/m or a serum albumin concentration below 30 g/L are typically considered malnourished. Additionally, tools such as the Subjective Global Assessment (SGA) and Nutritional Risk Screening 2002 (NRS-2002) are widely used to identify patients at high nutritional risk. These tools integrate patient history, physical examination findings,

and laboratory indicators to provide an objective basis for preoperative nutritional intervention decisions. Early intervention is particularly important for patients at risk of malnutrition. In summary, preoperative nutritional support significantly improves immune function and the capacity for recovery while reducing postoperative complications.

Personalized nutritional intervention strategies

Nutritional needs during postoperative recovery vary depending on individual patient characteristics and the type of surgery performed. Personalized nutritional intervention strategies should take into account factors such as nutritional status, basal metabolic rate, the severity of surgical trauma, and the risk of postoperative complications. For patients undergoing digestive system surgery, postoperative EN is often prioritized to support recovery of gastrointestinal function, given the direct impact of such procedures on food intake and nutrient absorption. For patients unable to tolerate EN, PN is necessary to provide essential energy and nutrients. In patients undergoing cancer surgery, among whom preoperative malnutrition is often highly prevalent, high-protein, high-calorie dietary interventions are particularly critical. Personalized interventions must also align with individual metabolic characteristics. For example, diabetic patients should follow low-sugar, high-fiber diets to control blood glucose fluctuations, while patients with obesity need to meet postoperative energy demands without excessive fat intake. Emerging precision nutrition approaches, such as those based on genomic and metabolomic data, also offer new insights into postoperative nutritional management. In summary, comprehensive preoperative nutritional assessment and personalized intervention strategies are key to optimizing postoperative recovery outcomes and providing patients with more scientifically grounded and effective therapeutic support.

Nutritional interventions in preoperative, postoperative, and recovery phases

Preoperative nutritional interventions

Preoperative nutritional status is crucial for postoperative recovery. Adequate preoperative nutritional reserves enhance the body's ability to cope with surgical trauma and reduce the incidence of postoperative complications. Nutritional interventions are particularly important for malnourished patients. High-protein, high-calorie diets are typically recommended preoperatively to enhance energy reserves and the capacity for protein synthesis. Protein

intake is central to preoperative nutritional interventions, as it supports immune function and provides essential substrates for tissue repair and cell proliferation. For patients requiring additional support, specialized medical nutrition formulas, such as immune-enhancing nutritional supplements, may be introduced. These products are rich in ω -3 fatty acids, glutamine, and antioxidants, which can effectively reduce postoperative inflammation and promote immune recovery. Studies have shown that the longer high-risk patients receive preoperative nutritional support, the more pronounced the effects of the intervention.⁶ Preoperative nutritional support for 7–14 days is generally recommended to sufficiently improve nutritional status and enhance postoperative resilience.

Postoperative nutritional support

Postoperative patients have significantly increased nutritional requirements due to trauma, inflammation, and metabolic disturbances. However, in the early postoperative period, patients often face challenges such as loss of appetite and reduced digestive and absorptive capacity, making the choice of nutritional support method crucial. Enteral nutrition is generally the preferred method of postoperative nutritional support, as it not only provides sufficient energy and nutrients but also helps maintain intestinal mucosal barrier function, thereby reducing the risk of bacterial translocation and infection.⁴⁴ The earlier EN is initiated, the better the recovery outcomes for patients. For patients with temporarily impaired gastrointestinal function or those unable to tolerate EN, PN is used to meet nutritional requirements. Parenteral nutrition provides necessary energy and nutrients directly via intravenous administration; however, long-term PN use may increase the risk of infection and metabolic disturbances.⁴⁵ Therefore, transition to EN as early as possible is recommended. Modern postoperative nutritional support also emphasizes immune-enhancing nutrients, such as glutamine, arginine, and ω -3 fatty acids. These nutrients regulate inflammatory responses, enhance immune function, and promote tissue repair, thereby accelerating postoperative recovery.

Nutritional management during the recovery phase

Nutritional management during the recovery phase is crucial for sustaining postoperative recovery outcomes and improving patients' QoL. While short-term postoperative nutritional support focuses on meeting basic needs, medium- to long-term management emphasizes the optimization of dietary composition and metabolic status. During recovery, patients should gradually resume a regular diet while ensuring sufficient protein and energy intake to maintain muscle mass and support tissue repair. Diets rich in vitamins and minerals can enhance immune function and improve overall health. Additionally, targeted

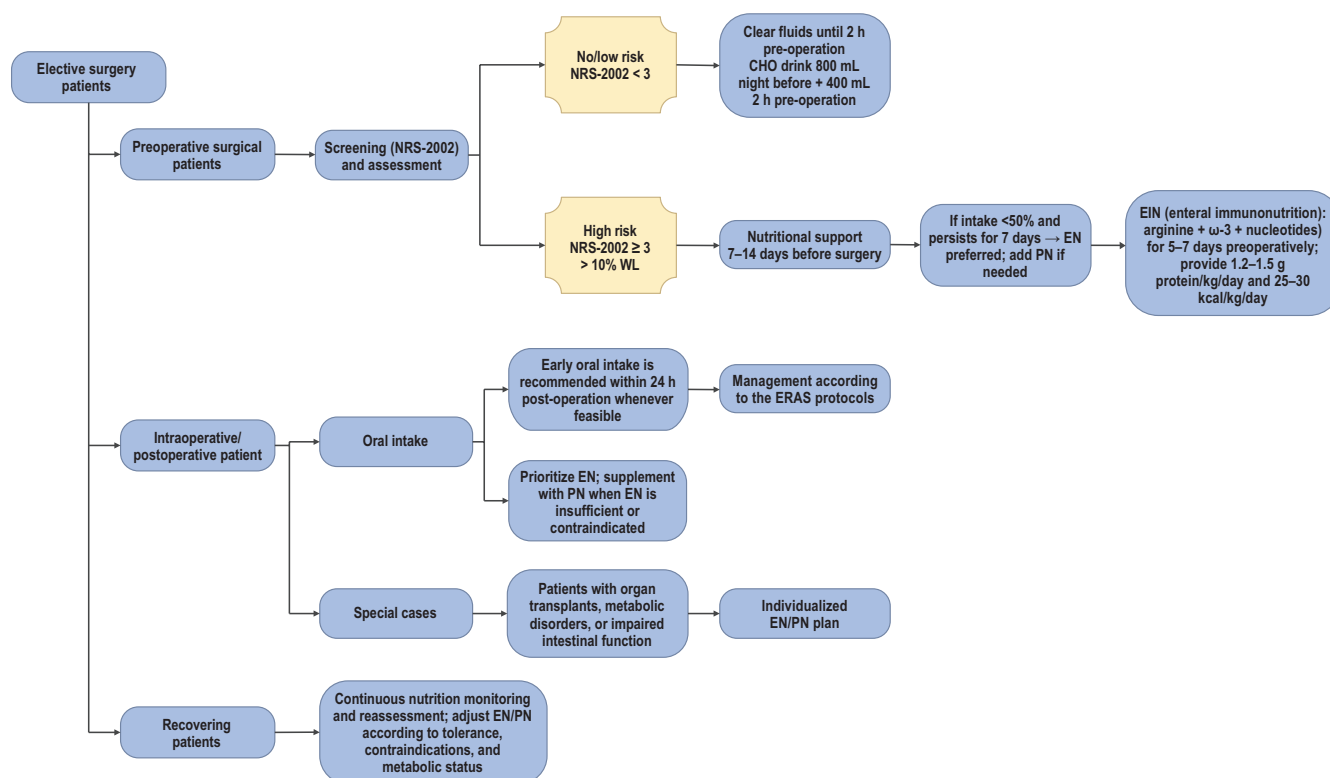


Fig. 2. Enhanced Recovery After Surgery (ERAS)-aligned perioperative nutritional management pathway

EN – enteral nutrition; PN – parenteral nutrition.

dietary supplementation is recommended to address potential postoperative nutrient deficiencies, including deficiencies in iron, zinc, and vitamin D. For postoperative patients with chronic diseases, such as diabetes or obesity, nutritional management during recovery must take their specific conditions into account and include individualized interventions. For instance, diabetic patients should strictly control carbohydrate intake, while patients with obesity should reduce total energy intake without compromising nutritional requirements. Wobith et al. highlighted that systematic nutritional management can significantly improve long-term recovery outcomes, including shorter recovery time, reduced recurrence rates, and better QoL.⁴⁶ Therefore, nutritional interventions during the recovery phase should be an integral component of postoperative care (Fig. 2).

Clinical research and efficacy analysis

Summary of clinical research findings

In recent years, clinical research into nutritional interventions for postoperative recovery has increased significantly, with substantial progress achieved, particularly through RCTs. Numerous studies have demonstrated the efficacy of various nutritional interventions in promoting postoperative recovery, providing robust evidence for

their use in clinical practice. An RCT involving patients undergoing abdominal surgery demonstrated that, compared with traditional postoperative dietary management, enteral immunonutrition (EIN) significantly reduced postoperative infection and complication rates and shortened hospital stays; however, EIN had no effect on noninfectious complications (Table 1).^{47–50} Another study found that a 2-week preoperative diet incorporating immune-enhancing formulas resulted in greater preoperative weight loss, reduced postoperative pain, and lower C-reactive protein (CRP) and liver enzyme levels compared with high-protein formulas or standard diets (Table 2).^{51–55} A prospective clinical trial reported that lipid emulsions containing ω-3 polyunsaturated fatty acids (PUFAs), administered to patients after liver resection, effectively controlled inflammation, preserved liver function, and reduced complication rates and length of hospital stay (Table 3).^{56–59} Collectively, these studies consistently support the positive impact of systematic nutritional interventions on postoperative recovery.

Efficacy evaluation metrics

Several key metrics are used to evaluate the effectiveness of nutritional interventions, including postoperative infection and complication rates. Postoperative patients often face an increased risk of infection due to reduced immune function, and nutritional interventions play a crucial role in mitigating this risk by improving immune function and reducing inflammation. For example, appropriate

Table 1. Effects of enteral immunonutrition (EIN) in gastrointestinal surgery

Population (n)	Formula (main nutrients)	Timing/duration	Main outcomes	Effect size (95% CI/p-value)	Quality/remarks	Reference
3,692 GI cancer patients (35 RCTs)	arginine + ω -3 PUFAs + nucleotides	perioperative period (5–7 days before and after surgery)	overall and infectious complications, LOS, mortality	overall RR = 0.79 ($p < 0.001$); infectious RR = 0.66 ($p < 0.001$); LOS MD = -2.36 days (95% CI: -3.76 to -0.97); no difference in non-infectious complications or mortality	high-quality meta-analysis; no increase in EN-related events	47
3,793 GI cancer patients (37 RCTs)	arginine + ω -3 PUFAs $\pm$ RNA	preoperative period (5–7 days) + post-operation (5–7 days)	infectious complications, SSI, anastomotic leak, LOS	infection OR = 0.58 (95% CI: 0.47–0.72); SSI OR = 0.65 (95% CI: 0.52–0.81); anastomotic leak OR = 0.67 (95% CI: 0.47–0.93); LOS MD = -1.94 days (95% CI: -3.00 to -0.87)	significant benefit with pre-/peri-operative IMN; no increase in adverse events	48
1,269 patients (13 RCTs)	arginine + RNA + ω -3 PUFAs	perioperative period (5–7 days before and after surgery)	infectious complications, LOS, immune function	infection OR = 0.41 ($p < 0.001$); LOS WMD = -3.48 ($p < 0.001$); $\uparrow$ CD4 (WMD = 11.39, $p < 0.001$); $\downarrow$ IL-6 (WMD = -201.83, $p < 0.001$)	no difference in mortality; improved immune parameters; no serious adverse events	49
705 hepatectomy patients (9 RCTs)	arginine + ω -3 PUFAs + nucleotides	postoperative period (7 days)	liver function, inflammatory markers, immune parameters, GI recovery	first flatus MD = -14.60 h (95% CI: -16.06 to -13.15, $p < 0.001$); ALT MD = -22.26 (95% CI: -28.42 to -16.10); IL-6 MD = -46.29 (95% CI: -50.24 to -42.34); IgG MD = 1.52 (95% CI: 1.22–1.82)	safe and effective; improved liver function, reduced inflammation, and enhanced immune recovery	50

EIN – enteral immunonutrition; GI – gastrointestinal; RCTs – randomized controlled trials; PUFAs – polyunsaturated fatty acids; RNA – ribonucleic acid; LOS – length of hospital stay; RR – relative risk; OR – odds ratio; MD – mean difference; WMD – weighted mean difference; 95% CI – 95% confidence interval; EN – enteral nutrition; IMN – immunonutrition; SSI – surgical site infection; CD4 – cluster of differentiation 4 (CD4⁺ T lymphocytes); IL-6 – interleukin-6; ALT – alanine aminotransferase; IgG – immunoglobulin G.

Table 2. Early enteral nutrition (EEN) across different surgical procedures

Timing of EEN and comparator	Main outcomes	Adverse events / safety	References
EEN $\leq$ 24 h vs delayed feeding $>$ 48 h	infectious complications reduced (RR = 0.50, 95% CI: 0.38–0.67; $p < 0.01$); earlier first bowel movement (MD = -4.10 h, 95% CI: -5.38 to -2.82)	improved albumin; no increase in leak or mortality	51
Early supplemental PN (E-SPN $\leq$ 24–48 h) vs EN, L-SPN, or TPN	reduced overall adverse events (OR = 3.02, 95% CI: 1.81–5.02); infectious events (OR = 3.12, 95% CI: 1.59–6.15); non-infectious events (OR = 2.47, 95% CI: 1.14–5.34); shorter time to resumption of flatus (SMD = 0.98, 95% CI: 0.59–1.36)	no safety concerns reported	52
EN $\leq$ 24 h vs standard management $\geq$ 72 h	major complications 19.2% vs 10.2% ($p = 0.030$); infectious events ($p = 0.047$); shorter LOS ($p < 0.001$)	safe and effective; reduced ICU mortality ($p = 0.018$)	53
EEN (immunonutrition $\leq$ 24 h) vs standard diet	infectious events 7.4% vs 20% ($p < 0.05$); anastomotic leak 3.7% vs 7.3% ($p < 0.05$); LOS 12.7 $\pm$ 2.3 vs 15.9 $\pm$ 3.4 days ($p = 0.029$)	improved cellular immunity; no difference in mortality	54
EN $\leq$ 48 h vs $>$ 48 h	28-day mortality 25.5% vs 50.0% ($p < 0.001$); mechanical ventilation 66.5% vs 80.6% ($p = 0.007$); ICU LOS 13.07 $\pm$ 16.44 vs 16.23 $\pm$ 13.57 days ($p < 0.001$); surgical subgroup: HR = 1.85 (95% CI: 1.02–3.35, $p = 0.043$)	early feeding lowered mortality and shortened ICU stay; no safety issues identified	55

EEN – early enteral nutrition; EN – enteral nutrition; PN – parenteral nutrition; E-SPN – early supplemental parenteral nutrition; L-SPN – late supplemental parenteral nutrition; TPN – total parenteral nutrition; RR – relative risk; OR – odds ratio; MD – mean difference; SMD – standardized mean difference; 95% CI – 95% confidence interval; LOS – length of hospital stay; ICU – intensive care unit; HR – hazard ratio.

supplementation with immune-enhancing nutrients, such as glutamine and ω -3 fatty acids, significantly reduces the incidence of surgical site infections and pneumonia, as well as severe complications, such as sepsis. Therefore, nutritional interventions are an indispensable adjunctive strategy for reducing postoperative infection rates.

In addition to infection rates, the duration of hospital stay and healthcare costs are important parameters for assessing the impact of nutritional interventions. The length of hospital stay is closely related to the rate of postoperative

recovery, and appropriate postoperative nutritional support has been shown to significantly shorten hospitalization. For instance, in patients undergoing surgery for gastrointestinal malignancies, early initiation of EN improved recovery efficiency, reduced postoperative complications, and decreased healthcare resource utilization.⁵⁴ Furthermore, such interventions can lower overall medical expenses, underscoring their value from an economic perspective. Nutritional interventions therefore help balance improved recovery efficiency with reduced financial burden.

Table 3. Clinical effects of ω -3 fatty acid–enriched EN or PN in postoperative and critically ill patients

Population (n)	Intervention (ω -3 dose and route)	Control	Main outcomes	Key results (effect size/p-value)	Safety	References
Gastric cancer patients undergoing Roux-en-Y reconstruction (n = 210; retrospective cohort)	PN with ω -3 fish oil emulsion 0.2 g/kg/day	standard PN	incidence of DGE; LOS; symptom score	DGE significantly lower (4.3% vs 15.5%, $p = 0.005$); shorter LOS (10 vs 13 days, $p < 0.001$); ω -3 an independent protective factor (OR = 0.32, 95% CI: 0.11–0.96, $p = 0.042$)	no adverse events reported	56
Gastric cancer patients after radical gastrectomy (n = 120, RCT)	EN + PN enriched with n-3 PUFA (postoperative days 1–5)	EN + PN without n-3 PUFA	immune and inflammatory markers (LYM, CD3 ⁺ , CD4 ⁺ , CD8 ⁺ , CD4 ⁺ /CD8 ⁺ , CRP, IL-6, TNF- α); nutritional status; complications	immune and nutritional indices $\uparrow$; CRP, IL-6, TNF- α $\downarrow$ ($p < 0.05$); complication rate 7% vs 20%; earlier first flatus / defecation	well tolerated; no differences in readmission or reoperation	57
Liver transplant recipients (n = 98, RCT)	PN containing ω -3 PUFA for 7 days post-operation	standard PN or oral diet	ALT, prealbumin (PAB), infection rate, LOS	ALT and PAB significantly improved ($p < 0.05$); infectious morbidity reduced; LOS shortened compared with PN group	no serious adverse effects	58
Mechanically ventilated ICU patients (n = 100, RCT, double-blind)	combined EN + supplemental PN enriched with ω -3 PUFA for ≤ 28 days	EN + PN without ω -3 PUFA	change in PaO ₂ /FiO ₂ (from day 1 to day 4); ICU outcomes; catecholamine-free days	no difference in PaO ₂ /FiO ₂ (-1.3 ± 83.7 vs 13.3 ± 86.1 , $p = 0.780$); more catecholamine-free days (~ 4 days, $p = 0.048$); higher EN tolerance at day 6 (51.0% vs 29.8%, $p = 0.034$)	safe; no ω -3-related adverse events	59

PN – parenteral nutrition; EN – enteral nutrition; PUFA – polyunsaturated fatty acid; DGE – delayed gastric emptying; LOS – length of hospital stay; OR – odds ratio; 95% CI – 95% confidence interval; RCT – randomized controlled trial; LYM – lymphocyte count; CD3⁺ – cluster of differentiation 3-positive T lymphocytes; CD4⁺ – cluster of differentiation 4-positive T lymphocytes; CD8⁺ – cluster of differentiation 8-positive T lymphocytes; CRP – C-reactive protein; IL-6 – interleukin-6; TNF- α – tumor necrosis factor alpha; ALT – alanine aminotransferase; PAB – prealbumin; ICU – intensive care unit.

Functional recovery metrics reflect improvements in patients' overall physical performance after surgery. These metrics include muscle strength, mobility, and the ability to perform activities of daily living, which are often key goals of postoperative recovery. Surgical trauma and postoperative inflammation can increase protein catabolism, whereas nutritional interventions that support protein synthesis and tissue repair can accelerate functional recovery. For example, adequate protein and amino acid supplementation in the early postoperative phase significantly improved muscle strength, which is critical for long-term recovery and improved QoL.⁶⁰

Finally, QoL scores serve as a comprehensive measure of postoperative recovery outcomes. These scores assess not only physical function but also psychological well-being and social participation, with commonly used tools including the 36-Item Short Form Health Survey (SF-36) and the EuroQol 5-Dimension Questionnaire (EQ-5D). Comprehensive nutritional interventions combining protein, micronutrients, and immune-enhancing nutrients significantly improve QoL, particularly by enhancing psychological wellbeing and restoring social engagement. These effects extend beyond the early recovery phase and can profoundly influence long-term patient outcomes. Thus, QoL scores are an indispensable component when evaluating the effectiveness of nutritional interventions.

Integrating these evaluation metrics allows clinical research to comprehensively reflect the practical effects

of nutritional interventions across multiple dimensions. This approach not only provides a scientific foundation for developing more precise and effective intervention plans but also offers new directions for optimizing postoperative nutritional support strategies.

Technologies and emerging trends in nutritional interventions

Nutrigenomics and personalized nutritional interventions

With the rapid development of omics technologies, nutrigenomics offers new tools for exploring personalized nutritional interventions. By integrating data from genomics, transcriptomics, metabolomics, and microbiomics, nutrigenomics reveals interindividual differences in nutrient requirements and metabolic responses, thereby enabling precision nutritional strategies. For instance, genomic data can identify genetic variations in key metabolic pathways and predict an individual's capacity to metabolize specific nutrients. Metabolomics enables real-time monitoring of postoperative patients' metabolic status, providing dynamic feedback for optimizing nutritional plans. Research indicates substantial potential for precision nutritional interventions in postoperative recovery. For example,

supplementation with specific micronutrients, such as folic acid or vitamin D, in patients with certain genetic variants can effectively improve postoperative immune and metabolic function.⁶¹ Additionally, dietary interventions targeting the gut microbiota, informed by microbiomics data, have been shown to significantly alleviate postoperative inflammation and improve immune function.^{62,63} These findings highlight that nutrigenomics provides a solid scientific foundation for developing personalized nutritional intervention strategies.

Development of novel nutritional formulations

Traditional postoperative nutritional support has primarily focused on meeting basic energy and nutrient requirements. However, recent advances in novel nutritional formulations have emphasized functionality and specificity. For instance, immune-enhancing formulas enriched with glutamine, arginine, and ω -3 fatty acids can effectively lower the risk of postoperative complications by modulating inflammatory responses and enhancing immune function. Nutritional formulations containing antioxidants, such as vitamins C and E, are intended to reduce oxidative damage, thereby promoting tissue repair. Functional foods and supplements derived from natural products are emerging as new areas of focus in postoperative recovery. For example, plant-derived polyphenols, which exhibit anti-inflammatory and antioxidant properties,⁶⁴ have been used to mitigate postoperative inflammatory responses. Similarly, the combined use of probiotics and prebiotics can optimize the balance of the gut microbiota, further enhancing postoperative immune recovery and metabolic regulation.⁶⁵ In the future, the development of novel nutritional formulations will increasingly focus on personalized approaches tailored to specific types of surgery and patient characteristics.

Digital technology in nutritional management

Digital technologies are revolutionizing postoperative nutritional management by enabling real-time monitoring and targeted interventions through wearable devices and artificial intelligence (AI). Wearable devices continuously monitor physiological parameters, such as body weight, temperature, heart rate, and activity levels, providing important reference data for adjusting nutritional support during recovery. For example, changes in body weight and fluid status can be used to assess hydration and energy balance, helping optimize nutritional support plans. AI technologies use big-data analytics to predict postoperative nutritional requirements and assess the risk of potential complications. AI models based on pre- and postoperative data can generate personalized nutritional recommendations, including energy intake, macronutrient ratios, and specific micronutrient supplementation.

Furthermore, smartphone applications and telemedicine platforms facilitate seamless communication between patients and healthcare teams, enhancing adherence to nutritional interventions and improving their effectiveness. As digital technologies continue to evolve, integrated nutritional management systems combining AI, the Internet of Things (IoT), and smart sensors are expected to be applied more widely in postoperative recovery. This technology-driven approach not only improves the precision and efficiency of interventions but also provides patients with higher-quality, personalized care.

Current status and challenges

Limitations of molecular mechanism research

Despite significant progress in understanding the molecular mechanisms underlying nutritional interventions, many unresolved issues remain. Current research primarily focuses on the effects of individual nutrients, such as glutamine or ω -3 fatty acids, with limited exploration of interactions among multiple nutrients and their combined impact on postoperative recovery. Additionally, most studies rely on *in vitro* experiments or animal models, leaving the applicability of their results to postoperative recovery in humans uncertain. For instance, the influence of different types of surgery and interindividual variability on molecular pathways remains unclear, hindering the translation of research findings into clinical practice. Moreover, studies at the molecular level often overlook the complex relationship between nutritional interventions and patients' overall physiological status. Postoperative inflammation and immune regulation involve intricate interactions among multiple signaling pathways, and the ways in which nutrients dynamically modulate these pathways to promote recovery remain inadequately understood. Future research should adopt systems biology approaches and integrate multi-omics technologies, such as metabolomics, proteomics, and transcriptomics, to comprehensively elucidate the mechanisms of nutritional interventions.

Challenges in clinical practice

In clinical practice, balancing the need for standardized nutritional intervention protocols with the demand for individualized care remains a major challenge. Although unified clinical nutrition guidelines, such as the ESPEN Practical Guideline for Clinical Nutrition in Surgery,⁴¹ are available, variation in their adoption and adherence across institutions remains a major obstacle. This lack of standardization not only limits the comparability of research outcomes but also reduces the efficiency of clinical implementation. On the other hand, postoperative nutritional needs vary significantly among individuals due to factors

such as age, basal metabolic status, and type of surgery, which influence specific nutrient requirements. Achieving personalized nutritional support within a standardized framework remains an unresolved issue.

Additionally, adherence to postoperative nutritional interventions poses a challenge in clinical settings. Some patients may find it difficult to fully comply with nutritional plans because of postoperative discomfort, psychological stress, or financial constraints, thereby limiting the effectiveness of interventions. Future clinical studies should focus on strategies to improve adherence and satisfaction, such as psychological support, digital technologies, and telemedicine.

Moreover, the quality and applicability of existing evidence vary across types of surgery. Parenteral nutrition may increase the risk of catheter-related infection and metabolic complications, while excessive micronutrient supplementation, such as zinc, copper, or antioxidants, can cause toxicity. The optimal dose and duration of immunonutrition remain uncertain. Larger, well-designed trials are needed to improve the quality of evidence and guide safe, standardized practice.

Future research directions

Integration of multidisciplinary research strategies

Research on nutritional interventions in postoperative recovery should emphasize multidisciplinary collaboration and integration, particularly by bridging basic research and clinical application. Multi-omics technologies, such as genomics, metabolomics, and microbiomics, offer powerful tools for analyzing the dynamic effects of nutritional interventions. For example, metabolomics can help identify metabolic changes in postoperative patients and clarify which nutrients play key roles in repair and recovery, while microbiomics can reveal how the gut microbiota regulate immune function and support strategies involving probiotics and dietary fiber. Systems biology methods can integrate these omics data to construct comprehensive models of nutritional metabolic networks, providing insights into the multilayered regulatory effects of nutritional interventions at the molecular level. Such approaches not only clarify complex mechanisms but also provide theoretical support for developing precise and individualized intervention strategies. Future research should also focus on dynamically monitoring postoperative metabolic states and integrating omics technologies to optimize intervention strategies, thereby enabling real-time, precision nutritional support.

Strengthening cross-disciplinary collaboration

Advancing the research and application of nutritional interventions requires combining expertise from clinical

nutrition, surgery, immunology, and data science to develop innovative and effective strategies. Surgery provides the clinical context and identifies the need for nutritional interventions, while clinical nutrition enables the design of scientifically sound dietary and nutritional support plans based on postoperative conditions. Immunology research sheds light on how specific nutrients regulate inflammation and immune responses, offering new insights into the prevention of postoperative infections and complications. Furthermore, data science and AI play critical roles in optimizing intervention plans. Nutritional models based on big-data analytics can predict postoperative recovery trajectories in different patients and generate personalized nutritional recommendations. For example, AI algorithms can dynamically adjust the energy and nutrient composition of nutritional formulas based on pre- and postoperative physiological parameters, thereby improving the precision of interventions. Future research should emphasize cross-disciplinary collaboration and the joint development of digital nutrition management platforms that enhance patient adherence and improve the overall effectiveness of postoperative interventions.

Limitations of the study

This review has several limitations. The included studies vary in terms of types of surgery, nutritional protocols, and outcome measures, which introduces heterogeneity and limits direct comparability. Some sections rely on mechanistic or observational data rather than randomized evidence, and no quantitative meta-analysis was conducted. These factors should be considered when interpreting the findings. Future large-scale, standardized RCTs are needed to validate the conclusions and strengthen the evidence base.

Conclusions

Nutritional interventions play an important role in postoperative recovery by reducing inflammation, enhancing immune function, promoting tissue repair, and optimizing metabolic balance. Existing studies suggest that nutritional interventions may help shorten recovery time, reduce complications, and improve patients' QoL. However, current research on nutritional interventions has several limitations, including an incomplete understanding of molecular mechanisms, a lack of standardized intervention protocols, and insufficient integration of individualized needs into clinical practice. Further high-quality, multidisciplinary research is needed to address these gaps. By integrating cutting-edge technologies, such as nutrigenomics, metabolomics, and microbiomics, future studies will be able to reveal the mechanisms of nutritional interventions more comprehensively and provide scientific evidence for precision nutritional support. In clinical practice, developing

more systematic and standardized postoperative nutritional management guidelines, together with individualized plans, will help optimize patient outcomes. This approach not only enhances the effectiveness of interventions but also advances the field of postoperative recovery, benefiting a broader range of patients. In summary, nutritional interventions represent a promising strategy for supporting postoperative recovery. Continued research and implementation will further improve patient outcomes and promote integration between medicine and nutritional science.

Use of AI and AI-assisted technologies

Not applicable.

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SALSA (Health, Nutrition and Sociability): Community networks for healthy eating, autonomy, and social interaction: Study protocol for a clinical randomized trial

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Conflict of interest

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Abstract

Loneliness is a highly relevant social determinant of health associated with depression, anxiety, reduced quality of life, malnutrition, cognitive decline, and increased morbidity and mortality. Social prescribing initiatives that connect individuals with community-based activities show potential to improve emotional and social wellbeing, although evidence, particularly regarding shared-meal programs, remains limited and inconsistent. This study aims to evaluate a community-based intervention consisting of group meals in restaurants, complemented by monthly healthy eating workshops, to improve emotional, nutritional, and social outcomes among adults experiencing loneliness and symptoms of anxiety and/or depression. We hypothesize that participation in restaurant-based group meals will significantly enhance emotional wellbeing, social support, and diet quality compared with usual care. A randomized controlled trial will be conducted with 2 groups, intervention and control, and a total of 98 participants aged ≥ 18 years who report loneliness and mild-to-moderate anxiety and/or depression. The intervention group will attend group lunches twice weekly for 4 months in local restaurants, accompanied by a facilitator and supported by monthly healthy eating workshops. The control group will receive usual care. Data collected will include sociodemographic, clinical, and emotional variables (BDI-II, GAD-7), social support (DUKE-UNC-11), quality of life (EQ-5D), adherence to the Mediterranean diet, and healthcare resource use. Analyses will compare pre–post and between-group changes, with logistic regression used to identify predictors of clinical remission.

This innovative intervention leverages existing community infrastructure, namely restaurants, as spaces for social and nutritional support, strengthening local networks while promoting equity and sustainability. It may enhance emotional wellbeing, diet quality, and social connectedness and potentially reduce healthcare utilization. Implementation challenges include adherence, restaurant adaptation, financial sustainability, and interinstitutional coordination. The SALSA (Health, Nutrition and Sociability) trial will provide rigorous evidence on the effectiveness of a social prescribing model based on shared meals. Its results could inform scalable and sustainable strategies to reduce loneliness, improve wellbeing, and promote healthier, more connected communities.

Key words: depression, malnutrition, anxiety, older adults, loneliness

Highlights

- The SALSA trial evaluates restaurant-based group meals to reduce loneliness and improve wellbeing.
- A randomized controlled trial will assess emotional, nutritional, and social outcomes in 98 adults.
- Shared meals and healthy eating workshops aim to improve social support and Mediterranean diet adherence.
- Findings may inform scalable social prescribing strategies for healthier, more connected communities.

Introduction

Social relationships are a key determinant of health and wellbeing. Their absence has been identified as a risk factor for physical and mental health outcomes comparable to factors such as smoking, physical inactivity, and obesity.^{1–3} In this context, loneliness is understood as the perceived discrepancy between existing and desired social relationships.⁴ In addition, it has been associated with a wide range of health problems, including depression,^{5–7} anxiety,⁵ poorer quality of life,⁸ risk of malnutrition,^{9,10} cognitive impairment and dementia,¹¹ increased morbidity and premature mortality,^{12,13} and greater use of health services.¹⁴

Systematic reviews and meta-analyses^{15,16} conclude that loneliness affects a considerable proportion of the population, especially older adults, with prevalence estimated at around 1 in 4 in high-income countries. However, its prevalence varies greatly by region, being lowest in northern Europe and highest in southern and eastern Europe. Loneliness has been recognized as an emerging public health problem with a clear impact on mental and physical health.^{17,18}

Faced with this reality, addressing loneliness has become a political imperative in several countries. A prominent example is the UK, which implemented a pioneering national strategy in 2018: *A Connected Society: A Strategy for Tackling Loneliness*.¹⁹ This line of action represents a turning point in public policy, as it was the first national strategy in the world explicitly aimed at combating chronic loneliness as a health and social cohesion problem. Among its areas of intervention is social prescribing, through which healthcare professionals can refer patients to community activities such as urban gardening, reading groups, arts activities, or shared meals. The British strategy is based on a preventive, community-oriented approach involving locally based solutions and promotes coordinated responses across the political, social, and health sectors. Thus, it is presented as an international reference model that is flexible and replicable elsewhere.

Social prescribing programs are a viable way to link primary care with nonmedical community resources in order to improve users' wellbeing.^{20,21} This intervention strategy is especially important for older people, who often have unmet social needs that affect their health, resulting in loneliness and social isolation. The systematic review and meta-analysis by Paquet et al.,²² which included 24 studies with 10,739 participants, concluded that social

prescribing interventions may have a modest beneficial effect on reducing loneliness in older adults. However, the wide methodological heterogeneity and limited quality of the studies make it difficult to draw firm conclusions.

Furthermore, Middleton et al.²³ reviewed 36 studies of community shared-meal programs and concluded that these programs have the potential to generate significant benefits for the physical, emotional, and social health of older people living in the community. In addition to improving nutrition and reducing the risk of malnutrition, they promote social inclusion and psychological wellbeing. However, the review identified a lack of longitudinal research and robust evaluations to confirm the causal relationship between participation in these programs and the observed outcomes. It also highlighted the need for more studies addressing cultural and socioeconomic diversity, as well as gender differences and equity in geographic access.

Despite the promising potential of social prescribing and shared-meal interventions, several operational challenges must be acknowledged from the outset. Maintaining participant adherence can be particularly difficult among individuals experiencing anxiety, depression, or limited social support, who may struggle to attend regularly despite the anticipated benefits. In addition, restaurants may need to adjust schedules, menus, and physical spaces to accommodate participants' needs, requiring flexibility and, in some cases, specific training. Ensuring financial sustainability and establishing effective coordination among social services, healthcare providers, community organizations, and restaurant owners are also essential considerations for successful implementation and long-term scalability.

Given this context, we propose a clinical trial to evaluate the effectiveness of a social prescribing program aimed at people who experience loneliness, have limited social support, or present with symptoms of anxiety and/or depression. The intervention consists of participation in group meals in a restaurant 2 days a week for 4 months, with the aim of promoting social interaction and improving emotional wellbeing, quality of life, and social support.

Ethics approval and consent to participate

This study complies with the principles of Good Clinical Practice, the Declaration of Helsinki of the World Medical

Association, as amended at the 75th General Assembly in Helsinki, Finland, in October 2024, and current legislation on data protection and research involving human participants. The study has been evaluated and approved by the Ethics Committee of the Jordi Gol i Gurina University Institute for Primary Care Research Foundation (Manresa, Spain; IDIAPJGol), under registration No. 23/285-P. Participants will receive verbal and written information about the study. If they agree to participate, they will be asked to sign an informed consent form voluntarily.

Data confidentiality is guaranteed in accordance with Regulation (EU) 2016/679 (GDPR) and Organic Law 3/2018 on the protection of personal data and the guarantee of digital rights. Personal data will be coded and accessible only to the lead investigator and authorized personnel. No data will be transferred to third parties or internationally, and the data will be retained for 5 years for verification and scientific dissemination purposes.

The Catalan Health Institute (ICS) acts as the data controller and guarantees the security and confidentiality of the stored data. Paper questionnaires and informed consent forms will be stored in locked cabinets at the Research Support Unit (USR) of the ICS in Central Catalonia.

Monitoring

No independent Data Monitoring Committee (DMC) is planned, given the minimal-risk nature of the intervention. Trial conduct, recruitment, adherence, and adverse events will be reviewed monthly by the principal investigator and the research team. No interim efficacy analyses are planned.

Protocol amendments and post-trial care

Substantial protocol amendments will be communicated to the IDIAPJGol Ethics Committee and the funder and will be updated on ClinicalTrials.gov within 30 days. Participants will continue to receive routine healthcare after study completion. If any harm related to participation occurs, compensation will be provided in accordance with institutional policies and national legislation.

Open science and dissemination

The full protocol and statistical analysis plan will be made publicly available on ClinicalTrials.gov (NCT06800027) and in the institutional repository of IDIAPJGol once recruitment begins. De-identified participant data and statistical code will be available upon reasonable request. After publication of the main results, anonymized data and a data dictionary will be deposited in an open-access repository in accordance with FAIR (findable, accessible, interoperable, and reusable) principles. Study results will be communicated to participants, professionals, and

the public through plain-language summaries, community presentations, and open-access publications. Results will be reported in the ClinicalTrials.gov results section within 12 months of study completion.

Objectives

Main aim: To evaluate whether attending group lunches in a restaurant 2 days a week, accompanied by a facilitator and combined with participation in healthy eating workshops, improves the emotional wellbeing, nutritional status, social support, and quality of life of people who experience loneliness, have limited social support, or present with symptoms of anxiety and/or depression. Secondary aims: To analyze the impact of the intervention on healthcare resource use by evaluating possible changes in the frequency of medical visits and the prescription of psychotropic drugs.

Materials and methods

Design

Randomized clinical trial with 2 groups (control and intervention).

Context of the study

Study population: Adults aged >18 years assigned to the basic health area (ABS) of Sant Joan de Vilatorrada (12,721 assigned individuals), a semi-urban area in Central Catalonia, Spain.

Inclusion criteria: Independent individuals with problems related to living alone or feeling lonely, defined by code Z60 according to the International Classification of Diseases, 10th Revision (ICD-10), including older people, migrants, people with functional diversity, and individuals at risk of malnutrition and/or emotional disorders. Additional inclusion criteria are a score ≥ 14 and < 28 on the Beck Depression Inventory-II (BDI-II), or a score ≥ 10 on the Generalized Anxiety Disorder-7 scale (GAD-7), or a score < 32 on the DUKE-UNC-11 Social Support Scale; the possibility of follow-up for 12 months; the ability to read and write in Spanish or Catalan; and the ability to attend the restaurant 2 days a week.

Exclusion criteria: A diagnosis of dementia or moderate cognitive impairment; a diagnosis of major depression, defined as a BDI-II score ≥ 28 ; problematic consumption or abuse of alcohol or other psychoactive substances; physical or mental impairment preventing attendance at the restaurant 2 days a week; participation in other social-service food support programs; not being responsible for managing one's own food, including institutionalized people or people in comprehensive assisted living; and not signing the informed consent form.

Intervention

The intervention group will attend lunch at a restaurant, selected in advance and with previously trained staff, 2 days a week for 4 months. A facilitator will accompany participants to the restaurant and supervise them. Once a month, a workshop on healthy habits and meal management will be held at the town's senior citizens' center. Participants will not be required to pay anything, either for attending lunch at the restaurant or for participating in the workshops; all costs will be covered by the funding obtained to carry out the study. The control group will receive usual care at their primary care center.

Criteria for discontinuation or modification: Participants may discontinue attendance at their own request or on the basis of the investigator's judgement for safety or health reasons.

Adherence strategies: The facilitator will maintain weekly contact and follow up on missed sessions by phone; adherence is defined as attendance at $\geq 75\%$ of scheduled sessions.

Concomitant care: All standard medical and psychosocial care is permitted without restriction.

Harms: Adverse events related to participation, such as emotional distress, conflict, or physical incidents at the restaurant, will be recorded and reviewed by the research team.

Intervention design

In the initial phase of the project, restaurants will be identified and evaluated according to predefined criteria to ensure feasibility, nutritional suitability, and accessibility. Candidate establishments will be required to: 1) offer menus aligned with the Mediterranean dietary pattern; 2) have sufficient capacity to host groups of more than 25 participants; 3) ensure physical accessibility for individuals with reduced mobility; and 4) provide a complete menu at a maximum cost of EUR 12 per person.

Once a restaurant has been selected, a structured training program will be implemented to ensure that it can provide meals suited to the needs and characteristics of the participants. This training will be delivered by professionals from the Alícia Foundation, a research center specializing in food, nutrition, and culinary innovation and a leading institution in culinary medicine. The training sessions will aim to ensure balanced, complete meals and appropriate adaptation to prevalent comorbidities, such as diabetes, hypertension, obesity, dyslipidemia, and chewing or swallowing difficulties. The training will consist of a 2-h theoretical session on the principles of a balanced diet and key dietary and culinary considerations for individuals with the above conditions. This will be followed by a practical session focused on planning the weekly menus to be offered to participants. Using the restaurant's usual daily menu as a starting point, staff will be

guided on how to select, modify, and adapt dishes to ensure that meals remain nutritionally appropriate, varied, sufficient, and adaptable to comorbidity-related needs. During the first weeks of the intervention, the menus will be reviewed before being served to ensure that the training is being implemented correctly.

Regarding long-term financial sustainability, the current study is fully funded for the implementation period. The results of the trial will inform the development of future models that may include mixed funding schemes, such as public funding, municipal council support, and potential partnerships.

Sample size calculation

The sample size was determined assuming a comparison between independent groups at the end of the intervention, with the ability to detect:

- a minimum difference of 4.5 points in the mean BDI-II score, with an estimated standard deviation (SD) of 7 points²⁴;
- a difference of 2.7 points in the mean GAD-7 score, with an SD of 4.5 points²⁵;
- and a 7-point difference in the DUKE-UNC-11 social support questionnaire score, with an SD of 10.6 points.²⁶

A 1 : 1 allocation ratio, with 1 participant in the control group for each participant in the intervention group, a statistical power of 80%, and a 2-sided significance level of 5% ($\alpha = 0.05$) were assumed. Taking into account an expected loss to follow-up or dropout rate of 10%, the required sample size was estimated at 49 participants per group, i.e., 98 participants in total. The calculation was performed using GRANMO software v. 7.12 (Hospital Del Mar Research Institute, Barcelona, Spain).

Recruitment

The selection screening process will begin with the identification of the target population through official and clinical sources available in the study area. The researchers will request from the technical department of Primary Care Management and the Community of Central Catalonia a list of patients assigned to the ABS of Sant Joan de Vilatorrada with diagnostic codes (ICD-10) compatible with the initial criteria: problems related to living alone or feeling lonely (Z60), anxiety (F41), and/or depression (F32).

Once the list has been obtained, it will be reviewed by health and social care professionals from the ABS, who will verify which patients meet the inclusion criteria established in the protocol. Community representatives and municipal social services contributed to the recruitment design and restaurant selection.

The ABS administrative staff, under the direction of the responsible professionals, will contact the selected individuals by telephone to invite them to participate

in the study. Those who agree to participate will be invited to a face-to-face visit, during which the study will be explained, study information will be provided, and informed consent will be obtained. They will also undergo a basic physical examination, a blood sample will be taken, and validated questionnaires will be administered. Individuals who meet at least 1 of the following criteria: BDI-II ≥ 14 , GAD-7 ≥ 10 , or DUKE-UNC-11 < 32 , will be included in the final sample and will move on to the next phase of the study.

Group assignment

Participants will be assigned to the study groups, control and intervention, using a computerized randomization procedure conducted by the research group's statistician in R v. 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria).

Participants who meet the inclusion criteria and present with symptoms of anxiety and/or depression and/or low social support will be allocated equally to the control and intervention groups. To increase external validity and ensure better comparability between groups, stratified randomization will be performed according to age and sex. This allocation design aims to minimize the risk of bias in group composition and ensure balanced representativeness, thereby facilitating the generalizability of the results.

Variables and measurement methods

Independent variables

Sociodemographic variables: gender (male, female, non-binary), age, marital status (single, married, separated, widowed), and educational level (no education, primary education, secondary education, higher education).

Dependent variables

Clinical remission of depression or response to the intervention will be assessed after completion of the intervention and at 12 months post-intervention. Clinical remission is defined as a score < 14 points on the BDI-II, and response to the intervention is defined as a reduction from the initial score.²⁴

Remission of clinical anxiety or response to the intervention will be assessed after completion of the intervention and at 12 months post-intervention. Clinical remission is defined as a score < 10 points on the GAD-7 scale, and response to the intervention is defined as a reduction from the baseline score.²⁵

Improvement in social support will be assessed after completion of the intervention and at 12 months post-intervention. Response to the intervention is defined as a decrease in the score on the DUKE-UNC-11 Social

Support Questionnaire, and a score > 32 points is considered to indicate good social support.²⁶

Improvement in health-related quality of life (HRQoL) will be assessed after the intervention and at 12 months post-intervention. An improvement in HRQoL after the intervention is defined as a decrease in the score on the EuroQol questionnaire (EQ-5D) compared with baseline.^{27,28}

Clinical variables: weight, height, body mass index (BMI), blood pressure, complete blood count with differential, glycated hemoglobin, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, albumin, iron, ferritin, vitamin B12, and folate.

Adherence to the Mediterranean diet will be assessed using the validated PREDIMED questionnaire (Prevention with Mediterranean Diet study).²⁹

Satisfaction with the intervention: At the end of the intervention, a satisfaction survey using a 5-point Likert scale will be conducted.

Adherence to the intervention: Attendance at the restaurant and healthy eating workshops will be recorded. Adherence will be calculated for the intervention variable, defined as attendance at $\geq 75\%$ of the scheduled days.

Number of visits to the primary care center: pre-intervention period (4 months), intervention period (4 months), and post-intervention period (4 months). Number of visits to accident and emergency services: pre-intervention period (4 months), intervention period (4 months), and post-intervention period (4 months). Number of referrals to hospital: pre-intervention period (4 months), intervention period (4 months), and post-intervention period (4 months). Variables that may act as confounding factors or effect modifiers will be taken into account.

Pharmacological treatment: The defined daily dose (DDD), as defined by the World Health Organization (WHO), will be calculated for each active ingredient, taking into account the number of days, the dose dispensed, and the route of administration of the medication. The active ingredients recorded will be those classified as psychotropic drugs, namely antidepressants and anxiolytics.

Data collection and information sources

Participant retention will be supported by reminder calls and flexible scheduling.

Pre- and post-intervention data will be obtained through:

- the questionnaires specified above;
- clinical variables collected through physical examination and blood testing. The clinical variables collected in the study are those commonly requested during routine check-ups of primary care patients;
- data such as the number of visits during a specified period and prescribed medications, obtained through data extraction by the technical department of the Territorial Management of Central Catalonia;
- participant feedback on their experience.

Data analysis

Blinding: This is an open-label trial due to the nature of the intervention. Outcome assessors and statisticians will remain blinded to group allocation during data analysis. Unblinding will occur only if necessary for participant safety.

Before the intervention begins, the baseline scores of the selected scales will be compared after randomization to assess initial equivalence between groups. These comparisons will serve to rule out statistically significant differences before the intervention.

First, the sociodemographic characteristics and other variables of participants in each group, intervention and control, will be described. Qualitative variables will be presented as absolute frequencies and percentages, while quantitative variables will be summarized as means and SDs or, in the case of non-normal distributions, as medians, ranges, and 25th and 75th percentiles. To evaluate the effect of the intervention, the mean scores of the different scales and variables will be compared within each group to assess pre–post intervention changes and between the 2 groups to assess differences before and after the intervention. Student's t-test for independent and paired samples will be used when the data are normally distributed; otherwise, the Mann–Whitney U test or Wilcoxon signed-rank test will be used, as appropriate. A secondary analysis will be performed using multiple logistic regression, including sociodemographic and confounding variables, for the clinical remission of depression variable, yes/no (yes: BDI-II < 14), the clinical remission of anxiety variable, yes/no (yes: GAD-7 < 10), and the social support variable, yes/no (yes: DUKE-UNC-11 > 32).

To address potential missing data, we will first examine the pattern and mechanism of missingness. When data are judged to be missing at random, multiple imputation using chained equations will be applied to the relevant variables. Sensitivity analyses will compare complete-case results with those obtained from the imputed datasets to evaluate the impact of missing data and protocol deviations on the study conclusions.

Results showing differences with $p < 0.05$ will be considered statistically significant, and 95% confidence intervals (95% CIs) will be reported. R v. 4.2.1 will be used for the statistical analysis.

Discussion

Loneliness is a social determinant of health with proven adverse effects on people's emotional, functional, and nutritional status, especially among older adults. Despite the existence of institutional schemes, such as home-delivered meals or soup kitchens, designed to meet basic needs in situations of severe vulnerability, many people remain excluded from this structured provision. This includes a significant proportion of the population experiencing unwanted loneliness, often linked to physical dependence,

mobility difficulties, or lack of financial means. This group, who are not institutionalized or part of conventional care systems, may experience feelings of disconnection and isolation, as well as nutritional risk, with negative medium- and long-term health consequences.

The intervention presented in this study proposes an innovative approach based on the use of existing community infrastructure, such as neighborhood or local restaurants, as spaces for food, interaction, socialization, and community support. Instead of creating new facilities, this initiative recognizes the value of traditional restaurants, which are often at risk of disappearing, and transforms them into partners within the local social and health network. This reinforces the sustainability and efficiency of the model while encouraging the economic revitalization of the local community and promoting the creation of healthy environments, as recommended by community-oriented health promotion policies.

One of the strengths of this study lies in its innovative and community-based nature, which leverages local infrastructure and promotes intersectoral collaboration. In addition, the project aligns with the principles of equal opportunities in health throughout life, addressing structural inequalities associated with age, gender, location, origin, and educational or socioeconomic level. The intervention design also incorporates monthly workshops on healthy eating and practical cooking, conceived as tools for nutritional empowerment and adherence to the Mediterranean diet. This is especially useful for groups that do not usually undertake the tasks of planning or preparing meals, such as older men, overworked caregivers, or people with limited food literacy.

Beyond the expected improvements in emotional and nutritional indicators, the intervention may have an indirect effect on reducing the frequency of health service use as a result of improved perceived wellbeing, reduced isolation, and better monitoring of some chronic health conditions. This approach may translate into reduced healthcare costs and contribute to the sustainability of the system.

Limitations of the study

Despite these strengths, several limitations and operational challenges associated with its implementation must be considered. A major limitation of the present study is its open-label design, which may introduce bias related to participants' expectations or the assessment of subjective outcomes. However, given the nature of the intervention, namely shared meals in a restaurant setting accompanied by a facilitator, blinding of participants and the professionals involved in delivering the intervention is not feasible.

To mitigate this potential bias, several strategies have been incorporated. First, outcome assessors and the biostatistics team will remain blinded to group allocation throughout the analysis. Second, the use of validated and widely accepted instruments, BDI-II, GAD-7, DUKE-UNC-11, and EQ-5D, helps reduce the risk of bias when measuring emotional,

social, and quality-of-life outcomes. Additionally, clinical variables, including anthropometric and laboratory parameters, will be collected according to standardized procedures by staff not involved in delivering the intervention. To further support this approach, objective secondary outcomes, such as healthcare use, will be obtained from electronic medical records, thereby minimizing the influence of participants' perceptions. Finally, stratified randomization by age and sex will ensure a balanced distribution between groups and reduce the risk of selection bias.

The implementation of the intervention involves several operational challenges, for which specific mitigation strategies have been established.

Adherence: Participant adherence may be challenging given the profile of the target population, who often experience symptoms of anxiety or depression and limited social support. To address this, the intervention incorporates the active role of a facilitator, who will provide personalized support through regular weekly contact and telephone follow-up when participants miss a lunch session. This ongoing support is intended to strengthen engagement and ensure continuity throughout the program.

Adaptation of restaurant establishments: Adjusting schedules, menus, and physical spaces to the needs of the group may create logistical demands. To facilitate implementation, structured collaboration mechanisms have been established with participating restaurants. The Alicia Foundation will maintain direct communication with these establishments, offering continuous guidance and training to ensure balanced and complete menus and adapt them to prevalent comorbidities, such as obesity, diabetes, hypertension, and chewing or swallowing difficulties.

Financial sustainability: The intervention is fully funded for the current implementation period. Looking ahead, long-term sustainability will require the development of mixed funding models combining public co-funding, municipal support, private-sector involvement, and potential access to grants. The trial results will guide the design of these future sustainability strategies.

Interinstitutional coordination: Effective collaboration among social services, healthcare providers, restaurants, community organizations, and the Alicia Foundation is essential for successful implementation. The lead investigator, who has dedicated protected time to conduct the study, will oversee coordination across all involved institutions, ensuring smooth communication, shared operational protocols, and regular feedback mechanisms.

Conclusions

This proposal constitutes a social prescribing strategy that is especially important in the context of demographic and social change. Its rigorous evaluation will allow its effectiveness

to be determined, identify areas for improvement, and establish the basis for its structural implementation in other community settings, thereby contributing to reducing loneliness, improving emotional wellbeing, social support, and quality of life, and reducing healthcare expenditure.

Use of AI and AI-assisted technologies

Not applicable.

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