

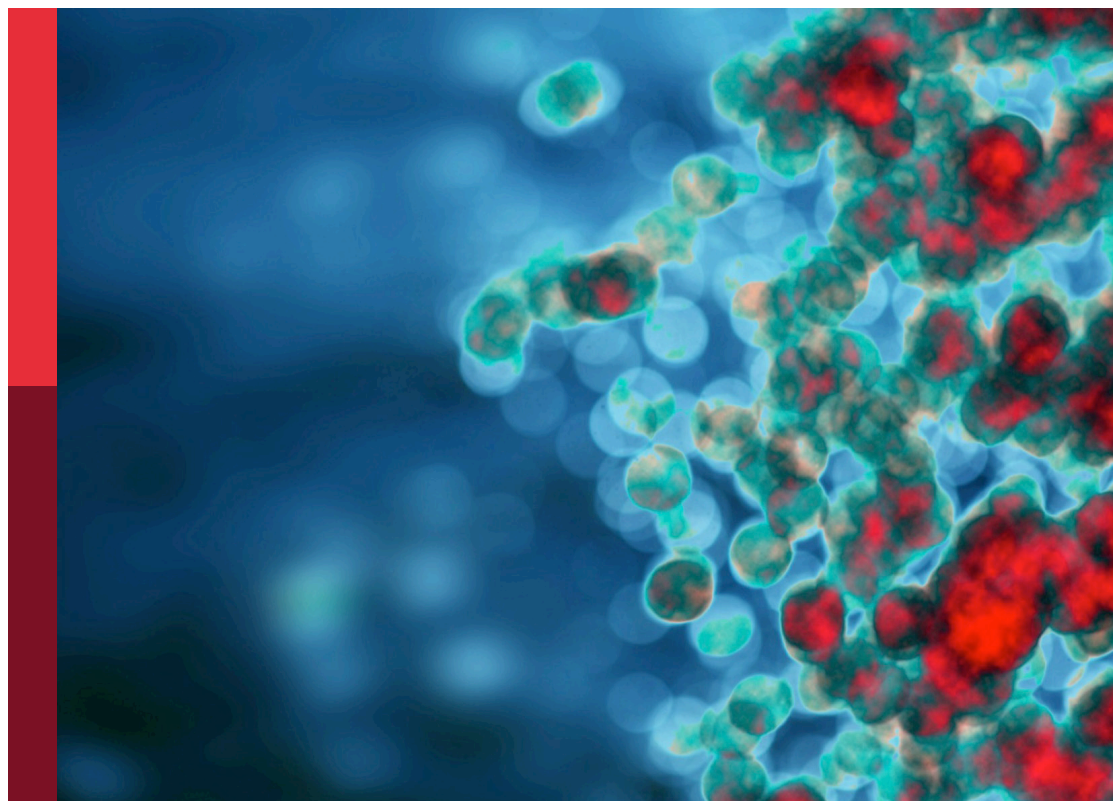
Immune system disorders: from molecular mechanisms to clinical implications

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Immune system disorders: from molecular mechanisms to clinical implications

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Table of contents

- 06 **Editorial: Immune system disorders: from molecular mechanisms to clinical implications**
Marisa M. Fernandez, Ruben D. Motrich, Matías Ostrowski and Mauricio De Marzi
- 10 **Revisiting regulatory T cells as modulators of innate immune response and inflammatory diseases**
Qifeng Ou, Rachael Power and Matthew D. Griffin
- 26 **Ankylosing spondylitis and psychiatric disorders in European population: a Mendelian randomization study**
Huifang Zuo and Min-Min Li
- 37 **Machine learning to characterize bone biomarkers profile in rheumatoid arthritis**
Giovanni Adami, Angelo Fassio, Maurizio Rossini, Camilla Benini, Riccardo Bixio, Denise Rotta, Ombretta Viapiana and Davide Gatti
- 47 **The neutrophil-lymphocyte ratio predicts all-cause and cardiovascular mortality among U.S. adults with rheumatoid arthritis: results from NHANES 1999-2020**
Erye Zhou, Jian Wu, Xin Zhou and Yufeng Yin
- 58 **Efficacy and safety of anakinra and canakinumab in PSTPIP1-associated inflammatory diseases: a comprehensive scoping review**
Juan Luis Sanz-Cabanillas, Francisco Gómez-García, Pedro Jesús Gómez-Arias, Ana Montilla-López, Jesús Gay-Mimbrera, Juan Ruano, Beatriz Isla-Tejera and Esmeralda Parra-Peralbo
- 75 **Causal role of immune cells in generalized anxiety disorder: Mendelian randomization study**
Zhen Ma, Min Zhao, Huanghong Zhao and Nan Qu
- 82 **Investigating the shared genetic architecture between hypothyroidism and rheumatoid arthritis**
Zhifang Peng, Weiping Huang, Mengjun Tang, Binbin Chen, Renqi Yang, Qing Liu, Chaoshui Liu and Panpan Long
- 92 **Complete Freund's adjuvant as a confounding factor in multiple sclerosis research**
Milica Lazarević, Suzana Stanisavljević, Neda Nikolovski, Mirjana Dimitrijević and Đorđe Miljković
- 100 **When inflammatory stressors dramatically change, disease phenotypes may transform between autoimmune hematopoietic failure and myeloid neoplasms**
Xi-Chen Zhao, Bo Ju, Nuan-Nuan Xiu, Xiao-Yun Sun and Fan-Jun Meng

- 122 **Prognostic significance of lymphocytic foci composition in minor salivary gland biopsies for severe disease flare and severity in Sjögren's syndrome: a 3-year follow-up cohort study**
Hye-Sang Park, Laura Martínez-Martínez, Berta Magallares López, Ivan Castellví, Patricia Moya, Helena Codes-Mendez, Nerea Hernandez Sosa, Cesar Diaz-Torne, Ana Laiz, Luis Sainz, Jose Luis Tandaipan, Anaís Mariscal, Teresa Franco-Leyva, Jordi Casademont, Candido Juarez and Hector Corominas
- 132 **Deep analysis of skin molecular heterogeneities and their significance on the precise treatment of patients with psoriasis**
Shengxiao Zhang, Minjing Chang, Leilei Zheng, Can Wang, Rong Zhao, Shan Song, Jiawei Hao, Lecong Zhang, Caihong Wang and Xiaofeng Li
- 144 **Association between systemic immune-inflammation index and psoriasis: a population-based study**
Xiya Zhao, Junqin Li and Xinhua Li
- 153 **Multiple environmental antigens may trigger autoimmunity in psoriasis through T-cell receptor polyspecificity**
Tatsushi Ishimoto, Yukiyasu Arakawa, Secil Vural, Julia Stöhr, Sigrid Vollmer, Adrian Galinski, Katherina Siewert, Geraldine Rühl, Yuri Poluektov, Marc Delcommenne, Orsolya Horvath, Mengwen He, Burkhard Summer, Ralf Pohl, Rehab Alharbi, Klaus Dornmair, Akiko Arakawa and Jörg C. Prinz
- 168 **Transcriptomics in idiopathic pulmonary fibrosis unveiled: a new perspective from differentially expressed genes to therapeutic targets**
Wenzhong Hu and Yun Xu
- 180 **Causal relationship between inflammatory cytokines and autoimmune thyroid disease: a bidirectional two-sample Mendelian randomization analysis**
Zhiwei Yao, Fengli Guo, Yanlu Tan, Yiyuan Zhang, Yichen Geng, Guang Yang and Song Wang
- 196 **Predictive factors of psychiatric syndrome in patients with systemic lupus erythematosus**
Wenqi Geng, Shangzhu Zhang, Jinya Cao, Xia Hong, Yanping Duan, Yinan Jiang and Jing Wei
- 204 **Clinical characteristics of patients with autoimmune nodopathy with anti-neurofascin155 antibodies**
Jiwei Zhang, Xiaotong Hou, Liting Wei, Jinshun Liu, Shibo Li, Yifan Guo, Hongbo Liu and Yan Jiang
- 212 **Investigation of the causal association between Parkinson's disease and autoimmune disorders: a bidirectional Mendelian randomization study**
Junyi Yang, Weiran Lin, Yumei Ma, Hui Song, Changqing Mu, Qian Wu, Chen Han, Jian Zhang and Xu Liu

- 224 **Discovery of biomarkers in the psoriasis through machine learning and dynamic immune infiltration in three types of skin lesions**
Xiao Zhou, Han Zhou, Xin Luo and Rui-Fang Wu
- 236 **CD8 T cells are dispensable for experimental autoimmune prostatitis induction and chronic pelvic pain development**
Floresencia C. Salazar, Maria S. Martinez, Daniela A. Paira, Yair A. Chocobar, Carolina Olivera, Gloria J. Godoy, Eva V. Acosta-Rodriguez, Virginia E. Rivero and Ruben D. Motrich
- 248 **Genetically predicted N-methylhydroxyproline levels mediate the association between naive CD8+ T cells and allergic rhinitis: a mediation Mendelian randomization study**
Zhengjie Chen, Ying Suo, Xintao Du and Xiaoyun Zhao
- 259 **Rheumatoid arthritis increases the risk of heart failure: results from the cross-sectional study in the US population and mendelian randomization analysis in the European population**
Kaisaierjiang Kadier, Diliyaer Dilixiati, Xu Zhang, Huan Li, Lirong Kuang, Jian Huang, Xintian Cai, Tao Ling, Fanqi Kong and Xiaozhu Liu
- 270 **Targeting autoimmune mechanisms by precision medicine in Myasthenia Gravis**
Paola Cavalcante, Renato Mantegazza and Carlo Antozzi
- 287 **The role of bilirubin as a biomarker of rheumatic diseases: a systematic review and meta-analysis**
Angelo Zinellu and Arduino A. Mangoni
- 300 **Deciphering the molecular landscape of rheumatoid arthritis offers new insights into the stratified treatment for the condition**
Min-Jing Chang, Qi-Fan Feng, Jia-Wei Hao, Ya-Jing Zhang, Rong Zhao, Nan Li, Yu-Hui Zhao, Zi-Yi Han, Pei-Feng He and Cai-Hong Wang
- 314 **Precision therapy for ulcerative colitis: insights from mitochondrial dysfunction interacting with the immune microenvironment**
Yi-fan Zhang, Meng-ying Fan, Qi-rui Bai, Rong Zhao, Shan Song, Li Wu, Jun-hui Lu, Jing-wei Liu, Qi Wang, Yuan Li and Xing Chen
- 331 **Exploring the impact of m⁶A modification on immune diseases: mechanisms and therapeutic implication**
Yutong Chen, Min Liu, Miao Lu, Linling Luo, Zhongyu Han and Xide Liu



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Editorial: Immune system disorders: from molecular mechanisms to clinical implications

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Editorial on the Research Topic

Immune system disorders: from molecular mechanisms to clinical implications

In recent years, our understanding of the immune system has expanded significantly, highlighting its crucial role not only in the defense against pathogens, toxins and tumor cells but also in controlling inflammation and inducing tolerance to self- and non-self-antigens (1, 2). Reported evidence during the last decades has allowed a better understanding of the pathogenic consequences of immune system dysfunction, including the development of immunodeficiencies, inflammatory and autoimmune disorders, among others (3). Moreover, current knowledge about immunoregulation has paved the way to better prevent or treat severe inflammatory conditions such as transplant rejection and hypersensitivities. However, mechanisms underlying immune deregulation are highly variable depending on the pathology (systemic chronic inflammatory diseases, autoinflammatory diseases, autoimmune disorders, immunodeficiencies, hypersensitivities) and the host. Given the diverse range of disorders resulting from excessive, inefficient, or inadequate immune responses, the study of factors involved in immune system deregulation has garnered significant attention in recent decades (Figure 1). In this Research Topic, some review articles have focused on different autoimmune diseases (AIDs) and treatments. Cavalcante et al. conducted a literature review on emerging biological drugs for treating Myasthenia gravis and emphasized the crucial importance of precision medicine for these patients, given the variability in the efficacy of current

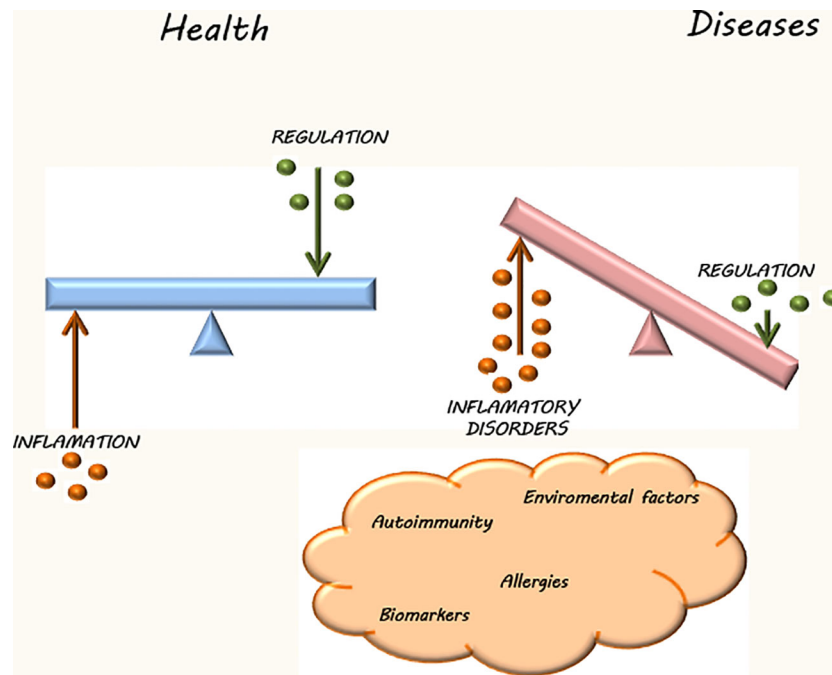


FIGURE 1

The immune system has a fundamental role in immune surveillance to prevent the development of tumor cells and inflammatory alterations or in immunological tolerance processes. The pathological consequences of its deregulation include inflammatory, autoimmune and immunosuppressive diseases, among others.

therapies. Moreover, [Zinellu et al.](#) performed a systematic review about the role of circulating concentrations of bilirubin as a biomarker of rheumatic diseases (RDs) revealing that patients show significantly lower concentrations of total bilirubin, conjugated bilirubin, as well as of the active antioxidant and anti-inflammatory unconjugated bilirubin. Consequently, they propose them as potential biomarkers of antioxidant and anti-inflammatory capacity in patients bearing rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), primary Sjögren syndrome, and myositis. Besides, [Chen et al.](#), described the composition, biological function, and regulation of N⁶-methyladenosine (m⁶A) in the immune microenvironment and its role in various immune diseases such as inflammatory enteritis and SLE, providing new targets and directions for their treatment. A mini review presented by [Lazarevic et al.](#) describes that complete Freund's adjuvant (CFA) used for the induction of experimental autoimmune encephalomyelitis (EAE) (a model of multiple sclerosis –MS–) is a confounding factor due to the multiple effects it causes in animals. For this reason, EAE variants without CFA are highlighted as valuable tools to study the pathogenesis of MS.

Another review by [Zhao et al.](#) highlights how significant changes in inflammatory factors can drive phenotypic transformations of the disease, shifting from autoimmune hematopoietic insufficiency (AHF) to myeloid neoplasms (MN). This suggests that the autoimmune responses observed in AHF may act as an antileukemic mechanism, and their suppression could potentially trigger the development of MN. In addition, the effectiveness of IL-1 pathway inhibitors for treating PSTPIP1-

associated inflammatory diseases (PAID) was examined in a systematic review by [Sanz-Cabanillas et al.](#) While anakinra and canakinumab have demonstrated potential in managing conditions such as sterile pyogenic arthritis, pyoderma gangrenosum and acne (PAPA), and PSTPIP1-associated myeloid proteinemia inflammatory (PAMI) syndromes, there is limited information available regarding the adverse effects of these treatments. As anticipated, regulatory T cells (Tregs) feature prominently in this Research Topic. [Ou et al.](#) provide a comprehensive summary of Tregs' modulatory effects, emphasizing their critical interactions with innate immune effectors and their influence on disease development. They also discuss the potential therapeutic applications of Tregs in the context of autoimmunity, allogeneic transplants, and various common medical conditions where organ and tissue damage is primarily driven by inflammatory processes.

This Research Topic also includes several original research articles that expand our current knowledge on various aspects of several different immune-mediated diseases, such as allergic rhinitis, rheumatoid arthritis, cardiovascular disease, autoimmune thyroid disease, idiopathic pulmonary fibrosis, ulcerative colitis, psoriasis, Sjögren's syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, systemic lupus erythematosus, and ankylosing spondylitis, among others.

[Chen et al.](#) performed a study utilizing Mendelian randomization (MR) to explore the relationship between immune cell phenotypes and metabolite levels in allergic rhinitis (AR). Notably, three immune cell phenotypes were identified as protective factors for AR: naïve CD8^{br} %CD8^{br} cells, CD3 in CD39⁺ activated Tregs, and HVEM in

CD45RA-CD4+ cells. Additionally, authors identified three metabolites as risk biomarkers for AR: N-methylhydroxyproline, N-acetylneuraminate, and 1stearoyl-2-arachidonoyl-gpc (Chen et al.).

Different attention-grabbing reports focused on rheumatoid arthritis (RA), a common and debilitating chronic systemic autoimmune disease. Given that heart failure (HF) has emerged as the second leading cause of cardiovascular death among RA patients, Kadier et al. investigated the association between HF and RA. While their cross-sectional study of the U.S. population revealed a significant link between the two, similar findings were not observed in the European population through Mendelian randomization analysis, suggesting that further research is needed on this topic. Additionally, Chang et al. utilized machine learning to analyze RA subtypes, identifying three distinct categories based on upregulated differentially expressed genes (DEGs): RA subtype A, which is enriched in pathways related to neutrophil activation; RA subtype B, associated with IFN signaling; and RA subtype C, driven by CD8+ T cells. These findings offer valuable insights for patient stratification, potentially paving the way for enhanced molecular diagnostics and therapeutic strategies in the future. It is well established that the neutrophil-to-lymphocyte ratio (NLR) serves as a biomarker for systemic inflammation and immune activation. In their study, Zhou et al. demonstrate that RA patients with a higher NLR face an increased risk of all-cause and cardiovascular mortality compared to those with a lower NLR. They suggest that NLR could be an inexpensive and readily accessible prognostic marker for RA. Disruption of bone metabolism is another important factor altered in RA. Adami et al. conducted a cross-sectional case-control study to explore associations between biomarker levels and clinical variables. The authors identified a distinct bone profile in RA, characterized by changes in bone density and unique bone biomarker patterns, which may provide valuable insights for enhancing the management of bone involvement in RA. Noteworthy, a controversial association between hypothyroidism and RA has been proposed. Interestingly, Peng et al. report a potential causal association of hypothyroidism with RA. Analyzing gene expression data in several tissues, authors observed a genetic associations between hypothyroidism and RA, particularly in local genomic regions. They identified TYK2, IL2RA, and IRF5 as shared risk genes for both hypothyroidism and RA.

Evidence suggests a potential relationship between Parkinson's disease (PD) and various autoimmune diseases (AIDs). Yang et al. conducted a bidirectional Mendelian randomization analysis to explore the causal associations between PD and a range of AIDs. Notably, their findings indicate a potential positive correlation between genetically determined PD and the incidence of type 1 diabetes (Yang et al.). Besides, another MR study revealed the close association between immune cells and generalized anxiety disorder (GAD) through genetic methods, thereby offering direction for future clinical research (Ma et al.). Autoimmune thyroid disease (AITD) is among the most common thyroid disorders. A MR analysis by Yao et al. identified seven causal associations between inflammatory cytokines and AITD. Elevated levels of TNF- β and reduced levels of SCGF- β were associated with an increased risk of Graves disease (GD). Conversely, high levels of IL-12p70, IL-13,

and IFN- γ , along with low levels of MCP-1 and TNF- α , indicated a higher risk of Hashimoto thyroiditis (HT) (Yao et al.).

Idiopathic pulmonary fibrosis (IPF) is a progressive lung function deteriorating condition associated with high mortality. By using microarray datasets, Hu and Xu analyzed the relationships between differentially expressed genes (DEGs) and IPF. Authors identified 486 highly expressed genes and 468 lowly expressed genes. However, MR analysis identified six significantly co-expressed genes associated with IPF that participate in essential biological processes and pathways, including macrophage activation and neural system regulation. Authors emphasize the potential of targeting specific molecular pathways for the treatment of IPF, laying the foundation for further research and clinical work (Wenzhong Hu and Yun Xu).

Another study using machine learning algorithms identified 108 differentially expressed mitochondria-related genes (DE-MiRGs) in the colonic mucosa of ulcerative colitis (UC) patients (Zhang et al.). Results revealed a significant enrichment in pathways associated with mitochondrial metabolism and inflammation. Moreover, valuable MiRGs diagnostic models with predictive capabilities and therapeutic implications for UC were developed based on 17 signature genes (Zhang et al.). Similarly, Zhou et al., employed machine learning and analysis of DEGs in the skin of patients with psoriasis and atopic dermatitis. They identified four potential diagnostic genes and validated the results using real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry. In this way, they propose the CCNE1 gene as the one with the greatest diagnostic value. Psoriasis is a highly heterogeneous autoimmune skin disease that affects approximately 2-3% of the global population and lacks effective treatment options. In their study, Ishimoto et al. identify environmental antigens that may trigger autoimmunity in psoriasis due to TCR polyspecificity, including peptides from wheat, *Saccharomyces cerevisiae*, microbiota, tobacco, and several pathogens. They discovered that the same CD8+ T cells can recognize both autoantigens and environmental antigens, suggesting that a wheat-free diet may alleviate psoriasis symptoms in some patients. Besides, Zhang et al. identified 163 differentially upregulated genes in lesional skin from psoriatic patients using microarray datasets. Interestingly, they described three skin subtypes (A-C subtypes). Subtype A is related to activated immune cells and pathways related to inflammation, subtype B was modestly activated in all the signaling pathways, and subtype C exhibited high levels of stromal cells and signaling pathways activation associated with tissue proliferation. Moreover, Xgboost classifier predicts that subtypes A and B would respond well to methotrexate and interleukin-12/23 inhibitor treatments, while subtype C would have excellent outcomes after tumor necrosis factor- α inhibitors (etanercept) and interleukin-17A receptor inhibitors (brodalumab) therapies (Zhang et al.). The systemic immune-inflammation index (SII), as measured by lymphocyte, neutrophil and platelet counts in peripheral blood, is regarded as a reliable indicator of inflammatory state. By multivariate linear regression analysis, Zhao et al. report a significant positive correlation between psoriasis and elevated SII. Nonetheless, authors are cautious about their findings and suggest the need for further large-scale prospective studies to validate their results (Zhao et al.).

Chronic pelvic pain syndrome is a prevalent urological condition affecting young adult men (4). Patients typically present signs and symptoms of chronic inflammation in the absence of a detectable infectious cause. In this Research Topic, Salazar et al. report evidence indicating that CD8 T cells do not play a major role in the pathogenesis of chronic prostatitis and pelvic pain development using an animal model of Experimental Autoimmune Prostatitis (EAP), which has been largely used to study CPPS pathogenesis (5). Sjögren's syndrome is another intriguing prevalent autoimmune condition. Park et al. report that patients with higher T cell counts in the infiltrates of lymphocytic foci may face a two-fold increased risk of severe disease flares. Additionally, they found a weak but positive correlation between B cell and CD4+ T cell counts and the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) (Park et al.).

According to the latest guidelines on chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), patients with CIDP with anti-neurofascin 155 (NF155) antibodies are referred to as autoimmune nodopathy (AN), an autoimmune disorder distinct from CIDP. In a very original study, Zhang et al. provide evidence indicating that patients with AN with anti-NF155 antibodies differed from serologically negative patients with CIDP in terms of clinical characteristics such as onset age, limb weakness, sensory disturbance, ataxia, and multiple motor (Zhang et al.).

A retrospective analysis of systemic lupus erythematosus (SLE) was performed by Geng et al. to assess putative correlations with neuropsychiatric systemic lupus erythematosus (NPSLE). Authors found that 82.4% of psychiatric manifestations were attributed to SLE. Remarkably, mood disorders positively correlated with anti-cardiolipin (ACL) and anti- β 2 glycoprotein I (- β 2GP1) antibodies (Geng et al.). Finally, Zuo and Li carried out a MR analysis to confirm causal relationships between ankylosing spondylitis (AS) and five mental health conditions including major depressive disorder (MDD), anxiety disorder (AXD), schizophrenia (SCZ), bipolar disorder (BIP), and anorexia nervosa (AN). The authors found that ankylosing spondylitis (AS) may be causally linked to an increased risk of developing schizophrenia (SCZ) and anorexia nervosa (AN), offering new insights for risk management and preventive interventions for mental disorders in patients with AS (Zuo and Li).

This Research Topic aims to enhance our understanding of the cellular and molecular mechanisms underlying diseases associated with immune dysregulation. Much of the current focus is on exploring the interactions between immune dysregulation and factors such as age, sex, genetics, nutritional status, past infections, and environmental influences. These insights are expected to pave the way for the development of effective, targeted therapies for these conditions. However, further knowledge is needed regarding the consequences of the various processes that impact the immune system.

Author contributions

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Conflict of interest

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Revisiting regulatory T cells as modulators of innate immune response and inflammatory diseases

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Regulatory T cells (Treg) are known to be critical for the maintenance of immune homeostasis by suppressing the activation of auto- or allo-reactive effector T cells through a diverse repertoire of molecular mechanisms. Accordingly, therapeutic strategies aimed at enhancing Treg numbers or potency in the setting of autoimmunity and allogeneic transplants have been energetically pursued and are beginning to yield some encouraging outcomes in early phase clinical trials. Less well recognized from a translational perspective, however, has been the mounting body of evidence that Treg directly modulate most aspects of innate immune response under a range of different acute and chronic disease conditions. Recognizing this aspect of Treg immune modulatory function provides a bridge for the application of Treg-based therapies to common medical conditions in which organ and tissue damage is mediated primarily by inflammation involving myeloid cells (mononuclear phagocytes, granulocytes) and innate lymphocytes (NK cells, NKT cells, $\gamma\delta$ T cells and ILCs). In this review, we comprehensively summarize pre-clinical and human research that has revealed diverse modulatory effects of Treg and specific Treg subpopulations on the range of innate immune cell types. In each case, we emphasize the key mechanistic insights and the evidence that Treg interactions with innate immune effectors can have significant impacts on disease severity or treatment. Finally, we discuss the opportunities and challenges that exist for the application of Treg-based therapeutic interventions to three globally impactful, inflammatory conditions: type 2 diabetes and its end-organ complications, ischemia reperfusion injury and atherosclerosis.

KEYWORDS

regulatory T cells (Treg), innate immune response, macrophages, dendritic cells, immune regulation, neutrophils, inflammatory diseases, cell therapies

Introduction

Regulatory T cells (Treg), classically identified in humans and other species as CD4⁺/Foxp3⁺ lymphocytes, comprise only 5–10% of total CD4⁺ T cells, but are essential for balancing immune system activity throughout life. The immunosuppressive and immune modulatory functions of Treg have been unequivocally established in the context of adaptive immunity and have become fundamental to our understanding of autoimmunity, transplant rejection, tumor antigen-specific immune responses and the distinction between harmful and commensal microorganisms (1). Less well appreciated has been the accumulating research evidence that Treg directly modulate activity of the innate immune system through cross-talk with mononuclear phagocytes (monocytes/macrophages), granulocytes (neutrophils), dendritic cells (DC) and innate lymphocytes (NK cells, NKT cells, $\gamma\delta$ T cells and other innate lymphocytic cells (ILC)) (2). The impacts of innate immune cell interactions with Treg may include modifications to cell survival, proliferation, cytokine production, phagocytosis, cytotoxicity and other effector functions. Although Treg-mediated suppressive effects on innate immune cells can be implicated in their regulation of antigen-specific T- and B-cell responses, they may also be more broadly relevant to immune-mediated inflammatory diseases (IMID) and disorders of tissue homeostasis, such as diabetes, atherosclerosis, Parkinson's disease, wound healing and aging (3). This suggests that a deeper understanding of the Treg phenotypic and functional properties that underlie their modulation of innate immune responses could open new translational pathways for the burgeoning field of regulatory immune cell therapies (4).

In this review, we aim to summarize current knowledge of the direct modulatory effects of Treg on diverse innate immune cells types with an emphasis on mechanisms and clinically-meaningful contexts in humans as well as experimental animals. We also discuss the relevance of these insights to common human diseases and health challenges for which non-autoimmune inflammation is a key component and reflect on the prospects for Treg-based therapies to be applied to their treatment.

Although the majority of the studies described here focus on the canonical CD4⁺CD25⁺CD127[−] Foxp3⁺ Treg phenotype, it is important to acknowledge that there is considerable heterogeneity in regard to Treg subtypes and functionality. Most clearly, CD4⁺ Treg may be divided into “natural” or thymic-derived (tTreg), peripherally-derived (pTreg) and *in-vitro* induced Treg (iTreg). In addition, however, sub-populations defined on the basis of specific surface markers, transcription factors, functional properties or disease associations have been described, including type 1 regulatory T cells (Tr1), Th3 cells, FoxA1⁺ Treg (5), CD39⁺ Treg, CD8⁺ Treg, Treg-of-B and others (6, 7). Where relevant, therefore, we highlight Treg subpopulations that have been described to regulate specific aspects of innate immune response.

Regulatory T cell interactions with mononuclear phagocytes and granulocytes

Mononuclear phagocytes

Accumulating evidence supports the existence of a biologically significant role for Treg in regulating monocyte- and macrophage-mediated inflammation in diverse contexts. For example, the co-occurrence of decreased Treg proportions and increased monocyte inflammatory mRNA expression was identified in the blood of adults with major depressive disorders (8). In contrast, in patients with acute HIV-1 infection, increased proportions of Foxp3⁺Helios⁺CD45RA⁺ Treg were reported along with decreased frequencies of CD14⁺⁺CD16⁺ (intermediate) monocytes and increased proportions of PD-1⁺ cells among both CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ (non-classical) monocytes (9). In scurfy mice, an animal model in which Foxp3 mutation results in profound Treg deficiency, increased myelopoiesis and monocyte homing receptor expression, phagocytic activity and cytokine production occurs (10). Recently, Hand et al. reported that diurnal variations of Treg numbers occur within joints of mice and that depletion of Treg resulted in increased joint inflammation at night in association with enhanced production of IL-1 β by Ly6C^{hi} monocytes (11). These observations of inverse relationships between Treg and monocyte activities indicate a potential role of Treg in directly regulating the inflammatory profile of mononuclear phagocytes. In both *ex vivo* human cell experiments and *in vivo* animal models, Treg have been demonstrated to promote monocyte differentiation toward alternatively activated/anti-inflammatory (M2) macrophage phenotypes characterized by high phagocytic activity and expression of related marker proteins (CD206, CD163, and heme oxygenase-1); low antigen-presentation capacity (CD40, CD80/86, and class II MHC); increased secretion of anti-inflammatory cytokines (IL-10) and decreased production of pro-inflammatory molecules (TNF, IL-6, nitric oxide (NO) and reactive oxidative species (ROS)). Such influences of Treg on mononuclear phagocyte differentiation have been reported to be both cell contact- (12, 13) and cytokine-dependent. In the case of soluble mediators, Treg IL-10 (14), IL-13 (15), TGF- β , IL-4 (16), arginase (17), and soluble fibrinogen-like protein 2 (18) have all been shown to play a role in various experimental settings. The transcription factor Krüppel-like factor 10 (KLF10) (19) and the signaling pathway mediated by mammalian target of rapamycin complex 1 (mTORC1) have also been reported to be necessary for Treg suppression of macrophage-mediated inflammation (20). Recently, exosome-based cross-talk between Treg and macrophages has also been documented. In a mouse model of acute myocardial infarction, Hu et al. reported that

infusion of Treg-derived exosomes reduced infarct size and improved cardiac function by promoting macrophage polarized towards M2 phenotypes in myocardial tissue (21). In a reverse interaction, Zhou et al. demonstrated that macrophage-derived exosomes transferred miR-29a-3p and miR-21-5p into CD4⁺ T cells to promote their differentiation into Treg, in a mouse tumor microenvironment (22). Furthermore, Treg-supported M2 macrophages can induce CD4⁺CD25⁻ T cell to acquire a suppressive phenotype, forming a positive feedback loop in immune regulation (23). Mechanistically, macrophage promotion of Treg induction, proliferation and migration has been linked to an IL-10 and TGF- β -secreting (M2c) phenotype, to the secretion of resistin-like molecule (Relm)- α and to the induction of CD62L on Treg (24–26). Figure 1 illustrates a range of reported interactions between Treg and mononuclear phagocytes along with their key mediators and outcomes.

From a therapeutic perspective, Romano et al. have reported that ex vivo expanded human Treg may be more potent in suppressing monocyte activation than freshly-isolated Treg. In this study, the effects of ex vivo expanded Treg on activated human monocytes were manifested as reduction of NF- κ B signaling, a higher proportion of CD14⁺CD206⁺CD163⁺CD86⁻

(M2-like) cells and compromised potency to induce Th17 cell responses (27). Using a CD28 super-antagonist, Cai et al. recently showed that *in vivo*-expanded endogenous mouse Treg adoptively transferred to diabetic animals undergoing ischemic stroke induced an increase in CD206⁺ M2 microglia/macrophages in ischemia tissue detected by real-time visualization using an optical imaging probe (28).

In addition to the initiation and resolution of inflammation, monocytes and tissue macrophages also play an important role in the development of post-inflammatory fibrosis through direct effects or via interactions with fibroblasts. In the short-term, the pro-fibrotic effects of alternatively-activated macrophages are beneficial to wound healing and tissue repair, whereas their prolonged pro-fibrotic activity contributes to the pathogenesis of a range of disease processes (29). For example, fibrosis of lung (30) and liver (31) as well as chronic kidney disease (32) and systemic sclerosis (33), have all been reported to be linked with macrophage-induced fibrosis. Currently, the potential influence of Treg interactions with mononuclear phagocytes on physiological and pathological tissue fibrosis is not well understood. Of interest, Song et al. recently reported that adoptively transferred Treg accelerated tissue repair and kidney function recovery in mice following

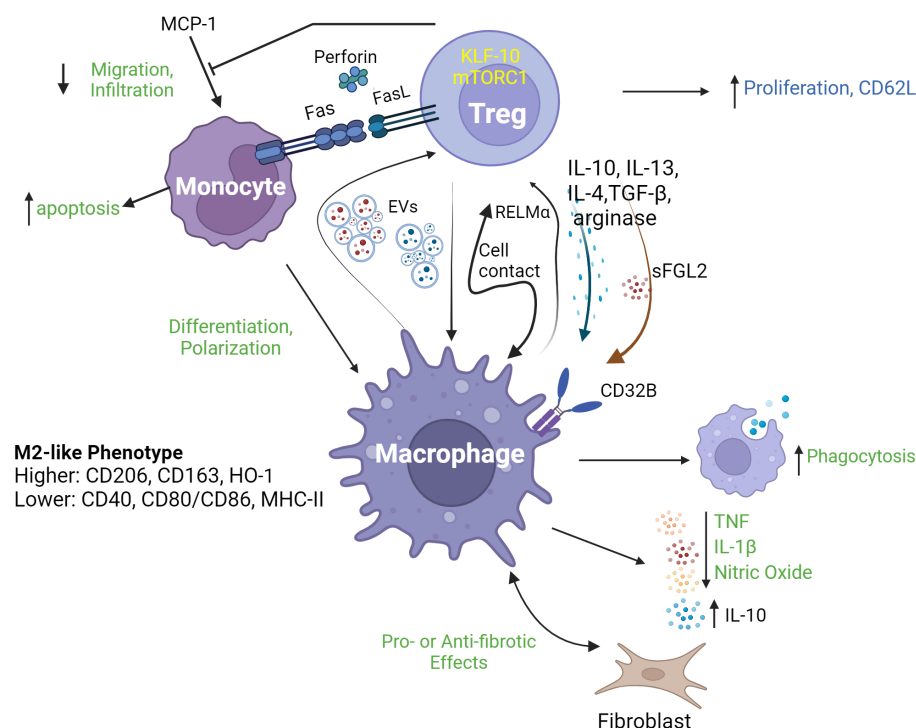


FIGURE 1

Regulatory T cell interactions with mononuclear phagocytes. Regulatory T cells (Treg) interact with mononuclear phagocytes including monocytes and macrophages, through a range of mechanisms leading to a diverse array of consequences. As shown in the upper part of the figure, Treg modulate several aspects of monocyte behavior, including their survival (through death receptor and cytolytic pathways), migration/infiltration (through inhibition of MCP-1) and differentiation into M2-type macrophages. Such functions may be dependent on specific Treg transcriptional (KLF-10) and signaling (mTORC1) programs. As depicted in the lower part of the figure, Treg and macrophages may engage in complex cross-talk including release/expression of signature modulatory cytokines (IL-10, IL-13, IL-4 and TGF- β), arginase, RELM α and sFGL2; and exchange of extracellular vesicles (EVs). Outcomes of this cross-talk may include enhanced macrophage phagocytosis, release of pro-inflammatory mediators (TNF, IL-1 β and nitric oxide) and modulation of the communication between macrophages and fibroblasts, as well as increased Treg proliferation and expression of CD62L. EVs, extracellular vesicles; RELM α , resistin-like molecule α ; MCP-1, monocyte chemoattractant protein-1; KLF-10, Krüppel-like factor 10; mTORC1, mechanistic target of rapamycin complex 1; sFGL2, soluble fibrinogen-like protein 2; HO-1, haemoxygenase 1.

reversal of unilateral ureteral obstruction in association with higher intra-renal proportions of M2 macrophages (34). Nonetheless, the concept of Treg ameliorating fibrosis through M2-like polarization of macrophages is not entirely in accordance with current understanding of M1/M2 balance in disease-associated fibrosis. For example, in animal models of diabetic kidney disease, pharmacological interventions which alleviated renal fibrosis and preserved kidney function have variously been reported to promote M1 (35) or M2 (36) macrophage phenotypes. Furthermore, Kim et al. demonstrated that M2c macrophages, induced with IL-10 and TGF- β from mouse bone marrow-derived macrophages, exacerbated renal fibrosis following acute kidney injury (32). Bhandari et al. also reported that a subset of human macrophages capable of activating fibroblasts and promoting fibrosis exhibited both M1- and M2-associated surface marker expression and cytokine production in the setting of systemic sclerosis (33), while Tan-Garcia et al. observed enrichment of TNF-secreting, CD206⁺ macrophages in fibrotic liver of hepatocellular carcinoma (31). Such results indicate that the classical dichotomy of macrophage M1/M2 polarization frequently fails to explain the various roles of macrophages in fibro-inflammatory disease and, by extrapolation, raise the possibility that Treg modulation of mononuclear phagocyte activation and differentiation could also exacerbate disease in some settings (37). In keeping with this, pro-fibrotic Treg subtypes have been identified as participating in ventricular remodeling after heart failure (38) and in lymphatic tissue fibrosis after HIV infection (39). In addition, adoptively transferred Treg have been reported to worsen the pro-fibrotic environment in bleomycin-induced lung injury models (40). In non-fibrotic diseases also, the effects of Treg on macrophage phenotype may be more ambiguous than simply modulating from M1 to M2. For example, Treg have been shown to decrease macrophage infiltration in lesional skin, resulting in reduced disease progression in a model of psoriasis (41). However, the macrophage phenotypes that predominate in psoriasis progression may include CD68⁺iNOS⁺ (M1), CD206⁺ (M2), or phenotypes distinct from M1/M2 (42). These studies highlight the complexity of predicting the outcome of Treg/mononuclear phagocyte cross-talk in different contexts as well as the need for carefully designed pre-clinical studies to guide the development of Treg-based therapies for non-autoimmune acute and chronic inflammatory diseases. Further investigation of the phenotypes, origin, migration and pro/anti-inflammatory function of monocytes and macrophages will help to develop a deeper understanding of how Treg modulate their activity in specific disease settings.

Regulatory T cells also express multiple mediators of programmed cell death and may suppress inflammation through induction of apoptosis in mononuclear phagocytes (Figure 1). While the killing of CD4⁺ T cells by Treg is thought to be based on TRAIL/TRAILR and galectin pathways (43), the mechanisms underlying monocyte killing by Treg is controversial. Venet et al. reported that human Treg induced CD14 down-regulation – a marker of apoptosis – in LPS-activated monocytes through a FasL/Fas interaction (44). In contrast, however, Jagger et al. concluded from co-culture experiments that FasL/Fas-mediated killing of activated human monocytes is mediated by effector T cells (Teff)

rather than Treg, and that the effects of Treg were predominantly suppressive rather than pro-apoptotic (45). Nevertheless, Grossman et al. have also reported Treg to be capable of inducing apoptosis via perforin in multiple cell types, including CD14⁺ monocytes (46). Thus, the significance of Treg-mediated killing in modulating mononuclear phagocyte-associated inflammation requires further investigations.

Effects of Treg on phagocytosis in the context of inflammatory disease have also been reported (Figure 1). In atherosclerosis, for instance, phagocytosis by macrophages contributes to the resolution of arterial wall plaques and, in this context, Sharma et al. have recently demonstrated, in mice, that Treg prime resident macrophages to be phagocytic and pro-resolving. Mechanistically, this effect was mediated by Treg-secreted IL-10 and macrophage-derived IL-13 (47). Finally, there is evidence that resident Treg have specialized functions for local regulation of mononuclear phagocyte activity in inflammation-sensitive tissues. For example, Xie et al., reported that cerebral, but not splenic, Treg modulated the phenotype (upregulated CD163, downregulated RT1B) and cytokine production (downregulated TNF, IL-1 β , and IL-6) of resident macrophages (microglial cells) in an IL-10-dependent manner, to maintain immune homeostasis in the central neuron system (14).

Granulocytes

In comparison to mononuclear phagocytes, there has been relatively limited investigation of Treg interactions with granulocytes. Nonetheless, it has been proposed that Treg can mediate anti-inflammatory and pro-tolerogenic effects through their influences on neutrophils. As described by Lewkowicz et al., Treg activated by LPS or CD3/CD28 ligation induced expression of a range immune suppressive pathways in neutrophils through different mediators in addition to promoting their apoptosis (48). In a subsequent study, these authors showed that human neutrophils co-cultured with activated Treg, had increased expression of anti-inflammatory molecules (IL-10, TGF- β 1, IDO and HO-1). Upregulation of IDO was dependent on Treg expression of CTLA-4, although the corresponding target on neutrophils was not clear (49). In a mouse model of sepsis complemented by human co-culture experiments, Okeke et al., demonstrated that a PI3K α -dependent Treg function limited severe inflammation by promoting neutrophil apoptosis (50). Adoptively transferred Treg have also been shown to improve intestinal barrier function in association with decreased frequency of neutrophils in the intestinal tissue in the setting of heatstroke (51). In a meticulous study of mouse autoimmune hepatitis, Umeshappa et al. observed that autoantigen-specific Tr1 cells together with B regulatory cells (Breg), orchestrated neutrophil recruitment to the liver and reprogrammed them to myeloid-derived suppressor cell (MDSC) subtypes via GM-CSF, IL-10, and TGF- β . These regulatory neutrophils subsequently protected liver tissue from autoimmune injury via cathelin-related anti-microbial peptide (CRAMP) (52). Of interest, human Treg have been shown to recruit neutrophils *in vitro* by secretion of CXCL8 (53). Furthermore, neutrophils have

been found to be spatially distributed closer to Foxp3⁺ Treg and to potentially modulate CD4⁺ T cell differentiation via PD-L1/PD-1 interactions (54). Less well understood, however, is the relationship of Treg with NETosis, a process by which neutrophil extracellular traps (NETs) are extruded by neutrophils prior to cell death. A positive correlation between Treg with high-risk NETosis score was identified in ANCA glomerulonephritis (55). However, it has also been reported that Treg tend to have no impact on NETosis of polymorphonuclear neutrophils stimulated by either immune complex or PMA (56). Inversely, in diverse animal model settings, NETs have been shown to promote Treg differentiation from naïve CD4⁺ T cells and to be supportive of Treg suppression potency (57). Nonetheless, NETs have also been reported to promote differentiation of naïve CD4⁺ T cells towards Th17-like phenotype either *in vitro* or *in vivo* (58).

As is clear from these studies, different Treg subtypes are capable of complex cross-talk with neutrophils during acute and chronic immune/inflammatory responses. How such interactions influence disease outcomes or could be exploited in therapeutic contexts remains unclear and it should be acknowledged that some of the published work in this area indicates a potential for detrimental effects. For example, Treg in skin expressing the integrin α V β 8 use latent-activated TGF- β in a cell contact-dependent manner to induce CXCL5 expression by keratinocytes, recruiting neutrophils and, as a result, contributing to a delayed epithelial regeneration (59). Figure 2 summarizes reported Treg

granulocyte interactions along with their key mediators and functional outcomes.

Mast cells (MC), as a type of tissue-resident granulocytes, play a pro-inflammatory role in parasite infection and anaphylaxis in addition to mediating regulatory effects on innate and adaptive immune responses under other conditions. In the past two decades, it has become increasingly clear that Treg modulate MC activation and migration in different clinical settings and by numerous mechanisms. A prototypical mechanism is cell-cell contact based on OX40 (on MC) and OX40L (on Treg) resulting in downregulation of the Fyn/Gab2/PI3K pathway and suppressed degranulation of MC, halting allergic inflammation (60). Treg-derived TGF- β has been reported to prime MC IL-6 production, which facilitates clearance of neutrophils in innate lung inflammatory (61) – an effect that is dependent on membrane-bound rather than soluble TGF- β (62). Treg-derived TGF- β is also reported to function in counterpart with IL-4 to modulate MC activation by reciprocal downregulation of their receptors (63). Unlike TGF- β , Treg-secreted IL-9, in a soluble form, promotes MC migration to skin allografts to promote immune tolerance (64), or to draining lymph node to promote renal protection in the nephrotoxic serum nephritis model (65). However, one study has reported that Treg suppressed rather than enhanced IL-9 production, resulting in abrogation of IL-9-dependent MC degranulation and aggravation of intestinal nematode burden in *S. ratti* infected mice (66).

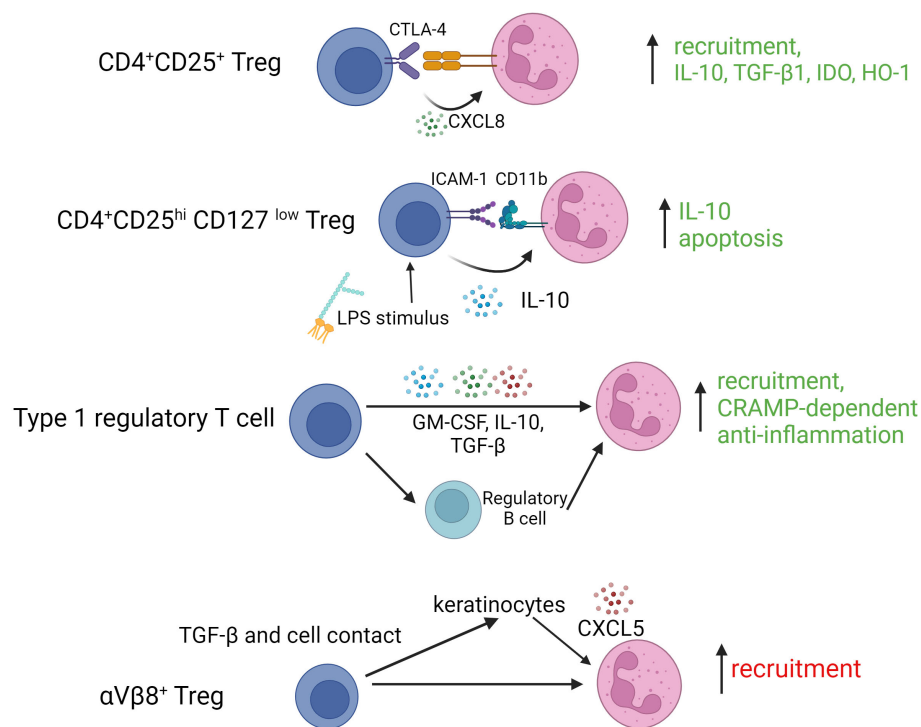


FIGURE 2

Interactions of various regulatory T cell subtypes with granulocytes. TGF- β 1, transforming growth factor beta 1; IDO, indoleamine-2,3-dioxygenase; HO-1, hemoxygenase 1; ICAM-1, intercellular adhesion molecule 1; LPS, lipopolysaccharide; GM-CSF, granulocyte macrophage colony stimulating factor; α V β 8, integrin alphaVbeta8.

Mast cells, in turn, influence Treg phenotype and function in similar fashion to Treg cross talk with monocytes and neutrophils. For example, MC-derived IL-2 is indispensable for maintaining resident Treg/Teff ratio for skin homeostasis in chronic allergic dermatitis (67). Similar effects on Treg were also observed for MC-secreted TGF- β (68). In contrast, MC-derived histamine muted Treg suppressive potency on effector T cells by signaling through the H1 receptor *in vitro* (69). Mast cells, in particular stimulated by IL-33 (70), may also drive Treg into Th17-like phenotypes via OX40/OX40L-mediated cell contact in presence of MC-derived IL-6 (71). Overall, therefore, the interplay between Treg and MC is clearly complex with diverse potential outcomes, necessitating further, context-dependent research before it can be directly exploited for therapeutic purposes. A recent comprehensive review focused on therapeutic opportunities for cholangiopathies provides a more detailed delineation of Treg/MC cross talk (72).

Regulatory T cell interactions with dendritic cells

Dendritic cells represent a key interface between innate and adaptive immunity, sensing microbial and other threats in tissues through pattern recognition and responding by producing soluble mediators of inflammation as well as by migrating to secondary lymphoid organs while processing and presenting foreign peptides to activate naïve antigen-specific CD4⁺ and CD8⁺ T cells (73). The interplay between DC and Treg has been extensively studied and Treg/DC cross-talk has been established to be an important pathway by which Treg support immune tolerance to self- and non-threatening foreign antigens (74). Although the focus of this review is on Treg effects on innate immunity, the complex mechanisms underlying Treg interactions with DC and their downstream influences on both innate and adaptive aspects of DC functions merit some description as they provide important insights into the potential for Treg-based therapies to be applied to a range of IMID.

In this regard, both surface interactions and exchange of soluble mediators between Treg and DC are of significant interest. As potent antigen-presenting cells, activated DC abundantly express the B7 family surface proteins CD80 and CD86 which mediate co-stimulation for initialization of Teff activation by binding to the CD28 receptor (75). In contrast, Treg inherently express higher levels of CTLA-4 (CD152) which competes with CD28 for binding to CD80/CD86 and, thereby, limits DC-stimulated Teff responses. In addition to competing with CD28, however, Treg are also known to directly downregulate DC expression of CD80/CD86 through CTLA-4 (76). Of interest, one mechanism for this downregulation has been directly visualized as a physical process by which CD80/CD86 molecules are removed from the DC surface by Treg and internalized (trans-endocytosis) (77) or incorporated into the Treg surface membrane (trogoncytosis) (78). Depletion of CD80 from the DC surface may lead to an increase of free PD-L1 surface expression, favoring co-inhibition of Teff activation (79). Thymic-derived Treg have also been shown to induce endocytosis of CD70 from the surface of DC, thereby inhibiting the CD70/CD27 pathway

which is required for DC-primed Th1 responses (80). It is also noteworthy that the mechanisms and outcomes of these interactions could vary between secondary lymphoid tissues and inflamed organs such as autoimmune target organs or transplants. For example, Dai et al. recently reported in an allogeneic islet transplant model that, while Treg contacts with DCs and suppression of DCs/T effector interactions within the inflamed allograft were dependent on adenosine generated by the ectonucleotidase CD73, contacts between Treg and DCs within the spleen were independent of CD73 (81).

Soluble factors secreted by Treg, in particular IL-10 and TGF- β , also suppress DC surface expression of key proteins including HLA-DR, CD80/CD86 and CD40 as well as their production of pro-inflammatory cytokines such as TNF and IL-12 (82, 83). Another cytokine, IL-35, produced predominantly by thymic Treg, has been reported, in mice, to induce tolerogenic DC characterized by increased expression of CD11b and IL-10 and decreased MHC-II (84). Zhang et al., have demonstrated the combined roles of human Treg cell surface proteins (CTLA-4) and soluble factors (IL-10 and TGF- β) in inducing a type of tolerogenic DC from primary monocytes in the presence of T helper cells (85). Another mechanism for non-contact-dependent modulation of DC by Treg occurs through release and uptake of extracellular vesicles (EVs). For example, Tung et al. reported that murine CD4⁺Foxp3⁺Treg-derived EVs induced increased IL-10 and decreased IL-6 production in LPS-activated DCs, and this modulated cytokine production is in association with evidence of EV-mediated trafficking of miR-150-5p and miR-142-3p (86).

The question of whether Treg modulation of DC functions is dependent on recognition of DC-presented MHC II/peptide complexes by the Treg remains under investigation and is of particular importance to the clinical translation of Treg therapies for immune-mediated diseases without a well characterized auto- or allo-immune basis. In this regard, it is reasonable to hypothesize that antigen-specific Treg mediate more potent modulation of inflammatory processes than polyclonal Treg if DCs presenting their cognate antigens are present. Indeed, in the setting of experimental autoimmune (type 1) diabetes, antigen-specific Treg, based on a unique TCR repertoire capable of recognizing specific class II MHC-presented peptide antigens, have been clearly shown to prevent disease more potently than polyclonal Treg (87, 88). Furthermore, adoptively transferred or *in vivo*-induced antigen-specific Treg are considered to have enhanced potency to contribute to disease-specific immune regulation, consequently necessitating a lower dose of infused Treg and reducing the potential for systemic immunosuppression (89). In keeping with this, Akkaya et al. observed, in co-cultures, that mouse antigen-specific iTreg formed stable compact clusters around cognate antigen-pulsed DCs and stripped off MHC-II peptide complexes from the DC surface to compromise DC-induced activation of naïve antigen-specific Teff. This interaction was not present in co-cultures of polyclonal Treg with DC. The findings were replicated in *in vivo* experiments, further supporting the conclusion that Treg capture of MHC/peptide complexes from DC was antigen-specific (90). In a study by Liang et al., Treg expanded by culture with antigenic peptide-bearing DCs, subsequently suppressed DC maturation through Lag-

3:MHC-II-based cell contact only in the presence of peptide (91). Of note, however, polyclonally expanded Treg were not used as controls in these studies. Furthermore, others, have provided evidence that Treg suppress DC antigen presenting potency in an MHC-independent manner. For example, Chen et al. and Yan et al, reported that polyclonal Treg suppress DC interaction with conventional T cells, by inducing changes in cytoskeletal polarization through stable integrin-mediated contact independent of antigen and MHC-II (92, 93). In the study of Yan et al., IL-2 conditional polyclonal Treg were shown to build stable connections with MHC-II-deficient DCs and such conditioned DCs were incapable of activating CD8⁺ T cells (93). Specific molecular interactions reported to be involved in the strong adhesion between Treg and DC include LFA-1/ICAM-1 (92, 94); integrin $\alpha 4\beta 1$ (95) and Nrp1 (96), regardless of MHC restriction. Overall, more investigation is needed to characterize and validate the mechanisms underlying Treg/DC physical interactions and to exploit non-antigen-specific Treg modulation of DC in inflammatory diseases that lack an auto- or allo-immune component. More broadly, whether TCR/MHC peptide engagement is necessary for or enhances Treg suppression of the pro-inflammatory functions of other MHC II-expressing innate immune effectors such as monocytes/macrophages and granulocytes merits additional study.

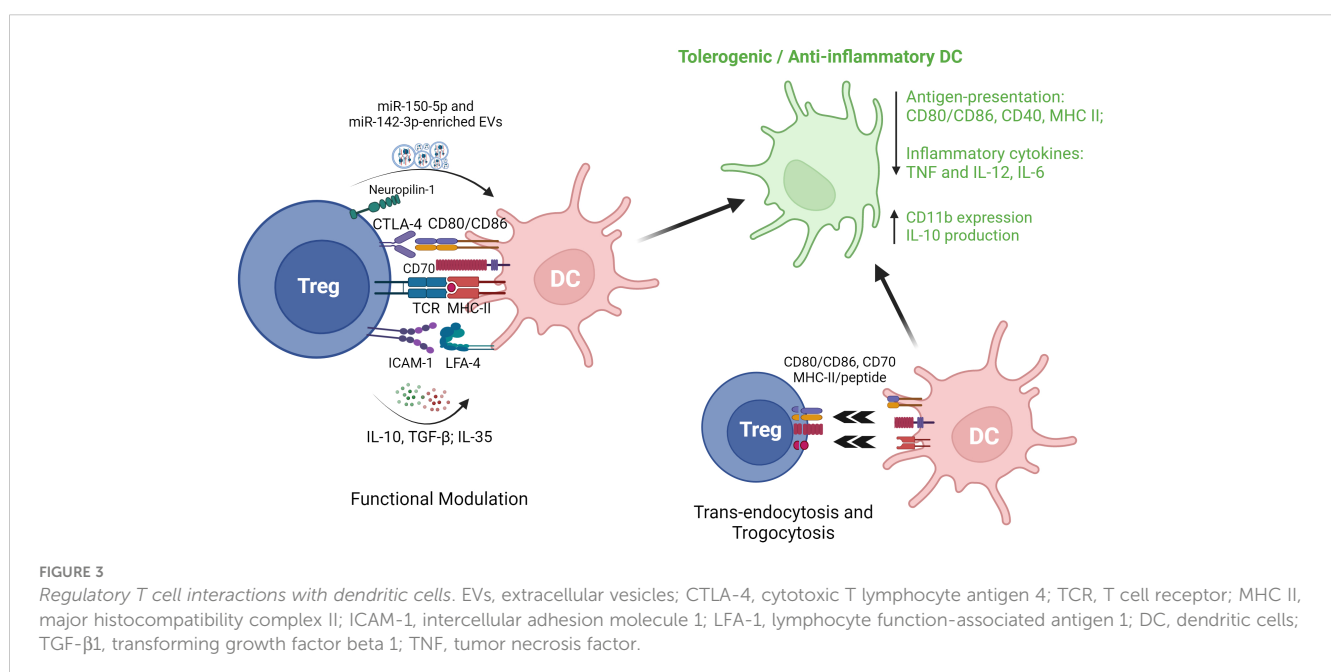
Besides canonical CD4⁺Foxp3⁺ Treg, regulation of DC by other types of Treg has also been documented. In a study involving mouse and human experiments, Liu et al. reported that FoxA1⁺ Treg with a deficiency in Foxp3 expression yet a higher expression of PD-L1, reduce IL-12 and IL-17 production by LPS-activated HLA-DR⁺ antigen presenting cells (APC) (5). Other studies have investigated the cross-talk between IL-10-secreting DC (DC-10) and Tr1 cells (97), demonstrating that co-culture of human DC-10 with CD4⁺ T cells results in the induction of antigen-specific Tr1 in the setting of autoimmune disease (98) and organ transplantation (99). Initially

identified and purified as CD14⁺CD11c⁺CD83⁺ cells (100), DC-10 could also be generated from bead-selected CD14⁺ monocytes by culture in presence of GM-CSF and IL-4 (99). Recently, a better-defined DC-10 population (CD14⁺CD16⁺CD141⁺CD163⁺) has been purified from blood and shown to exhibit similar IL-10 production and Tr1 induction potency (101). Importantly, ex vivo generated Tr1 have been recognized as a promising anti-inflammatory cell therapy and may modulate DC to more tolerogenic phenotypes characterized by higher expression of ILT-3, ILT-4 and HLA-G. Induced Tr1 can also release granzyme B and perforin through CD2/CD58- and CD226/CD155-based cell contact, to induce cell death in myeloid cell-derived APC. Despite lack of Foxp3 expression, Tr1 express key Treg-associated receptors (e.g. CTLA-4, PD-1 and ICOS) and are capable of similar suppressive regulation of DC through cell-cell contact (102). A range of the reported mechanisms of Treg modulation of DC phenotype and function are summarized in Figure 3.

Regulatory T cell interactions with innate or innate-like lymphocytes

Natural killer cells

Natural killer cells, a subset of innate immune cells derived from lymphoid progenitors, play important roles in the control of viral infection and tumorigenesis in an MHC-independent manner. The effector functions of NK cells include cytotoxicity and induction of programmed cell death in target cells as well as production of pro-inflammatory cytokines. Numerous studies have provided evidence that Treg counteract NK cell effector functions by decreasing cytotoxic activity and proliferation, downregulating activation receptors (NKG2D and NKp44), upregulating inhibitory receptors (CD158a, CD158b, and NKG2A) (103), and suppressing



production of IL-12 and IFN- γ (104). Mechanistically, membrane-bound TGF- β on Treg has been shown to be an important mediator of Treg suppression of NK cells response in the setting of cancer (104). In a mouse model of hepatitis B, Treg-mediated inhibition of NK cell cytotoxicity was attenuated by blockade of OX-40L, identifying a role for the OX-40/OX-40L pathway in protecting against NK cell-mediated chronic liver injury in this infection (105). Intrahepatic Treg also suppressed NK cell degranulation and NKG2D expression in a CTLA-4-dependent manner, contributing to decreased liver fibrosis by regulating NK cell interactions with hepatic stellate cells (106). Secretion of IL-37 by Treg is reported to suppress NK cell proliferation, cytotoxicity and IFN- γ production through its receptor IL-1R8 in association with NK cell downregulation of TIM-3 and upregulation of PD-1 (107). In a mouse model of autoimmune diabetes, an indirect mechanism of suppression of NK cells by Treg has been described by which Treg consumed IL-2 produced by islet-infiltrating CD4⁺ cells, thereby curbing the localized expansion of NK cells (108). A recent study also suggested that IL-10⁺ Treg suppress cytolytic activity of NK cells in acute retroviral infection. In this study, Littwitz-Salomon et al. showed that Treg were activated with increased expression of ICOS, IL-10 and TGF- β during the early, acute phase of Friend Retrovirus (FV) infection in mice. Depletion of Treg or neutralization of IL-10 (but not TGF- β) resulted in increased NK cell anti-viral activity (109). Figure 4A illustrates various reported mechanisms of Treg modulation of NK cell cytolytic and pro-inflammatory functions.

Modulation of NK cells by Treg has also been reported to contribute to the maintenance of immune tolerance in models of allo-transplantation and autoimmune disease. For example, in mouse cardiac allografts, depletion of Treg resulted in increased graft infiltration by both NK cells and CD4⁺ T cells in association with more severe transplant vasculopathy. Of interest, the exacerbation of vasculopathy following Treg depletion was ameliorated by depletion of NK cells, implying an important role

for Treg suppression on NK cells in allograft survival (110). A similar protective role for Treg-mediated NK cells suppression has been reported in bone marrow transplantation (111) and autoimmune hepatitis (112).

In contrast to the settings described above, the crosstalk between Treg and NK cells within the uterus during pregnancy appears to form a positive feedback loop in which depletion or dysfunction of either cell type can result in miscarriage (113). The canonical pro-inflammatory role of NK cells has also been challenged in autoimmune disease. While NK cells can be characterized as pathogenic through direct cytotoxicity and production of inflammatory cytokines, they may regulate adverse immune response by eliminating autoreactive immune cells and by serving as a source of anti-inflammatory mediators such as IL-10, TGF- β , and IL-4 (114). Thus, the combined immunological roles of NK cells and Treg are likely to be complex and highly contextual, necessitating more disease-specific research in human subjects to better understand the likely effects of Treg-based therapies on NK cell-mediated innate immune responses. This may be of particular interest in auto-inflammatory diseases in which sterile inflammation and dysfunctional innate immunity occur in the absence of autoantibodies and autoreactive T cells (115, 116).

Natural killer T cells

Natural killer T (NKT) cells are a specialized type of innate-like lymphocytes, yet have the property of both NK cells and T cells by recognizing glycolipid ligands bound to the MHC class I-like protein CD1d via a restricted T cell receptor (TCR) repertoire and by widely participating in immune responses to infection, cancer, transplantation and fetal-maternal immunity (117). It was shown 20 years ago that human CD4⁺CD25⁺ Treg suppressed V α 24⁺NKT cell proliferation, cytotoxicity to tumor cell lines and cytokine production (IFN- γ , IL-4, IL-10, and IL-13) upon

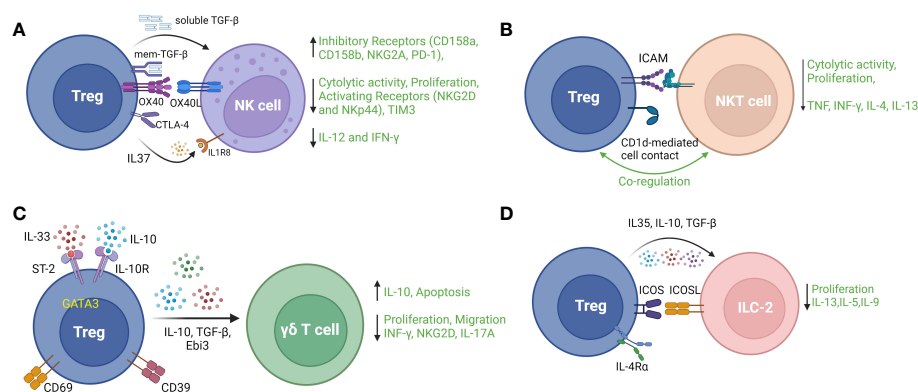


FIGURE 4

Regulatory T cell interactions with four main types of innate or innate-like lymphocytes: (A) Natural Killer (NK) cells, (B) Natural Killer T (NKT) cells, (C) Gamma delta ($\gamma\delta$) T cells (D) type 2 innate lymphoid cells (ILC-2). TGF- β – transforming growth factor beta 1; NKG2A, natural killer group 2A; PD-1, programmed cell death 1; NKG2D, natural killer group 2D; TIM3, T cell immunoglobulin and mucin domain-containing protein 3; Nkp44, natural killer p44; IFN γ , interferon gamma; ICAM – intercellular adhesion molecule; TCR, T cell receptor; TNF, tumor necrosis factor; ST-2, suppression of tumorigenicity 2; IL-10R, interleukin 10 receptor; Ebi3, Epstein Barr virus-induced; ICOS, inducible T cell costimulatory; ICOSL, inducible T cell costimulatory ligand; IL-4R α , interleukin 4 receptor alpha.

stimulation by α -galactosylceramide (α GalCer)-pulsed Mo-DCs. This suppression was dependent on direct contact between Treg and NKT cells and could be neutralized by antibody against ICAM but not IL-10 or TGF- β (118). Subsequently, Venken et al. reported that Treg suppressed proliferation and modulated cytokine production of human invariant (i)NKT cells in response to glycolipid or to innate stimuli. Conversely, Treg were induced to produce high amounts of IL-10 following contact with stimulated iNKT cells, indicating cross-talk between the two cell types (119). Evidence for direct Treg/NKT cell cross-talk *in vivo* has been reported by Hua et al., who demonstrated that CD1d deficient mice had reduced Treg number and function in the liver and that adoptively transferred Treg ameliorated NKT cell-mediated liver injury in a CD1d-dependent manner (120). In co-cultures of blood-derived human cells, induced Treg were shown to increase the proportion of CD4⁺ NKT cells with lower cytotoxicity and pro-inflammatory cytokine production than their CD4⁺ counterparts. Of interest, α GalCer-stimulated PBMC cultures from cancer patients contained higher proportions of both CD4⁺ NKT cells and effector Treg than those of patients with benign tumors (121).

In addition to the findings from studies such as these that Treg suppress NKT cell activation and function, predominantly through contact-dependent interactions, there is also experimental evidence that activated NKT cells delete or suppress the counter-regulatory functions of Treg in settings such as allergic asthma and pregnancy loss (122, 123). Of interest, NKT cells were also reported to promote Treg expression of negative costimulatory receptors PD-1 and IL-10 production through secreting IL-4 (124), and NKT cell-secreted IL-2 helped sustain Treg survival (125). Type 2 NKT cells, a subset with a more diverse TCR repertoire, have been documented to have immunomodulatory potential and to protect from autoimmune disease (126). Figure 4B summarizes aspects of Treg/NKT cell cross-talk described above. Overall, there is compelling evidence for direct, clinically-relevant interactions between Treg and NKT cells that merit further investigation – particularly in relation to diseases and treatment strategies in which NKT cells participate in dictating the nature of tissue inflammation.

Gamma Delta T cells

$\gamma\delta$ T cells represent a T cell subset which express TCRs consisting of γ and δ chains rather than the canonical TCR composed of α and β chains. They are typically categorized as innate immune cells because their activation via the TCR is MHC-independent and because they also express a range of surface NK receptors which modulate their responses to stress-associated antigens (127). The effector functions of $\gamma\delta$ T cells include target cell cytotoxicity and production of inflammatory cytokines including IFN γ , TNF, IL-17A and IL-6. Accumulating evidence indicates that Treg exert regulatory effects on $\gamma\delta$ T cells. For example, in a mouse model of ischemic stroke, the beneficial effect of intracerebral administration of IL-10 was shown, in part, to be mediated by induction of FoxP3⁺ Treg to suppress IL-17A production by $\gamma\delta$ T cells (128). Similarly, IL-10-dependent Treg effects have been shown to reduce $\gamma\delta$ T cell proliferation in a model of spontaneous colitis

(129), and to inhibit human $\gamma\delta$ T cell IFN γ secretion in response to the *Mycobacterium tuberculosis* antigen ESAT-6 (130). Direct Treg suppression of proliferation and production of IL-17A and IFN γ by $\gamma\delta$ T cells was also observed in a mouse model of mucosal inflammation, although the mechanism was not elucidated (131). Along with IL-10, TGF- β has also been reported to participate in Treg suppression $\gamma\delta$ T cell responses. In patients with hepatocellular carcinoma (HCC), compromised anti-tumor potency of $\gamma\delta$ T cells was observed and Treg isolated from HCC tissue were suppressive of IFN γ production and tumor cell cytotoxicity by $\gamma\delta$ T cells. Importantly, these effects were abrogated by anti-IL-10 or anti-TGF- β 1 antibodies (132).

The role of specific Treg subpopulations in suppressing $\gamma\delta$ T cells has been investigated in some disease settings. In a mouse model of myocardial infarction (MI), Blanco-Domínguez et al. used depletion and adoptive transfer of Treg to demonstrate that CD69⁺ Treg, through CD39 ectonucleotidase activity, protected against myocardial inflammation by suppressive effects on $\gamma\delta$ T cells including induction of apoptosis, inhibition of IL-17A production and reduced migration to infarcted myocardium. In human subjects, higher proportions of circulating CD69⁺ Treg were shown to be associated with reduced risk of re-hospitalization for heart failure following acute MI (133). In a model of respiratory mucosal injury, Faustino et al. observed that depletion of GATA3⁺ Treg expressing the IL-33 receptor, ST2, resulted in increased IL-17-secreting $\gamma\delta$ T cells and exacerbation of allergic lung inflammation. Mechanistically, the suppressive effect of ST2-expressing Treg on pulmonary $\gamma\delta$ T cells was dependent on Treg exposure to allergen peptide and IL-33 resulting in Treg-secretion of Ebi3 (a component of IL-35). The inhibition of $\gamma\delta$ T cells was associated with decreased eosinophil recruitment to the lungs (134). Figure 4C provides a summary of Treg markers, pathways and mediators shown to be relevant their modulatory interactions with $\gamma\delta$ T cells.

As for other innate immune effectors, $\gamma\delta$ T cells may also compete with and inhibit Treg functions. For example, IL-23 secretion by $\gamma\delta$ T cells was shown to reduce Treg numbers and suppression of CD4⁺ $\alpha\beta$ T cells during mouse autoimmune encephalomyelitis (135). Similarly, secretion of IFN γ by $\gamma\delta$ T cells may suppress the induction of Treg differentiation from CD4⁺ T cells as well as Treg expansion and suppression of effector T cells *in vitro* and *in vivo* (136–138). Importantly, in some settings, $\gamma\delta$ T cells may also mediate regulatory effects on immune responses (139). Nonetheless, the weight of experimental evidence to date indicates that Treg can serve an important role in regulating the potent pro-inflammatory effects of $\gamma\delta$ T cells in a range of diseases and that development of Treg-based therapies that replicate this function may be a promising translational goal.

Innate lymphoid cells

Although NK cells have been characterized as innate lymphoid cells for several decades, additional subtypes of bone marrow-derived cell types with lymphoid morphology and non-antigen specific immunological functions have been described more

recently and termed ILC1, 2 and 3. These cells are present in lymphoid organs and blood but occur at higher concentrations at epithelial barriers and other tissue sites and play important roles in regulating the responses to infection or injury and in maintaining tissue homeostasis. They serve as a potent source of cytokines and can be viewed as serving as “helper cells” during evolving immune/inflammatory responses. The analogy to helper cells is strengthened by the associations of ILC1, 2 and 3 with production of signature cytokines that match those of CD4⁺ Th1, Th2 and Th17 cells respectively (140, 141).

Investigation of the interactions between Treg and ILC is in its infancy and we focus here on recent studies related to ILC2 which represent the most numerous ILC and serve to control extracellular parasitic infection and widely participate in mucosal immunity, by secreting type 2 cytokines including IL-4, IL-5, IL-9, IL-13. Rigas et al. have shown that human TGF- β -induced Treg suppressed IL-5 and IL-13 secretion by ILC2 through contact-dependent (ICOS/ICOS-L) and soluble (TGF- β and IL-10) mediators to control airway inflammation in a humanized model of ILC-2-dependent asthma. Interestingly, this effect was absent in naïve Treg (142). Similarly, in a mouse model of allergic asthma, Khumalo et al. demonstrated that Treg expression of the IL-4 receptor alpha was necessary for Treg-mediated suppression of ILC2 proliferation and secretion of IL-5 and IL-13 (143). In co-culture experiments informed by a mouse model of asthma, Krishnamoorthy et al. observed dose-dependent Treg suppression of IL-13 secretion by ILC-2, which was enhanced by Treg priming with the fatty acid derivative maresin 1 (144). Lui et al. observed increased proportions of ILC2 and related type 2 cytokines in PBMC from patients with allergic rhinitis which was accompanied by reductions in IL-35-producing Treg. Subsequently, IL-35 and induced IL-35-producing Treg were shown to suppress ILC2 in activation cultures. A role for ICOS/ICOSL in Treg suppression of ILC2 was also shown in this study (145). In another recent study of PBMC from allergic rhinitis patients, Treg-secreted IL-10, induced by co-culture with induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSC), was shown to suppress ILC-2-secretion of IL-13, IL-9, and IL-5 in an ICOS/ICOSL-dependent manner (146). The key mediators of Treg/ILC2 interactions that have been described to date are summarized in Figure 4D.

The impact of Treg on ILC2 has been investigated in non-allergic pathological contexts as well. For example, in a mouse model of chronic pancreatitis, Treg depletion resulted in increased proportion of ILC2 cells, but not ILC1, in spleen and pancreatic tissue and concomitantly aggravated pancreatic fibrosis (147). Finally, in contrast to other studies described here, Treg and ILC2 were shown by Gao et al. to play complementary roles in reducing the severity of atherosclerosis in a mouse model. In this study, Treg tended to promote rather than suppress ILC2 and ILC2-derived IL-13 in a cell-contact and cytokine-based (IL-10 and TGF- β) manner (148). Notably, ILC2, much like Treg cells, express CD25 and are consumers of IL-2. A consequence of this is that ILC2 may be inadvertently expanded by administration of low-dose IL-2 intended to enhance Treg numbers *in vivo* (149). Whether there is natural antagonism between Treg and ILC2 during immune responses through competition for IL-2 remains to be directly

investigated. Interestingly, it has been reported that IL-2 produced by ILC3 aids in supporting regulatory functions of Treg cells to maintain gut tissue homeostasis (150), while also serving as a cofactor to ILC2 survival/proliferation in lung inflammation (151). Thus, in common with other innate immune cell types reviewed here, the interactions described to date between ILC and Treg reflect a range of mechanisms and outcomes. Nonetheless, consistent striking observations of direct Treg-mediated suppression of maladaptive ILC2 activity in asthma and allergy speak to a distinct area of clinical potential.

Relevance to human diseases and translational opportunities

In contrast to the growing number of clinical trials of Treg-based therapies in patients with autoimmune diseases and organ transplants, little clinical translation has occurred to date of the capacity of Treg to modulate innate immune responses. Nonetheless, as we have reviewed in the preceding sections, there is a wealth of basic and pre-clinical evidence that specific Treg functional characteristics could be exploited as novel treatment strategy for innate immune-mediated inflammatory diseases (152). We conclude this review with short appraisals of evidence in favor of the development of Treg-based therapies for three common inflammatory conditions in which innate immune responses play a key role: type 2 diabetes (T2DM), ischemia reperfusion injury (IRI) and atherosclerosis.

Firstly, in the case of T2DM and its major complications, some human studies have observed numerical defects in circulating Treg in association with the presence or progression of diabetic nephropathy, retinopathy (153) and diabetic foot ulcer (154). In addition, animal studies have demonstrated that adoptive transfer or *in vivo* expansion of Treg ameliorate the severity of T2DM and its macro- and micro-vascular complications. For example, in diabetic pigs, intravenously injected Treg were shown by Guo et al. to protect tissue-engineered blood vessels from intimal hyperplasia by inhibiting inflammation and cell apoptosis and promoting endothelial progenitor cell mobilization (155). Other studies in rodent models of diabetes have demonstrated that adoptively transferred Treg migrate to retina and reduce neovascular retinopathy (156) or suppress obesity-linked insulin resistance and preserve kidney function (157). In a model of stroke in T2DM, endogenous expansion of Treg by a CD28 superagonist was found to increase M2-type macrophages and to alleviate ischemic brain injury (28). Similarly, *in vivo* expansion of Treg in mice with T2DM by injection of a novel hybrid cytokine bearing IL-2 and IL-33 activities, reduced diabetic kidney damage (158). Importantly, functional as well as numerical Treg deficits may also occur in T2DM. For example, Zhu et al. observed increased proportions of IL-17-producing Foxp3⁺Treg in T2DM patients compared to healthy patients which associated with higher body mass index (BMI) and the HbA1c levels (159). Similarly, Sheikh et al. reported that CD4⁺CD25⁺CD45RA⁺ Treg have comparable frequency but reduced suppressive potency in T2DM compared to non-diabetic controls (160). Overall, these and other studies

provide evidence that augmentation of the autologous Treg repertoire in T2DM, either through ex vivo or endogenous expansion, has potential to prevent or reverse multiple severe diabetic complications.

Ischemia reperfusion injury refers to an acute compromise to the blood supply of an organ or tissue resulting in cellular damage or necrosis followed by a restoration of blood flow which further aggravates tissue injury by activating inflammatory processes. A significant role for Treg in alleviating inflammation has been demonstrated through Treg depletion and adoptive transfer in animal models of intestinal and renal IRI and of ischemic stroke (161, 162). In keeping with modulatory effects on mononuclear phagocytes summarized in an earlier section, ex vivo expanded Treg have also been shown to modulate the production of TNF and IL-1 β by Kuffer cells and, thereby, protect against acute liver injury due to IRI (163). More widely studied has been the impact of Treg on myocardial IRI. A recent detailed review of this topic highlights that Treg, migrating to myocardial tissue after infarction through CCR5, the CXCR4-CXCL12 axis, and the Hippo pathway, may promote myocardial tissue repair through multiple mechanisms including modulation of macrophage phenotypes, direct interactions with cardiomyocytes and increasing collagen content and maturation in the infarct zone (164). A distinct heart-resident Helios^{high}Nrp-1^{high} Treg population, which expresses Sparc and is sustained and expanded by IL-33/ST-2 interaction was also identified and proved to be the primary mediator of Treg-mediated repair following myocardial infarction (165). In acute kidney injury, a CXCR3⁺ Treg subpopulation was found to be recruited to kidney tissue following reperfusion and to correlate with lower renal tissue injury and better kidney function (166). As evidenced by this predominantly pre-clinical literature, therapeutic strategies aimed at expanding or delivering pro-repair Treg to the site of IRI-related tissue injury have promise for limiting the consequences to brain (162), heart, kidney, liver, intestine and other organs in clinical settings. However, successful translational strategies in this area will likely require highly stable Treg-based therapies with distinct migratory and effector functions.

Atherosclerosis refers to the formation of cholesterol-containing plaques in arterial blood vessels, which may result in severe acute or chronic cardiovascular conditions due to reduced blood flow to tissue or rupture leading to thrombosis. Following initial changes to endothelial cells, increasing evidence indicates that progression of atherosclerotic plaques is driven by inflammatory responses. Indeed, three recent clinical trials of anti-inflammatory agents have proven beneficial effects in patients with atherosclerosis, including reductions in cardiovascular events and mortality (167–169). A number of studies have provided evidence for anti-inflammatory and athero-protective roles for Treg in atherosclerosis, including correlations between disease progression and defects of Treg numbers (170) or function (171). In animal models, Sharma et al, provided more direct evidence that Treg protect from rupture and promote regression of atherosclerotic plaques through modulatory effects on macrophages (47). Adoptively transferred Treg have also been found to contribute to reduced size and contraction of atherosclerotic plaques in hypercholesterolemic

mice (172, 173). Studies such as these provide convincing evidence that Treg-enhancing approaches could be of value as a therapeutic strategy for reversing atherosclerosis. It is worth noting, however, that challenges will arise in maintaining the stability and adaptability of Treg in the pro-inflammatory setting of atherosclerosis (174, 175). In this regard, Treg within atherosclerotic aortas have been reported to have a high expression of Foxp3 but also low CD25 levels, expression of Th1-associated factors IFN- γ and T-bet and compromised suppressive potency (176). Understanding Treg metabolism under hypoxic conditions may help to address this issue.

Conclusion

As we have described here, the capacity for Treg to suppress innate immune responses and to ameliorate acute and chronic diseases involving non antigen-specific inflammation is well established. Furthermore, many mechanistic details of the specific modulatory effects of Treg and Treg subpopulations on individual innate immune effectors have been identified in experimental animals as well as human cells. That this body of knowledge could be exploited to develop Treg-enhancing strategies for a much broader range of conditions than that which they have, to date, been applied seems self-evident. Importantly, Treg cell therapies have proven to be safe in early phase clinical trials and are now in the process of overcoming several important obstacles to their more effective use in autoimmune diseases and organ transplants. While this progress should provide a valuable platform for extending Treg therapies to diseases driven primarily by innate immune responses, additional challenges related to tissue targeting, *in vivo* stability and selection for the required effector mechanisms will almost certainly require an extensive amount of focused research and innovation. Nonetheless, we propose that the existing evidence is sufficient to support a greater investment of time and resources in the development of interventions to augment Treg modulation of innate immunity.

Author contributions

QO: Conceptualization, Funding acquisition, Writing – original draft, Writing – review and editing. RP: Funding acquisition, Writing – original draft. MG: Conceptualization, Funding acquisition, Supervision, Writing – review and editing.

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Conflict of interest

MG reports honoraria from the American Society of Nephrology; Hebei Medical University, China; Théa Pharma Ltd., Ireland; and Novo Nordisk as well as advisory roles as an Editorial Board member for the journal Transplantation and an Associate Editor for Mayo Clinic Proceedings.

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Ankylosing spondylitis and psychiatric disorders in European population: a Mendelian randomization study

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Background: Epidemiologic evidence has demonstrated a correlation between ankylosing spondylitis and psychiatric disorders. However, little is known about the common genetics and causality of this association. This study aimed to investigate the common genetics and causality between ankylosing spondylitis (AS) and psychiatric disorders.

Methods: A two-sample Mendelian Randomization (MR) analysis was carried out to confirm causal relationships between ankylosing spondylitis and five mental health conditions including major depressive disorder (MDD), anxiety disorder (AXD), schizophrenia (SCZ), bipolar disorder (BIP), and anorexia nervosa (AN). Genetic instrumental variables associated with exposures and outcomes were derived from the largest available summary statistics of genome-wide association studies (GWAS). Bidirectional causal estimation of MR was primarily obtained using the inverse variance weighting (IVW) method. Other MR methods include MR-Egger regression, Weighted Median Estimator (WME), Weighted Mode, Simple Mode, and Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO). Sensitivity analyses are conducted to estimate the robustness of MR results.

Results: The findings suggest that AS may be causally responsible for the risk of developing SCZ (OR = 1.18, 95% confidence interval = (1.06, 1.31), $P = 2.58 \times 10^{-3}$) and AN (OR = 1.32, 95% confidence interval = (1.07, 1.64), $P = 9.43 \times 10^{-3}$). In addition, MDD, AXD, SCZ, AN, and BIP were not inversely causally related to AS (all $p > 0.05$).

Conclusion: Our study provides fresh insights into the relationship between AS and psychiatric disorders (SCZ and AN). Furthermore, it may provide new clues for risk management and preventive interventions for mental disorders in patients with AS.

KEYWORDS

ankylosing spondylitis, psychiatric disorders, Mendelian randomization, major depression, anxiety disorder, schizophrenia, bipolar disorder, anorexia nervosa

1 Introduction

Ankylosing spondylitis (AS) is a chronic progressive inflammatory disease with the axial skeleton as the main site of development, leading to typically inflammatory low back pain, as well as severe structural and functional deficits (1). The prevalence of the disease is reported to be between 0.07% and 0.32% worldwide and is 2–3 times more prevalent in men than in women (2). The etiology of AS has not been elucidated. Genetics, infection, gut microbiota, mechanical stress, gender, and environmental and lifestyle factors have been identified as risk factors for AS (3).

A study on the worldwide burden of diseases showed that the burden caused by psychiatric disorders is worsening (4). Psychiatric disorders are one of the major causes of death all over the world (5). Notably, in addition to chronic pain and disability, people with AS often suffer from psychiatric disorders, with depression and anxiety being the most common psychological traits (6, 7). A previous study showed that patients with AS had an increased risk of affective disorders (depression and bipolar disorder [BIP]) and anxiety disorder (AXD), while the prevalence of schizophrenia (SCZ) was not above that of the general population (8). Another retrospective study found that AS was not associated with subsequent new diagnoses of BIP and SCZ (9). Conversely, Kang et al. reported a significantly increased prevalence of comorbid psychoses in individuals with AS (10). Moreover, a population-based cross-sectional study suggests that AS reduces the risk of SCZ (11). Evidently, these clinical observational studies have yielded conflicting outcomes. Furthermore, it is worth noting that an earlier diagnosis of AS or psychiatric disorders does not invariably signify an earlier onset in many cases. Additionally, it is imperative to acknowledge that observational studies may encounter potential confounding factors such as reverse causation and selection bias, which can impact their findings. Consequently, the precise relationship between AS and psychiatric disorders remains elusive.

Since alleles are randomly assigned as they are inherited from parents to offspring, the process can be considered analogous to randomized grouping in a randomized controlled trial. Moreover, genotypes are innately determined and are not interfered with by confounding factors such as growth environment, socioeconomic status, and behavioral traits. In the Mendelian randomization (MR) investigation, genetic variants closely associated with risk factors were utilized as instrumental variables (IVs) to provide evidence of a causal relationship between exposure and outcome (12). This

approach is being increasingly adopted because it addresses the primary limitation of evidence from observational studies: the lack of measurement of confounding factors (13). In the present study, two-sample bidirectional MR and multivariate MR analyses were conducted utilizing genome-wide association studies (GWAS) statistics to investigate the potential role of AS as a risk factor for psychiatric disorders and the possibility of reverse causality between psychiatric disorders and AS.

2 Materials and methods

2.1 Mendelian randomization study

Three core hypotheses for MR studies should be met (14): (i) Genetic IVs are robustly and strongly associated with AS (association hypothesis); (ii) Genetic IVs are independent of confounders influencing the exposure-outcome relationship (independence hypothesis); and (iii) Genetic IVs affect psychiatric disorders exclusively through AS (exclusivity hypothesis) (15). We examined potential pleiotropy using a range of MR methods to validate the second and third MR hypotheses. Horizontal pleiotropy exists if the variants affect the target outcome through other traits than the exposure pathway, or if the variants have a direct effect on the outcome (16). Similar hypotheses were applied to reverse MR analysis. A schematic diagram of the MR causality study design is shown in Figure 1, which explains the basic principles of MR studies.

2.2 Sources of statistical data

GWAS summary data is an umbrella term for a series of data that respond to the effect of single nucleotide polymorphisms (SNPs) on phenotype. GWAS data associated with AS were collected from the IGAS (International Genetics of Ankylosing Spondylitis) Consortium comprising 22,647 subjects of European ancestry (9,069 cases and 13,578 controls) (17). Diagnostic criteria for AS are based on the modified New York (mNY) criteria (18). Genetic data associated with the four psychiatric disorders MDD, BIP, SCZ, and AN were sourced from the Psychiatric Genomics Consortium (PGC) database (<https://www.med.unc.edu/pgc/download-results>) to data overlap to ensure independence between two samples. Additionally, we have extracted GWAS summary statistics for AXD from the FinnGen consortium as part of our analysis (<https://www.finnngen.fi/fi>) (19). MDD GWAS summary statistics were gathered from the largest GWAS meta-analysis of depression, which included 246,363 cases and 561,190 controls. It is worth mentioning, however, that the GWAS summary statistics provided by the 23andMe company have not yet been made accessible to the public. Consequently, our present MR analysis relied on a cohort comprising 170,756 individuals diagnosed with MDD and 329,443 control subjects (20). Details of the GWAS data are shown in Supplementary Table S1. Case definitions for AS and the 5 mental disorders are in Supplementary Table S2.

Abbreviations: GWAS, genome-wide association studies; MR, Mendelian randomization; IVs, instrumental variables; SNPs, single nucleotide polymorphisms; LD, linkage disequilibrium; MAF, minor allele frequencies; OR, odds ratio; CI, confidence interval; WR, Wald Ratio; IVW, inverse variance weighted; WME, weighted median estimate; MR-PRESSO, Mendelian random pleiotropy residual sum and outliers; LOO, leave-one-out; SCZ, schizophrenia; AN, anorexia nervosa; MDD, major depression disorder; BIP, bipolar disorder; AXD: Anxiety disorder; ClpB, caseinolytic protease B homologue; NMDA, N-methyl-D-aspartic acid receptor; IL, interleukin; TNF, tumor necrosis factor.

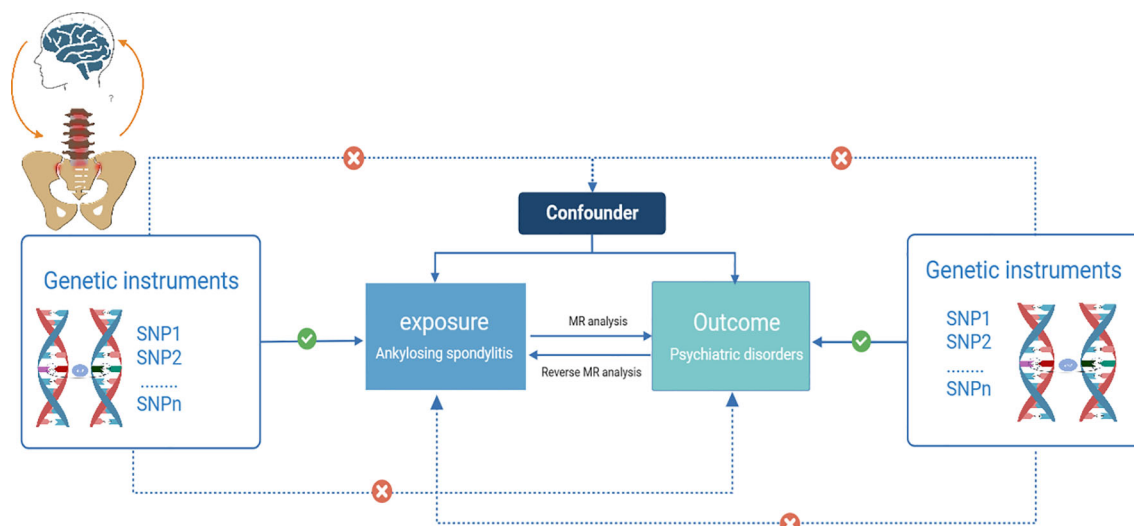


FIGURE 1

Schematic diagram of a two-sample Mendelian randomization study (Visualization of causal relationships between genetic instrumental variables, exposures, and outcomes. Solid arrows indicate permissible relationships between variables under the assumptions of Mendelian randomization, and dashed lines indicate prohibited relationships for valid IVs).

In our current MR study, to minimize potential bias due to population heterogeneity, individuals with AS or psychiatric disorders were restricted to European ancestry (21). No additional ethical approvals were required as data from public databases allowed open access to researchers.

2.3 Selection of IVs

Effective IVs were identified through careful filtering of the GWAS data, adhering to the core assumptions of MR. Initially, we searched for significant SNPs that were strongly correlated with AS at a genome-wide significance threshold of $p\text{-value} < 5 \times 10^{-8}$. Subsequently, SNPs with linkage disequilibrium (LD) were removed to ensure independence between IVs ($r^2 = 0.001$, $KB = 10,000$), as LD signifies a non-random association between alleles. Furthermore, we excluded allele frequencies that were less than 0.01 in terms of minor allele frequency (MAF). In cases where an exposure SNP was absent in the outcome-related GWAS summary data, it was substituted with a highly correlated SNP ($r^2 > 0.8$). IVs lacking alternative SNPs as well as outcome-related IVs ($p < 0.05$) were excluded from the analysis. After that, we employed harmonized effects to ensure effect allele alignments for exposure and outcome IVs, eliminating palindromic SNPs (defined as SNPs with A/T or G/C alleles) with moderate allele frequencies ($MAF > 0.42$). Finally, we estimated the strength of the association between SNPs and exposure using the F-statistic, calculated as follows: $F = \beta^2 / SD^2$, where β denotes the effect size of SNPs on exposure and SD denotes the standard deviation (22). To avoid bias in effect estimation, genetic variants with an F value below 10 were excluded, indicating their weak ability to explain exposure (23). The effective SNPs for the estimation of the causal effect of AS on psychiatric disorders, which were derived after a rigorous screening process, are presented in [Supplementary Table S3](#).

2.4 Statistical methods

As the primary and most accurate causal effect estimate, the inverse variance weighted (IVW) approach presumes that all SNPs are valid IVs. It estimates the overall causal effect by calculating the Wald Ratio (WR), which is the regression coefficient of the IV on the outcome divided by the regression coefficient of the IV on the exposure, for each SNP (15). The multiplicative random-effects IVW approach yields more conserved causal inferences than fixed-effects IVW and takes into account the uncertainty associated with pleiotropy. All “IVW” in this study refers to random-effects IVW unless otherwise stated (24). The IVW method yields robust findings when all IVs exhibit validity and there is no presence of horizontal pleiotropy. For genetic instruments involving a single SNP, we employed the WR. Additionally, other methods including MR-Egger regression, Weighted Median Estimator (WME), Weighted Mode, Simple Mode, and Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) were employed to supplement the results of MR to evaluate reliability and stability. The MR-Egger method, which considers that all genetic variants are null IVs, provides a valid test of the null causality hypothesis (14). This method performs weighted regressions with an intercept term, allowing the assessment of pleiotropy among the IVs, with the slope being the estimate of the causal effect (25). The MR-Egger regression model is the only straight line in the axes that does not automatically pass through the origin. When the proportion of invalid IVs is as high as half and the precision of the estimates varies considerably between IVs, the WME approach still provides credible effect estimates (26). Simple mode, which is a less accurate method than weighted mode, uses causal effect estimates from individual SNP to form clusters. Weighted mode method uses the same process but assigns weights to each SNP. While they may not be as robust as the IVW and WME methods in detecting causal effects, they do outperform MR-

Egger regression (27). The MR-PRESSO method was utilized to identify and remove outliers (SNPs with potential pleiotropy) and to estimate the corrected results (16).

Subsequently, we conducted a meticulous sensitivity analysis to identify and correct possible violations of the MR assumptions. Firstly, the IVW and MR-Egger methods were used to perform separate Cochran's Q-tests and Rücker's Q-tests to assess whether there was heterogeneity among IVs. A significance level of $P < 0.05$ was utilized to indicate potential heterogeneity, which was further visualized through a funnel plot. Notably, the presence of heterogeneity did not affect the results of the random-effects IVW estimation when the overall heterogeneity was balanced (24). The Steiger directionality test can be used to determine direction by calculating the proportion of variance explained by the IVs on the pair of exposure and outcome (28). To assess the validity of the overall direction of causality, we conducted an MR-Steiger directionality test for the validity of the hypothesis that exposure leads to outcomes (28). It is important to note that Steiger analyses can only provide preliminary evidence to verify directionality, not to establish causality. Additionally, the MR Egger intercept was employed for the horizontal polynomiality test, where a Y-intercept of zero would indicate the absence of any horizontal polynomial effect. Horizontal polynomiality is also detected by the MR-PRESSO global test, which estimates global heterogeneity to detect polynomiality (14, 16). A p-value of below 0.05 indicates the presence of pleiotropy in the selected IVs. Finally, for a comprehensive evaluation of the robustness of MR results, leave-one-out (LOO) analyses were employed, which evaluate whether the exclusion of one SNP had an impact on the overall effect of the remaining SNPs. It should be noted that the IVW method yields a statistically significant result while other complementary approaches do not exhibit statistical significance, and no evidence of pleiotropy was observed, one can deem it a favorable result, provided that the beta values of the other complementary approaches exhibit concordant directionality (29).

In this study, we corrected the p-values using the Bonferroni calibration method. As a consequence, we considered a Bonferroni-corrected threshold of $p < 0.01$ ($p < 0.05/\text{five exposures/one outcome}$) to be statistically significant as evidence that a causal relationship exists. It was considered suggestive evidence of causality if the P-value was between 0.05 and 0.01. The statistical analysis procedure described above was performed using the TwoSampleMR package (version 0.5.6) as well as the MRPRESSO package (version 1.0) within the R software (version 4.2.1). The TwoSampleMR code is freely available on this website under the following link: <https://mrcieu.github.io/TwoSampleMR>.

2.5 Reverse Mendelian randomization analysis

In the reverse MR analysis, we set the significance threshold of the p-value for IVs in psychiatric disorders more leniently to less than 5×10^{-6} to obtain sufficient SNPs. However, AN and AXD still failed to find usable SNPs in AS-associated GWAS despite a lower significance threshold of $p < 5 \times 10^{-6}$ after a series of stringent

screenings. Therefore, for AN and AXD we relaxed the threshold of the p-value of exposure-associated IVs in the screening condition to $< 5 \times 10^{-5}$ and the linkage disequilibrium [LD] r^2 to 0.01. The settings above were previously also used in many MR studies of psychiatric disorders (30, 31). The causal effect of psychiatric disorders on AS is currently analyzed using the MR analysis methods described above except for phenotype "AN", which applies only to the WR method because only one SNP is available. The valid SNPs obtained after rigorous screening for estimation of the MR causal effects of mental disorders on AS are listed in [Supplementary Table S4](#).

3 Results

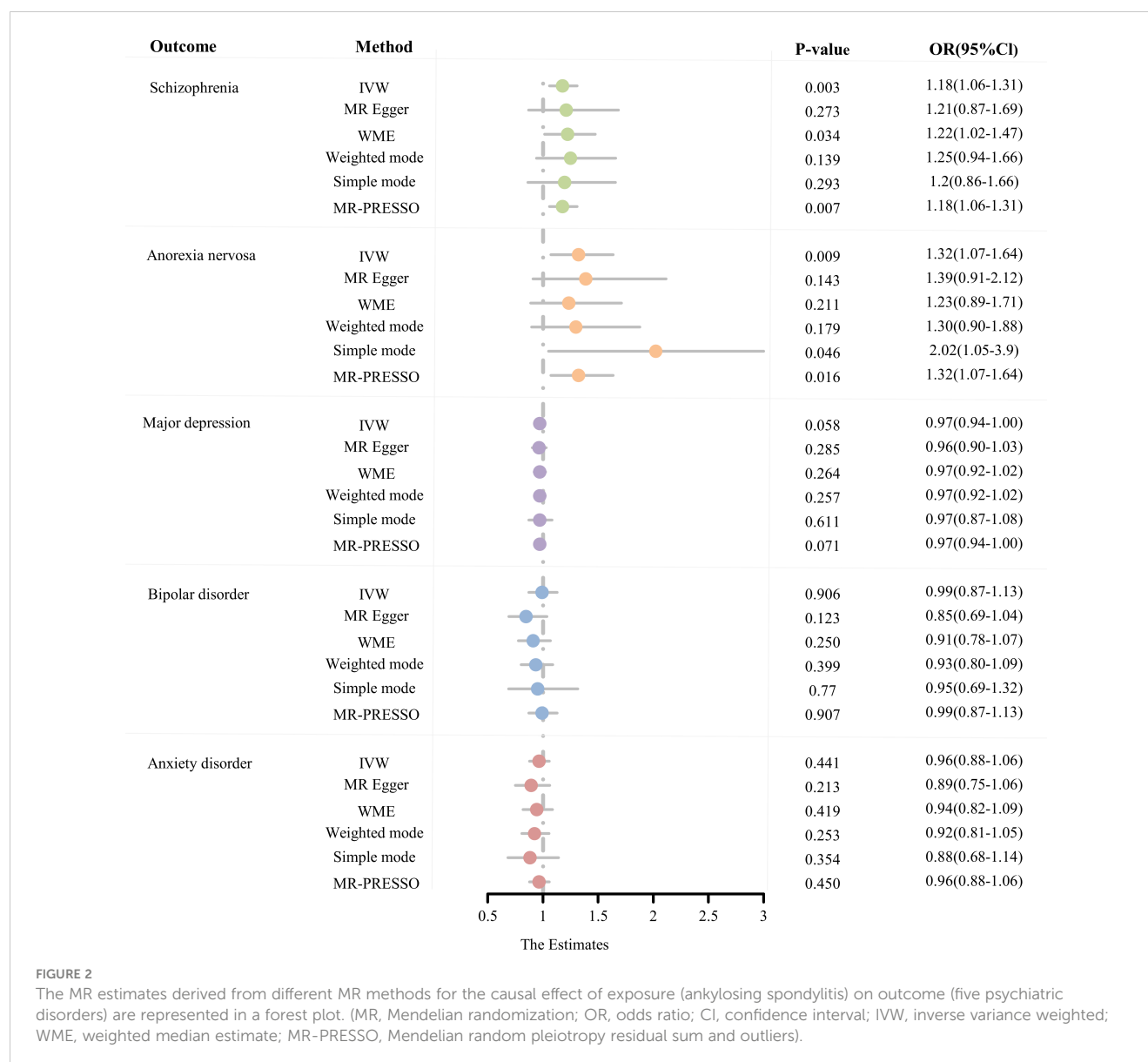
3.1 Research overview

The number of SNPs for AS as an exposure factor for each psychiatric phenotype ranged from 21 to 24 after a rigorous screening procedure. Based on the F-statistics, all of them were greater than 10, indicating that there is no bias caused by the utilization of feeble IVs ([Supplementary Table S3](#)). MR estimates obtained from the different methods are presented in [Figure 2](#), refer to [Supplementary Table S5](#) for more details.

3.1.1 MR analysis for the causal effects of AS on SCZ and AN

From the MR estimates we observe a causal effect of AS on SCZ and AN. To be specific, the causal-effect estimates of the IVW model suggested that AS significantly enhanced the risk of SCZ (OR = 1.18 95% CI 1.06-1.31, $P = 2.58 \times 10^{-3}$). Moreover, analogous causality estimates were also obtained from MR-PRESSO (OR = 1.18 95% CI 1.06-1.31, $P = 6.85 \times 10^{-3}$) and WME (OR = 1.22 95% CI 1.02-1.47, $P = 3.36 \times 10^{-2}$) models in the AS on SCZ MR analysis. Of these, the MR estimates were reliable for the MR-PRESSO method, with $p = 6.85 \times 10^{-3}$ remaining significant at the Bonferroni-corrected significance threshold ($p < 0.05/5 = 0.01$). Results from the WME method ($P = 3.36 \times 10^{-2}$) offered suggestive evidence of a potentially cause-effect association. Furthermore, as shown in [Figures 3A, 3D](#), the overall trend is consistent even though the estimates from the other complementary MR methods do not achieve statistical significance. In the MR analysis of AS on AN, the causal estimates of the IVW model indicated that AS was significantly associated with an elevated risk of developing AN (OR = 1.32, 95% CI: 1.07-1.64, $P = 9.43 \times 10^{-3}$). Consistent causality estimates were also obtained from MR-PRESSO (OR = 1.32, 95% CI 1.07-1.64, $P = 1.62 \times 10^{-2}$) and the simple mode (OR = 2.02, 95% CI 1.05-3.90, $P = 1.43 \times 10^{-2}$) approaches. As the p-values were above the calibrated p-value, both provided suggestive causality. As well, other complementary MR methods offer consistent estimates in the direction of causality ([Figures 3A, D](#)).

Then, sensitivity analyses demonstrated that our MR conclusions were robust ([Supplementary Table S7](#)). Cochran's Q-test and Rücker's Q-test showed that there was no heterogeneity in IVs ($p > 0.05$). The MR-PRESSO model did not detect SNPs with potential pleiotropy ($p > 0.05$). Additionally, the MR-PRESSO



global test and the MR-Egger intercept results revealed that there exists no horizontal pleiotropy in the selected IVs ($p > 0.05$). Asymmetry of SNP estimation accuracy scales in IVW and MR Egger methods is not shown in funnel plots (Figures 3B, E). LOO analysis reflects the robustness of conclusions independent of individual SNPs (Figures 3C, F). The Steiger directionality test showed that AS exhibits a positive directional in causality with SCZ and AN ($p < 0.05$).

3.1.2 MR analysis for the causal effects of MDD on BIP and AXD

Based on Bonferroni correction thresholds, the IVW methods of MR estimation showed that genetically predicted AS did not have a statistically significant effect on the risk of MDD (OR = 0.97, 95% CI: 0.94-1.00, $P = 5.77 \times 10^{-2}$), BIP (OR = 0.99, 95% CI: 0.87-1.13, $P = 9.06 \times 10^{-1}$), and AXD (OR = 0.96, 95% CI: 0.88-1.06, $P = 0.41 \times 10^{-1}$). Results from other MR analysis methods similarly support

this conclusion (Figure 2, Supplementary Table S5). Furthermore, no outliers were observed for MR-PRESSO based on the results of sensitivity analyses (all $p > 0.05$), and MR estimates were not affected by heterogeneity and horizontal pleiotropy (all $p > 0.05$). LOO analysis of overall effects shows no influence from a single SNP (Supplementary Figure S1). The results of the Steiger directionality assessment support the validity of the causal relationship's orientation (Supplementary Table S7). In conclusion, we did not find a significant causal relationship between AS and MDD, BIP, or AXD.

3.2 Reverse MR analysis

To explore the reverse causality of psychiatric disorders on AS, we performed a reverse MR analysis. In this study, we obtained IVs ranging from 1-20, all of which had F-statistics > 10 , suggesting that

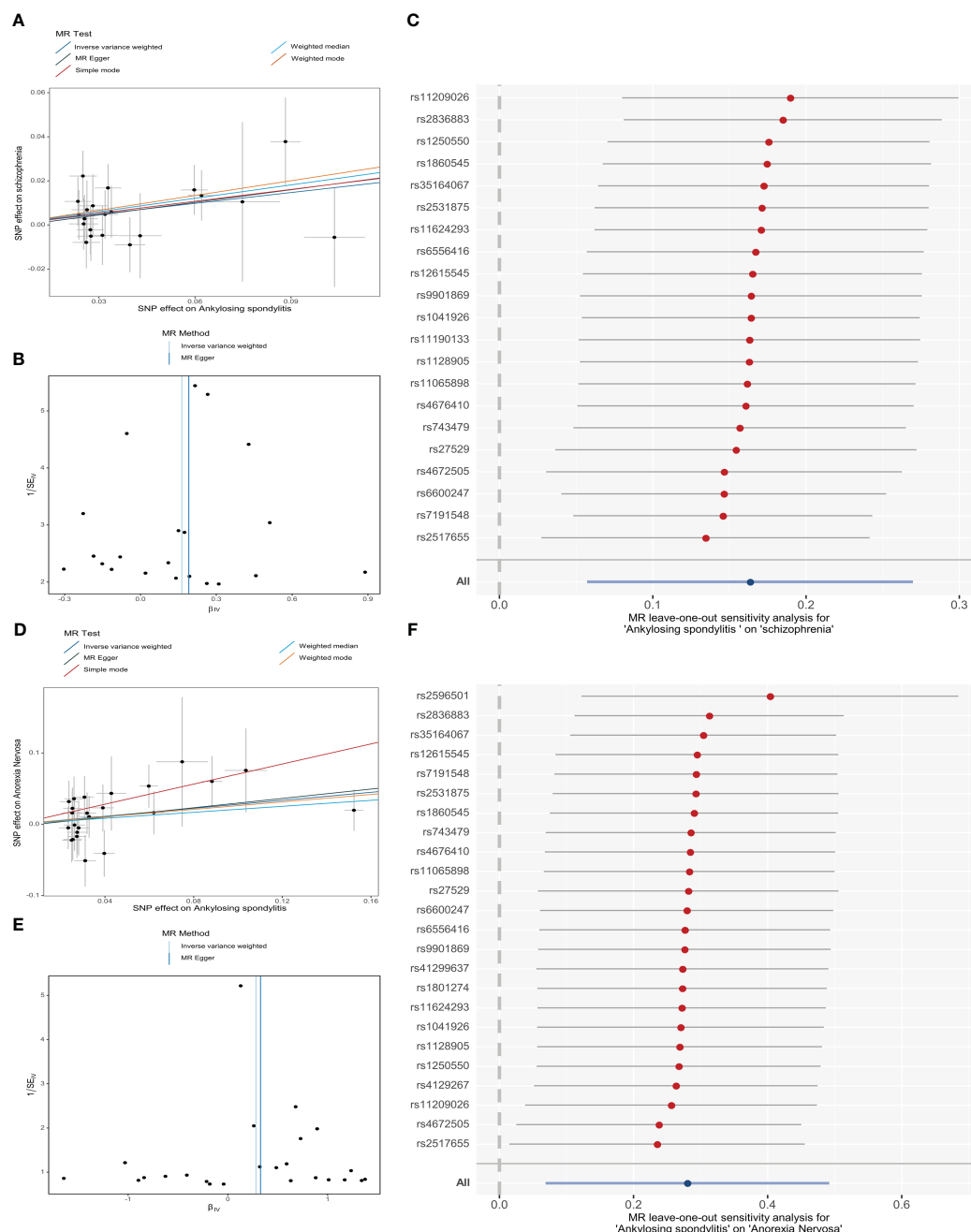


FIGURE 3

MR analysis of genetically predicted associations of ankylosing spondylitis on schizophrenia (A–C) and anorexia nervosa (D–F) visualized by Scatter plots (the slopes of the lines represent causal associations), Funnel plots, and Leave-one-out forest plot, respectively. (SNP, single-nucleotide polymorphism).

bias due to weak IVs could be neglected (Supplementary Table S4). The results of the MR estimation of the IVW method show that SCZ (OR = 1.01, 95% CI: 0.99–1.04, $P = 0.30$), AN (OR = 1.03, 95% CI: 0.97–1.10, $P = 0.32$), MDD (OR = 1.03, 95% CI: 0.94–1.12, $P = 0.53$), BIP (OR = 1.01, 95% CI: 0.94–1.08, $P = 0.75$), and AXD (OR = 0.97, 95% CI: 0.90–1.08, $P = 0.76$) did not have a statistically significant effect on the risk of AS. As depicted in Figure 4, none of the MR causal effect estimates for any of the complementary methods were statistically significant in terms of the causal association of mental

illness on AS (all $p > 0.01$) (Supplementary Table S6). Hence, the hypothesis of reverse causation between mental illness and AS fails to hold. Importantly, our sensitivity analysis, employing MR-PRESSO, failed to detect any SNPs exhibiting pleiotropic effects (all $p > 0.05$). Additionally, we observed neither heterogeneity nor horizontal pleiotropy among the genetic instruments (all $p > 0.05$) (Supplementary Table S8, Supplementary Figure S2). The funnel plot exhibits a roughly symmetrical distribution (Supplementary Figure S2). Notably, in the LOO analysis, no specific SNP unduly

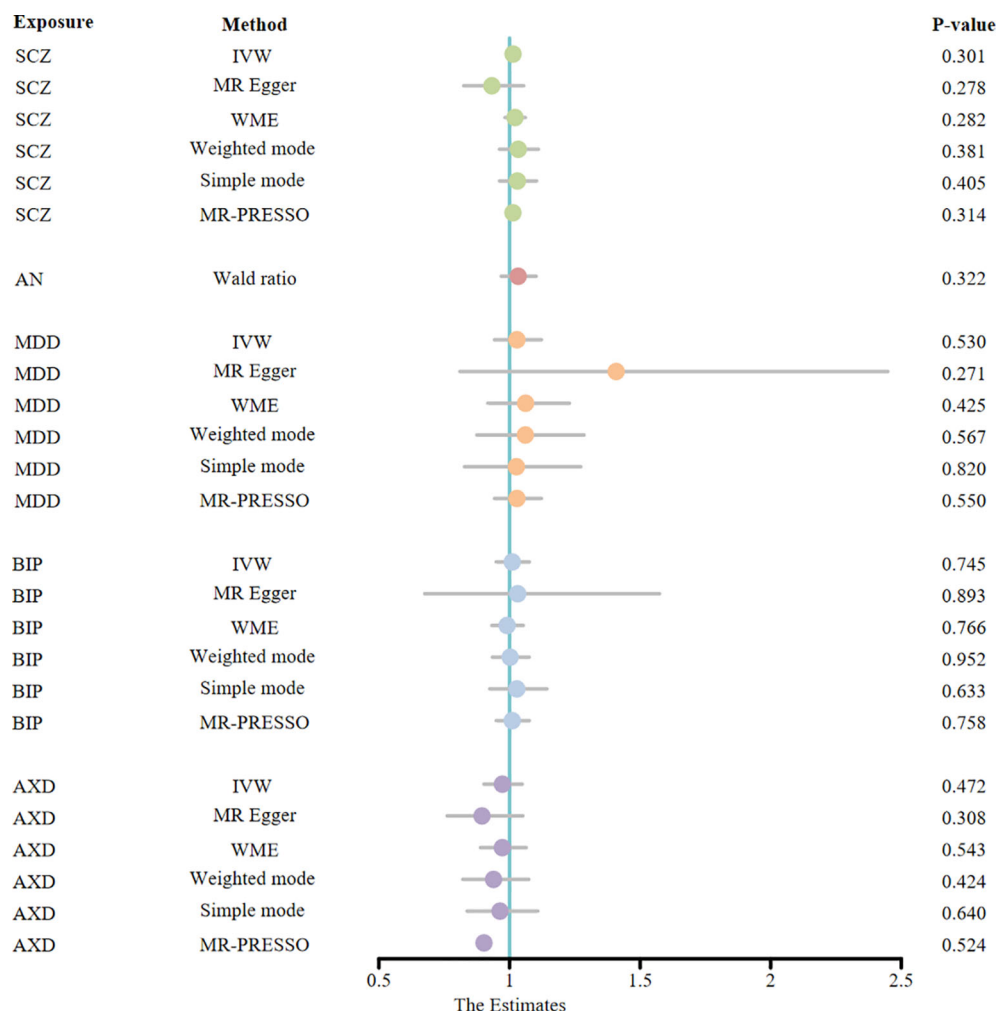


FIGURE 4

The MR estimates derived from different MR methods for the causal effect of exposure (five psychiatric disorders) on outcome (ankylosing spondylitis) are represented in a forest plot. (MR, Mendelian randomization; OR, odds ratio; CI, confidence interval; IVW, inverse variance weighted; WME, weighted median estimate; MR-PRESSO, Mendelian random pleiotropy residual sum and outliers; SCZ, schizophrenia; AN, anorexia nervosa; MDD, major depression disorder; BIP, bipolar disorder; AXD, Anxiety disorder).

influenced the MR estimates (Supplementary Figure S2). Moreover, the findings from the Steiger directionality test affirm the veracity of the causal directionality. It is noteworthy that the p-values for MDD and AXD did not attain significance (both $p > 0.05$). However, the fundamental causal direction remains true (Supplementary Table S8). We must acknowledge that, in the reverse MR study investigating AN as the exposure and AS as the outcome, we obtained only one usable SNP despite relaxing the criteria for IV selection. Consequently, we did not use MR methods such as IVW and MR Egger to analyze heterogeneity and horizontal pleiotropy.

4 Discussions

While potential causal relationships between AS and psychiatric disorders have been demonstrated in previous studies, as yet, there have been no conclusions drawn regarding the causal association between AS and psychiatric disorders (especially BIP and SCZ). It is

possible that the interference of external confounding factors has not been excluded in most studies, leading to some conflicting discoveries. Researchers frequently employ statistical techniques to address confounders in observational studies by incorporating known and measured confounders into regression models. Nonetheless, when confounders remain unobserved or unknown, or when their number is considerable, regression methods may fail to produce unbiased estimates of the genuine connection between an exposure and outcome. As a result, the interpretation of findings can potentially introduce biases (32). For instance, an observational study in Sweden on the relationship between rheumatic diseases and mental disorders, in which many unmeasured or unknown potential confounders, such as environmental risk factors and substance use, were included in the study's limitations (8). The presence of confounding variables can disrupt the estimation of exposure-outcome correlations and undermine the precision of causal determinations about study outcomes. Consequently, the demonstration of a direct causal relationship is rendered

unattainable. Moreover, it should be noted that some observational studies, especially cross-sectional studies, may not be able to definitively identify temporal associations between AS and psychiatric disorders. To overcome this constraint and elucidate causation, longitudinal analyses are of utmost importance. Essentially, the prospect of reverse causality must be taken into account, whereby the occurrence of psychiatric disorders may plausibly impact the genesis of AS (11, 33).

In this study, we performed a two-sample MR analysis using publicly available GWAS summary data for the first time to assess causal associations between genetically predicted AS and five psychiatric disorders by bidirectional assessment. SNPs were utilized as instrumental variables that were not subject to confounders or reverse causality (34). The present findings suggest a potential causal effect of AS on SCZ and AN. More specifically, AS was significantly associated with an increasing risk of developing SCZ and AN. However, there were no psychiatric traits identified that showed reverse causality with AS, which included SCZ, AN, BIP, AXD, and MDD. This study provides genetic evidence for a cause-and-effect correlation between AS and psychiatric disorders. Which plays a crucial role in the development of psychiatric disorders.

So far, the exact mechanisms linking AS and mental disorders have not been fully understood. The following assumptions deserve to be considered. In recent findings, immune dysregulation has been shown to be one of the primary risk factors in the pathogenesis of psychiatric disorders (35–39). Genetic studies have shown that genes involved in immune modulation (major histocompatibility complex loci) are significantly correlated with SCZ (40). Thus, there may be a shared risk immunogenetic link between AS and SCZ. Furthermore, cytokines function in the periphery and brain as key signaling molecules of the immune system. Cytokines produced by the AS autoimmune process reach the brain through a variety of mechanisms, including brain barrier leakage, active transport, and bound specific cytokine receptors, which play a crucial role in mediating crosstalk between the immune system and the brain, and thus may be a potential contributor to psychiatric disorders (41, 42). A previous study showed higher levels of interleukin (IL)-1 β and IL-6 production in patients with AS compared to normal subjects (43). Solmi et al. found that an association of elevated circulating concentrations of the cytokines tumor necrosis factor (TNF)- α , IL-1 β , and IL-6 with AN (44). In addition, several meta-analyses have found that cytokine abnormalities such as (altered levels of inflammatory factors such as IL-1 β , IL6, IL10, and TNF- α) are associated with SCZ (45). Cytokines such as TNF- α have been shown to have anorexic effects on the hypothalamus and peripheral anorexic effects *via* leptin (41, 46, 47). The SCZ cytokine model indicates that there is increased inflammation to be found in the brains of SCZ patients (42). Proinflammatory cytokines induce increased production of kynurenine through activation of the tryptophan metabolic pathway, which is involved in the regulation of the glutamate and serotonin systems, and kynurenine is converted to the N-methyl-D-aspartic acid receptor (NMDA) antagonist kynurenine in astrocytes. NMDA receptor hypofunction is associated with the pathophysiology of SCZ (48). As well, there are other biological events that can be triggered by cytokine activity, such

as the hypothalamic-pituitary-adrenal activation axis (49), increasing oxidative stress (50, 51), diminished hippocampal volume, and promotion of dopaminergic sensitization (42). Hyperactivation of the HPA axis can be reliably observed in AN and SCZ (42, 52). Besides, imbalances in the gut microbiota may also be a potential mechanism for AS and AN. One pathophysiological model of AN may stem from altered signaling between the microbe-brain axis that regulates feeding behavior. The model suggests that the caseinolytic protease B homologue (ClpB) produced by *Escherichia coli* is a mimic of the α -melanocyte-stimulating hormone, a neuropeptide expressed by neurons in the arcuate nucleus of the hypothalamus, which produces a feeling of satiety (53). It was found that levels of ClpB were higher in patients with AN compared to healthy controls (54). Studies have shown that the onset and development of AS are associated with changes in the composition of microorganisms (55). Ciccio et al. found a significantly increased abundance of *Escherichia coli* and *Prevotella* in the terminal ileum of AS individuals (56). This may increase levels of ClpB and may increase the risk of developing AN. However, the precise mechanisms by which AS leads to SCZ and AN still need to be further explored and more thoroughly researched.

In this analysis, we employed the IVW method as the primary statistical approach, which demonstrated superior statistical efficacy in comparison to alternative methods like MR-Egger. MR-Egger, due to its limited statistical efficacy, exhibited wider confidence intervals and insignificant P values when compared to IVW. Thus, IVW is commonly employed as the principal criterion for identifying potentially significant outcomes. To ensure the reliability of the IVW estimates, this study implemented additional MR methods and conducted sensitivity analyses. Additionally, it is crucial to ensure the absence of horizontal pleiotropy, wherein IVs utilized in MR analyses solely impact the target outcome through the intended exposure. Horizontal pleiotropy manifests when genetic variations influence traits unrelated to the pathway of interest but still affect the target outcome, or when the variations directly impact the target outcome (57). The presence of horizontal pleiotropy introduces distortions to the MR test, resulting in erroneous estimations of causality, diminished statistical power, and the potential for false-positive causal associations. Should horizontal pleiotropy be detected, it is advisable to refer to the results obtained from MR-Egger, as this method adjusts IVW analyses by accounting for imbalanced or directed horizontal pleiotropic effects across all SNPs (22). Consistent with the conventions of most MR analyses, our study adheres to the requisite of consistent β -direction for all MR methods (58).

In our study, we have several strengths: Firstly, it is a two-sample MR study with large-scale GWAS statistics, which minimizes the likelihood of reverse causality and potential confounding in observational studies. This considerably enhances the reliability and persuasiveness of our study. Secondly, summary statistics were obtained from samples of European ancestry. It effectively reduces bias due to population stratification. Third, due to the fact that the data were collected from different organizations, there was little overlap between exposures and outcomes in the sample. Fourthly, multi-sensitivity analysis results demonstrated that there was the non-existence of potential pleiotropy in the

instrumental variables indicating the robustness of our results. In the fifth place, the prevalence of AN in individuals with AS has not been investigated epidemiologically to our knowledge. The causal effect of AS on AN obtained in this study may guide future epidemiology. Lastly, we also performed a reverse MR analysis to determine that there is no reverse causality of psychiatric disorders on AS. There are inevitably some limitations to our study. The first concern is the possibility of bias in the GWAS summary statistics, such as coding errors, misclassification of cases, etc. Next, stratified analyses based on factors such as age and gender were not possible owing to the limitations of the GWAS summary data. Thirdly, currently, there have been fewer GWAS studies on AS, the reliability of the findings could be further validated by obtaining a larger exposure sample and extracting SNPs more closely associated with AS. Furthermore, in our MR analysis of reverse causality, we relaxed the significance threshold setting for p-values for IVs associated with mental disorders, which means that the proportion of variance explained by some valid IVs is relatively small. This may have contributed to the nonsignificant difference between the proportion of variance explained by IVs for MDD and AXD and the proportion of variance explained for AS in the Steiger directionality test. An insufficient number of IVs were available for AN. It was not possible to perform a sensitivity analysis to verify the robustness of the results. Moreover, given that we used GWAS summary data from individuals of European ancestry, caution should be exercised in generalizing the findings to other populations due to potential inter-ethnic genetic variations. It is imperative that data from other populations are analyzed and compared to make the results more trustworthy. Finally, while the MR-PRESSO examination or MR-Egger intercept evaluation did not discern significant pleiotropy and confounding elements, the potentiality of these factors cannot be entirely dismissed. Such is a pervasive predicament encountered in the realm of MR analyses.

5 Conclusions

In summary, the evidence presented in the two-way MR studies suggests that there may be a causal correlation between genetically predicted AS and the development of SCZ and AN. Our investigation provides valuable insights into intervening in psychiatric disorders (SCZ and AN) among individuals with AS. Further research in the future is imperative to unravel the intrinsic mechanisms that underlie the association between ankylosing spondylitis and SCZ and AN.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the present analysis was based on publicly available summary-level data, which did not need additional approval. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

HZ: Writing – original draft, Writing – review & editing. MML: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1277959/full#supplementary-material>

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Machine learning to characterize bone biomarkers profile in rheumatoid arthritis

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Background: Bone metabolism is disrupted in rheumatoid arthritis (RA); however, the bone metabolic signature of RA is poorly known. The objective of the study is to further characterize the bone metabolic profile of RA and compare it to psoriatic arthritis (PsA), systemic sclerosis (SSc) and healthy controls.

Methods: We did a cross-sectional case-control study on consecutively enrolled patients and age-matched controls. We collected clinical characteristics, serum biomarkers related to bone metabolism and Bone Mineral Density (BMD). A multiple correlation analysis using Spearman's rank correlation coefficient was conducted within the RA patient group to investigate associations between biomarker levels and clinical variables. Machine learning (ML) models and Principal Component Analysis (PCA) was performed to evaluate the ability of bone biomarker profiles to differentiate RA patients from controls.

Results: We found significantly lower BMD in RA patients compared to PsA, and Systemic Sclerosis SSc groups. RA patients exhibited higher Dkk1, sclerostin and lower P1nP and B-ALP levels compared to controls. No significant differences in CTX levels were noted. Correlation analysis revealed associations between bone biomarkers and clinical variables. PCA and ML highlighted distinct biomarker patterns in RA which can effectively discriminate bone biomarkers profile in RA from controls.

Conclusion: Our study helped uncover the distinct bone profile in RA, including changes in bone density and unique biomarker patterns. These findings enhance our comprehension of the intricate links between inflammation, bone dynamics, and RA activity, offering potential insights for diagnostic and therapeutic advancements in managing bone involvement in this challenging condition.

KEYWORDS

bone metabolism, bone markers, rheumatoid arthritis, bone mineral density (BMD), inflammation

Introduction

Rheumatoid arthritis (RA), a chronic autoimmune disorder, exerts a profound influence on skeletal health, extending beyond its well-documented effects on joint structures (1, 2). The intricate interplay between RA and osteoporosis is of substantial clinical significance, necessitating a comprehensive understanding of the underlying mechanisms. Within this context, the exploration of bone turnover markers and modulators (BTMs), encompassing a spectrum of biochemical entities reflective of bone formation and resorption dynamics, has emerged as pivotal in deciphering the intricate relationship between RA and osteoporosis. BTMs offer dynamic insights into the equilibrium between bone formation and resorption, shedding light on the intricate processes governing skeletal metabolism. These markers can be categorized into those reflecting bone formation and those indicative of bone resorption (3, 4). In the context of RA, where the chronic inflammatory milieu poses a multifaceted challenge to bone health, BTMs assume particular significance (5). The Wnt signaling pathway stands as a pivotal regulator of BTMs and plays a central role in the maintenance of bone homeostasis (6). In particular, two key antagonists within this pathway, sclerostin and Dickkopf-1 (Dkk1), have garnered substantial scientific attention (7). The upregulation of sclerostin and Dkk1 is thought to hinder bone formation, thereby exacerbating the risk of osteoporosis and fragility fractures in RA patients. The mechanistic insights into the interplay between sclerostin and RA pathogenesis continue to be a subject of active research, with implications for therapeutic targeting.

Psoriatic arthritis (PsA) primarily characterized by bone apposition (8), and systemic sclerosis (SSc), a non-inflammatory disease known to cause osteoporosis (9), offer distinct models for understanding osteoporosis etiology in autoimmune diseases. The main goal of this study is to examine RA's biomarker profile, comparing it with healthy controls and two other representative autoimmune diseases.

Materials and methods

Study population

We conducted a cross-sectional study involving a total of 1883 consecutively enrolled participants, which included 1462 patients diagnosed with Rheumatoid Arthritis (RA), 60 patients with Psoriatic Arthritis (PsA), 62 patients with Systemic Sclerosis (SSc), and 359 age-matched healthy controls. All participants were recruited from the Rheumatology Section of the University of Verona Hospital between January 2012 and December 2019. The study was conducted according to the protocol REUMABANK approved by the University of Verona local Ethic Committee, in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards, and all participants provided informed consent.

Data collection

For the RA patient group, we collected extensive clinical data at the time of serum sample collection, including disease duration, Disease Activity Score 28-C-Reactive Protein (DAS28-CRP), C-Reactive Protein (CRP) levels, Erythrocyte Sedimentation Rate (ESR) levels, Health Assessment Questionnaire (HAQ) scores, glucocorticoid (GC) cumulative dose, GC ongoing dose and disease modifying anti-rheumatic drugs (DMARDs).

Serum biomarker analysis

Blood samples were drawn at morning fasting. Serum samples were aliquoted and stored at -80°C until they were assayed for C-terminal telopeptide of type I collagen (CTX, a marker of bone resorption), Procollagen I Intact N-Terminal Peptide (P1NP, a marker of bone formation), Bone Alkaline Phosphatase (B-ALP, a marker of bone formation), Dkk1 (a Wnt inhibitor), Sclerostin (a Wnt inhibitor), 25OH-Vitamin D (25OHVitD), Parathyroid hormone (PTH). CTX, P1NP and B-ALP were measured by the IDS-ISYS Multi-Discipline Automated Analyzer (Immunodiagnostic System, Boldon, UK) based on chemiluminescence technology. The coefficients of variation (CV) intra-assay measured were 3.0 % for P1NP, 2 % for CTX, and 4 % for B-ALP. Serum Dkk1 and sclerostin were measured by ELISA (Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria) with sensitivities of 0.89 and 8.9 pmol/L and intra-assay coefficients of variation of 7.8 and 5.6 %, respectively. Inter-assay variabilities were 8.2 and 6.9 %, for Dkk1 and sclerostin, respectively. PTH was measured by ELISA (IDS Ltd. Boldon, UK) with intra-assay variability of 6 % and inter-assay variability of 7%. 25OHVitD was measured with LIAISON® 25OHVitD assay (DiaSorin, Italy), the intra-assay variability was 8% and the inter-assay variability was 12%. All samples were measured in a single batch in order to limit inter-assay variability.

Bone mineral density measurement

BMD was measured at femoral neck and lumbar spine (L1–L4) by dual-energy X-ray absorptiometry (DEXA) (QDR Hologic Delphi) in patients with RA, PsA and SSc. BMD levels were not available in healthy controls. The variation coefficient was 1 % for vertebral site and 1.2 % for femoral neck.

Group comparisons

To assess differences in biomarker levels and BMD among the study groups, we performed analysis of covariance (ANCOVA) adjusting for the following co-variables (cumulative glucocorticoid intake, age, gender, CRP levels, csDMARD and b/tsDMARD use) with Tukey's multiple comparisons *post-hoc* test.

Correlation analysis

A multiple correlation analysis using Spearman's rank correlation coefficient was conducted within the RA patient group to investigate associations between biomarker levels and clinical variables. A heatmap was generated to visualize these correlations. p 0-0.19 indicates very weak correlation, 0.2-0.39 weak, 0.40-0.59 moderate, 0.6-0.79 strong and 0.8-1 very strong correlation. To account for multiplicity, we used the False Discovery Rate (FDR) approach with the two-stage step-up method of Benjamini, Krieger and Yekutieli (Q value 5% of FDR).

Machine learning models

We employed machine learning (ML) techniques to evaluate the ability of biomarker profiles to differentiate RA patients from individuals with PsA and SSc. We ran three different ML models considering the following features in addition to serum biomarkers: cumulative glucocorticoid intake, age, gender, CRP levels, csDMARD and b/tsDMARD use: 1) Random Forest that ensemble learning method that combines multiple decision trees to make more accurate predictions. Each tree in the forest is constructed from a random subset of the data and a random subset of features. The final prediction is determined by aggregating the predictions of all individual trees. We optimized hyperparameters such as the number of trees ($n=10$), maximum depth (unlimited) to achieve the best classification performance. 2) Neural Network, which consist of interconnected layers of artificial neurons that process and transform data. In our case, a feedforward neural network with multiple hidden layers was employed. Hyperparameter optimization included tuning the number of hidden layers ($n=100$), regularization ($\alpha=0.001$), maximal number of iterations ($n=200$). 3) Logistic Regression, which estimates the relationship between a set of independent variables (in this case, biomarker levels) and the probability of a specific outcome (RA, PsA, or SSc). We selected a Ridge (L2) regularization with strength $C=1$.

To assess the performance of these machine learning models, we employed standard evaluation metrics, including classification accuracy (CA), precision, recall, and receiver operating characteristic (ROC) curves with area under the curve (AUC) analysis. We implemented cross-validation (10 folds, stratified) to ensure the robustness of our models and mitigate overfitting.

Principal component analysis

A Principal Component Analysis (PCA) was performed using all biomarkers to identify patterns and reduce dimensionality in the data. PCA was applied to reduce dimensionality of the dataset and find clusters of variables recording largely redundant information. PCs were selected based on eigenvalues explaining >75% of total variance.

Missing values

Missing values (less than 2% of the overall dataset) were imputed using the most common value for that specific variable. We also conducted sensitivity analyses with complete dataset analyses (excluding subjects with missing values).

Statistical analyses

Statistical analyses were performed using SPSS Version 26 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, CA, USA). The machine learning models were implemented using Orange: Data Mining Toolbox in Python Version 3.35.0. Hyperparameter tuning was conducted systematically to optimize each model's performance. PCA results were analyzed using PCA package on GraphPad Prism version 9.5.0 for Windows, GraphPad Software, San Diego, California USA.

Results

Characteristics of study participants

Table 1 summarizes the demographic and clinical characteristics of the study participants. A total of 1883 participants were included in the analysis, comprising 1462 Rheumatoid Arthritis (RA) patients, 60 Psoriatic Arthritis (PsA) patients, 62 Systemic Sclerosis (SSc) patients, and 359 age-matched healthy controls. RA patients had a mean disease duration of 99 months, with an average Disease Activity Score 28-C-Reactive Protein (DAS28-CRP) of 2.8. The mean C-Reactive Protein (CRP) and Erythrocyte Sedimentation Rate (ESR) levels were 0.9 mg/L and 20 mm/h, respectively. The Health Assessment Questionnaire (HAQ) scores in RA patients averaged 0.750.

Bone mineral density

Figure 1 displays the results of BMD measurements at the femoral neck and lumbar spine. The BMD at the femoral neck was significantly lower in RA (0.687 ± 0.118 g/cm²) compared to PsA (0.747 ± 0.099 g/cm², $p < 0.001$) and SSc (0.785 ± 0.118 g/cm², $p < 0.0001$). Similarly, BMD at the lumbar spine was reduced in RA (0.882 ± 0.133 g/cm²) compared to PsA (0.963 ± 0.136 g/cm², $p < 0.01$) and SSc (0.990 ± 0.173 g/cm², $p < 0.0001$).

Biomarker levels comparison

Figures 2 and 3 present the results of the analysis comparing biomarker levels among the different study groups P1nP levels in

TABLE 1 Study population characteristics.

Characteristic	Rheumatoid Arthritis (RA) (n = 1462)	Psoriatic Arthritis (PsA) (n = 60)	Systemic Sclerosis (SSc) (n = 62)	Healthy Controls (n = 359)
Age (years)	58.9 ± 12.0	57.9 ± 12.3	63.9 ± 8.8	59.3 ± 15.6
Gender (female/male)	1241 / 221	42 / 18*	62 / 0*	192 / 167*
Disease Duration (months)	99 (48–178)	85 (35–165)	106 (68–200)	N/A
DAS28-CRP	2.8 ± 4.6	N/A	N/A	N/A
CRP (mg/L)	2.9 (0.3–5.0)	0.4 (0.1–1.2)	2.8 (0.7–3.8)	N/A
ESR (mm/h)	20 (12–30)	N/A	N/A	N/A
HAQ Score	0.750 (0.250–1.250)	N/A	N/A	N/A
Median GC cumulative dose (g)	11.5 (0–15.7)	0 (0–1.0)	0.3 (0–0.9)	N/A
csDMARD (yes/no)	1257 / 205	24 / 36*	19 / 43*	N/A
b/tsDMARD (yes/no)	603 / 859	30 / 30	2 / 60*	N/A

Values are presented as mean ± standard deviation unless otherwise specified. DAS28-CRP, Disease Activity Score 28-C-Reactive Protein; CRP, C-Reactive Protein; ESR, Erythrocyte Sedimentation Rate; HAQ, Health Assessment Questionnaire; GC, Glucocorticoid. * p<0.05 vs RA.

the RA group ($46.6 \pm 25.2 \mu\text{g/L}$) were significantly lower than those in healthy control ($52.9 \pm 23.0 \mu\text{g/L}$, $p<0.001$) groups, PsA ($54.4 \pm 25.3 \mu\text{g/L}$, $p<0.01$) and SSc ($53.6 \pm 25.3 \mu\text{g/L}$, $p<0.01$). Bone ALP levels were lower in RA patients ($9.9 \pm 5.4 \text{ U/L}$) compared to healthy controls ($16.3 \pm 9.1 \text{ U/L}$, $p<0.001$), PsA ($14.8 \pm 11.1 \text{ U/mL}$, $p<0.0001$) and SSc ($14.6 \pm 6.5 \text{ U/mL}$, $p<0.001$). CTX levels did not differ between groups. Sclerostin levels were increased in RA patients ($35.5 \pm 15.6 \text{ pmol/mL}$) compared to healthy controls (32.8 ± 13). Dkk1 levels were significantly higher in the RA group ($31.8 \pm 17.4 \text{ pmol/L}$) compared to healthy controls ($25.1 \pm 17.2 \text{ pmol/L}$, $p<0.0001$) and SSc ($27.2 \pm 12.4 \text{ pmol/L}$, $p<0.01$) but not to PsA ($27.3 \pm 18.8 \text{ pmol/L}$, $p=0.051$) but were not different in PsA and SSc. PTH levels in RA patients ($26.6 \pm 16.2 \text{ ng/mL}$) were significantly lower than those in SSc ($46.8 \pm 20.6 \text{ ng/mL}$,

$p<0.0001$) but did not differ from healthy controls and PsA. 25-OH-vitamin D serum levels were significantly higher in SSc compared to other groups but did not differ between RA and PsA or healthy controls. We did not have access to data on anti-resorptive treatment for the overall cohort. To control for the confounding introduced by anti-resorptive we have conducted a sub-analysis on patients with CTX $>0.2 \text{ ng/mL}$ (in whom bone turnover is not suppressed and active treatment with anti-resorptive is unlikely). Sub analysis on these patients is presented in **Supplementary Material Figure 1**. CTX was significantly higher in RA compared to healthy controls, P1nP and B-ALP lower in RA compared to healthy controls, BMD was lower in RA compared to PsA and SSc. Sensitivity analyses with complete dataset (excluding subjects with missing values) yielded similar results (data not shown).

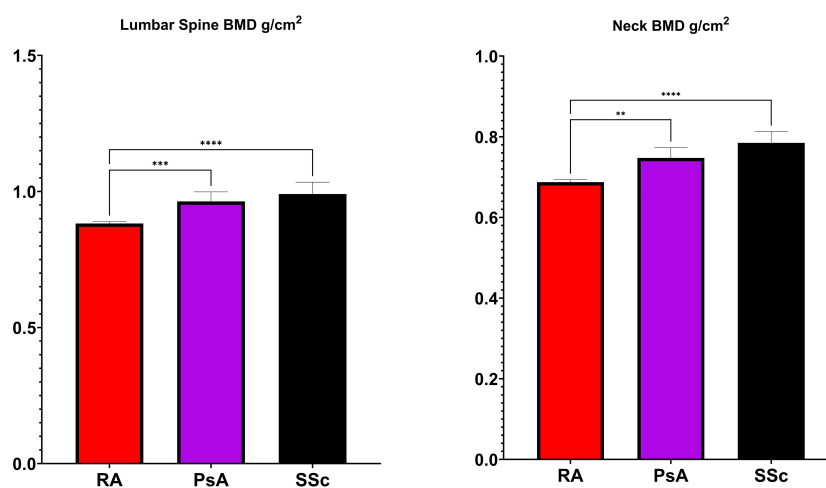


FIGURE 1

Bone Mineral Density (BMD) levels in rheumatoid arthritis (RA), psoriatic arthritis (PsA) and systemic sclerosis (SSc). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001.

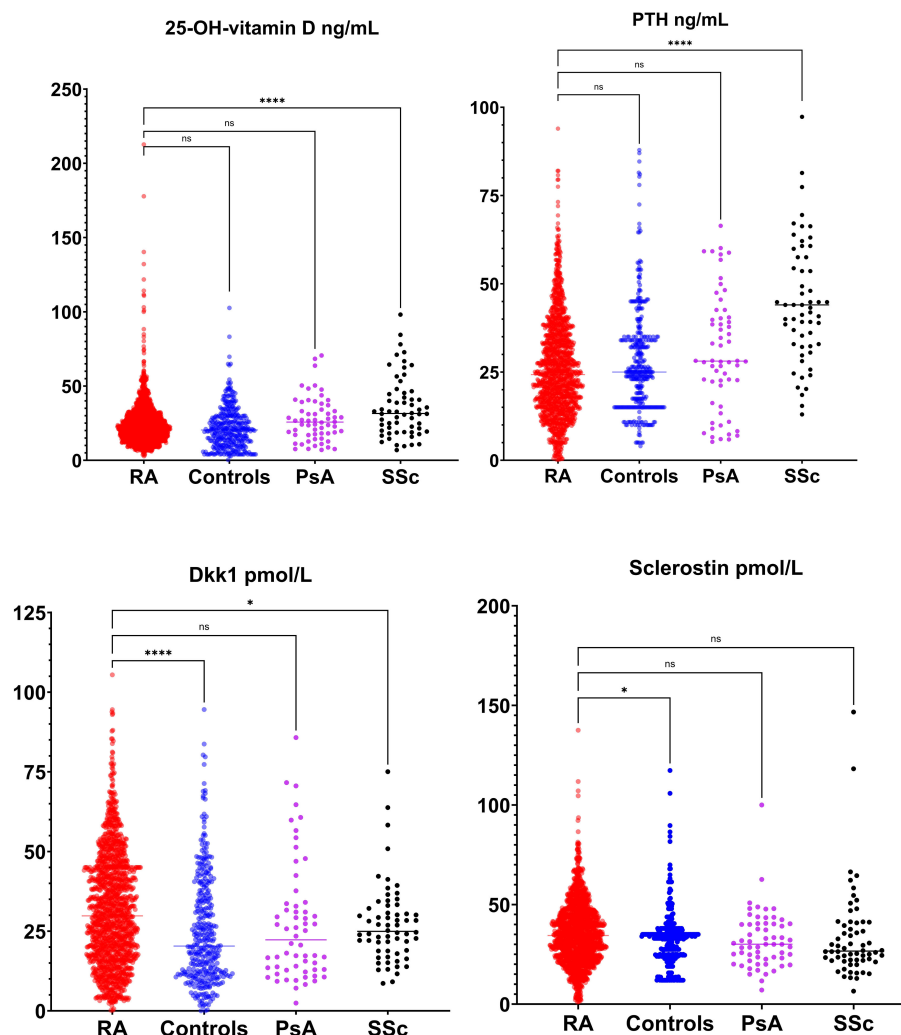


FIGURE 2

Bone modulators levels (25-OH-vitamin D, parathyroid hormone [PTH], dickkopf 1 [Dkk1] and sclerostin) in rheumatoid arthritis (RA), healthy control, psoriatic arthritis (PsA) and systemic sclerosis (SSc). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

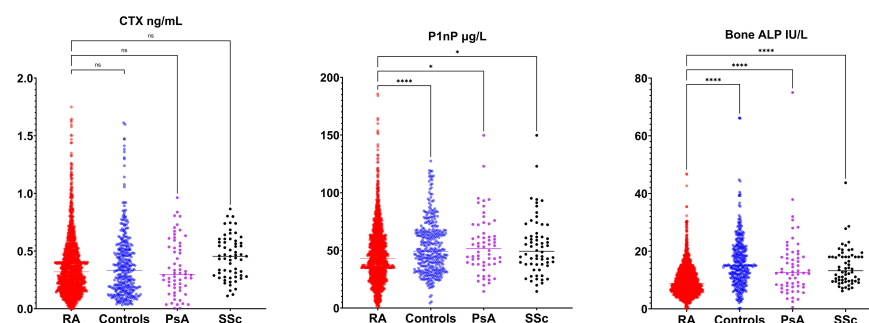


FIGURE 3

Bone turnover markers (C-terminal telopeptide [CTX], amino-terminal propeptide of type 1 procollagen [P1nP], bone alkaline phosphatase [ALP]) in rheumatoid arthritis (RA), healthy control, psoriatic arthritis (PsA) and systemic sclerosis (SSc). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Correlation analysis

In RA patients, Spearman's rank correlation analysis (Figure 4) revealed several significant associations between biomarkers and clinical variables. In particular, 25-OH-vitamin D was negatively associated with HAQ levels (p -0.131, p value 0.038), P1nP serum levels were positively associated with CRP levels (p 0.320, p value <0.001), PTH serum levels were positively associated with DAS28-CRP score (p 0.134, p value 0.034), sclerostin serum levels were positively associated with GC cumulative dose (p 0.145, p value 0.029) and Dkk1 serum levels were positively associated with RA disease duration (p 0.129, p value 0.045).

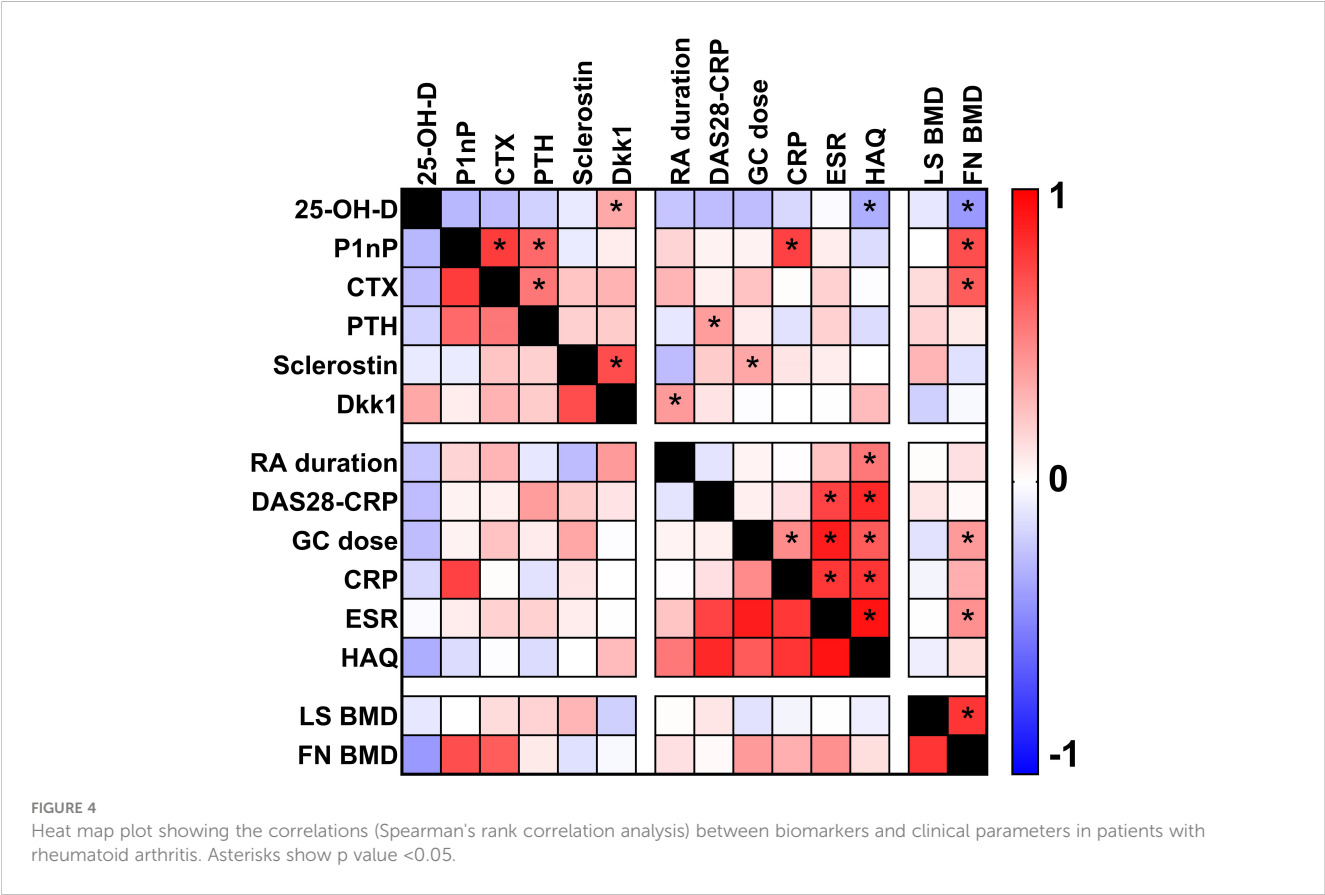
Principal component analysis

The Principal Component Analysis (PCA) results are illustrated in Figure 5. This PCA plot demonstrates the distribution of study participants in the multidimensional space defined by the biomarkers. The clustering patterns and relationships among participants are evident, providing valuable insights into the differentiation of RA from other rheumatic diseases and healthy controls. The x-axis of the PC Score graph represents the first principal component (PC1), which is the most influential linear combination of the biomarkers. The y-axis, corresponding to the

second principal component (PC2), captures the second-largest source of variation orthogonal to PC1. Each data point on the PC Score graph corresponds to an individual study participant, with their position determined by their biomarker profile's projection onto the PC1 and PC2 axes. Data points that cluster closely together on the graph share similar biomarker profiles. This clustering reflects the presence of distinct subgroups within our study population, characterized by specific patterns of biomarker expression. The PC Score graph allows us to explore whether the biomarker profiles of patients with RA form distinct clusters compared to controls. Moreover, it aids in identifying any potential overlap or separation between disease groups, shedding light on the discriminative power of these markers.

Machine learning models

Figure 6 summarizes the performance metrics of the machine learning models in discriminating RA patients from controls based on their biomarker profiles. The results indicate that ML models can effectively discriminate RA from age-matched controls and Neural Network model performed better than the other two models. More complete set of performance metrics is shown in Supplementary Materials (classification accuracy curve, calibration curve and F1 curve).



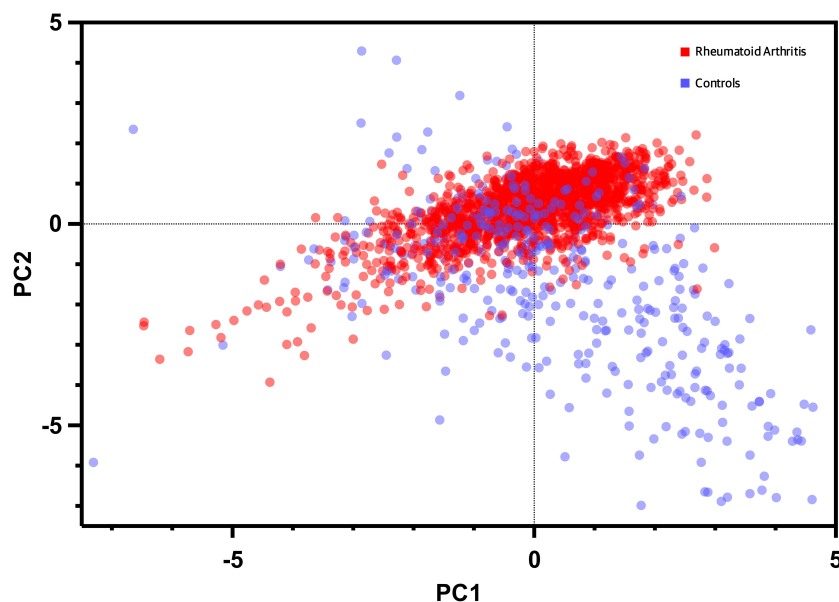


FIGURE 5

Dot plot of principal component analysis (PCA) based on biomarkers expression and bone mineral density levels. Each data point represents an individual participant, with the x-axis denoting the first principal component (PC1) and the y-axis representing the second principal component (PC2). The distribution of data points reflects the multidimensional clustering patterns based on the combined information of the biomarker, BMD, and clinical variables enabling visualization of distinctive subgroups and relationships within the study population.

Discussion

Herein, we aimed to comprehensively characterize BTMs, bone modulators and clinical parameters in a large cohort of RA patients and compare them with individuals with PsA, SSc, and age-matched healthy controls. Our findings shed light on the distinctive bone signature of RA and provide valuable insights into its pathophysiological mechanisms.

Osteoporosis is a key feature of RA, and it has been shown to be, at least in part, independent from GC use (10–12). Indeed, the pathogenesis of systemic and local bone loss in RA is complex and many metabolic pathways are likely to play a role. Among these mechanisms the Wnt system has emerged as a crucial regulator (7, 13–15). We found that Dkk1 levels were significantly higher in RA patients compared to healthy controls and SSc. Of note, we also found a trend toward difference with the PsA group ($p=0.051$). Similarly, sclerostin, another antagonist of the Wnt pathway, was elevated in RA patients compared to healthy controls, potentially contributing to the altered bone homeostasis observed. In line with these observations, P1nP and B-ALP levels were markedly reduced in RA patients compared to both healthy controls and PsA. The impaired bone formation might be central in RA-associated bone pathology, possibly due to the suppressive effects of low-grade chronic inflammation on osteoblastic activity (16, 17). Interestingly, CTX levels did not differ significantly among groups, this seemingly counterintuitive observation could potentially be attributed to the inclusion of patients with, on average, long-term disease duration and most of them experiencing a state of low disease activity. Moreover, further investigations into specific CTX subtypes and their association with RA disease activity may reveal additional insights. In

aggregate we can speculate that, in long standing RA, bone loss is mainly driven by impaired bone formation which is governed by altered Wnt system.

Our correlation analysis in RA patients unveiled intriguing relationships between biomarkers and clinical variables. 25-OH-vitamin D displayed a negative association with HAQ scores, emphasizing the role of vitamin D in functional status and disease severity in RA which has been reported in other cohorts (18–21). However, this association cannot prove a causal relationship but at least support a role of 25-OH-vitamin D as a proxy of severe disease and worse outcomes. Positive associations between P1nP and CRP levels and between PTH and DAS28-CRP scores suggest intricate interactions between inflammation, bone turnover, and disease activity in RA. We previously showed that PTH levels are the most important single determinant of Dkk1 levels (14, 22, 23), and their simultaneous elevation might predispose to bone erosions (13, 14, 24). Nonetheless, inflammation levels of the RA cohort were low on average, and we cannot generalize the present results to patients with active disease and more prominent inflammation. Similarly, the positive association of sclerostin levels with glucocorticoid cumulative dose despite being thought-provoking from a pathophysiological point of view, might have been triggered by the long duration of the disease which is, not surprisingly, highly correlated with GC cumulative dose. Additionally, the positive association between Dkk1 levels and RA disease duration suggests that Wnt pathway dysregulation may be linked to disease progression. Notwithstanding that, romosozumab, a sclerostin inhibitor, yielded encouraging results in preventing and treating GC induced osteoporosis (GIOP) in RA patients corroborating our finding (25–28).

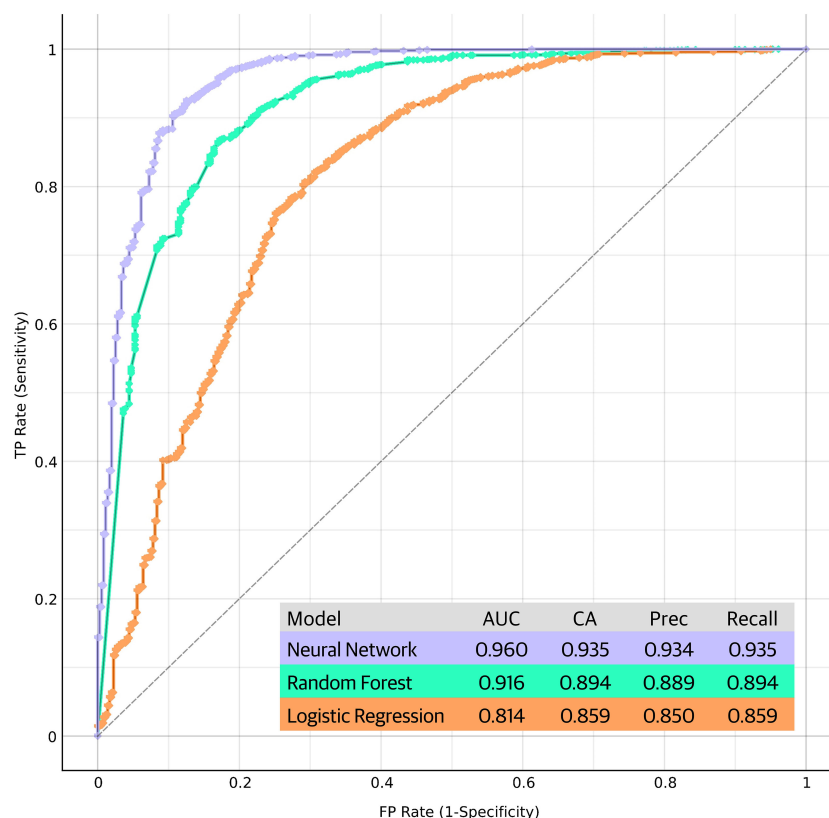


FIGURE 6

Performance metrics and receiver operating characteristic (ROC) curves of the machine learning models in discriminating RA patients from controls based on their biomarker profiles. The ROC curves plot the true positive rate (sensitivity) against the false positive rate (1-specificity) at various threshold settings, providing a comprehensive visualization of the models' discriminative power. Each curve represents the model's performance in distinguishing RA cases from controls, with the area under the curve (AUC) serving as an indicator of the overall predictive accuracy. Performance of the models are shown in the figure. CA, classification accuracy; Prec, precision.

Our PCA analysis revealed distinct clustering patterns among the study participants, reflecting unique biomarker profiles associated with RA group. The PCA approach allowed us to visualize the multidimensional nature of the biomarker data, highlighting potential biomarker combinations that contribute to the differentiation of RA from other rheumatic diseases and healthy controls. This supports the notion that RA possesses a characteristic bone signature, distinct from other rheumatic conditions. Furthermore, the ML analysis demonstrated promising results in the discrimination of RA patients from age-matched healthy controls based on their biomarker profiles. The Neural Network model exhibited superior performance, suggesting that complex relationships among biomarkers may hold key information for disease classification. These findings suggest that ML approaches could serve as valuable tools for the development of diagnostic or prognostic biomarker panels in RA.

Our study has strengths and limitations. We employed a cross-sectional design, which limits our ability to establish causality or determine the temporal relationships between biomarkers, clinical parameters, and disease progression. RA is a highly heterogeneous

disease with varying clinical manifestations and disease courses. In this study, we focused on a specific subset of RA patients with long-term disease duration and low disease activity. Consequently, the results may not fully capture the diversity of the RA population, particularly those with more active disease or shorter disease duration. Although we collected extensive clinical data, including disease duration, DAS28-CRP, CRP levels, ESR levels, HAQ scores, and GC dose, other relevant clinical variables, such as radiographic joint damage, were not included in our analysis. These factors can potentially influence biomarker levels and bone health in RA patients. While we aimed to age-match healthy controls to the RA patient group, other factors such as lifestyle, dietary habits, and comorbidities were not accounted for in the control group. These uncontrolled variables could introduce confounding effects on biomarker levels and BMD measurements. Biomarkers assessed in this study represent only a subset of the intricate pathways involved in bone remodeling. Additional bone-related biomarkers and factors may contribute to the overall understanding of bone metabolism in RA. The machine learning models used for disease classification were trained and tested on the same dataset. External

validation on an independent cohort of RA patients and controls is essential to assess the generalizability and robustness of the models in real-world clinical settings.

In conclusion, our study has provided comprehensive insights into the bone signature of RA, characterized by alterations in bone density and distinctive biomarker profiles. These findings contribute to our understanding of the complex interplay between inflammation, bone turnover, and disease activity in RA, with potential implications for diagnostic and therapeutic strategies in the management of bone involvement in this debilitating disease.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethic committee of the Verona Hospital protocol REUMABANK. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

GA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. AF: Investigation, Validation, Visualization, Writing – review & editing. MR: Investigation, Supervision, Validation, Visualization, Writing – review & editing. CB: Investigation, Validation, Visualization, Writing – review & editing. RB: Investigation, Validation, Visualization, Writing – review & editing. DR: Investigation, Validation, Visualization, Writing – review & editing. OV: Investigation, Validation, Visualization, Writing – review & editing. DG: Investigation, Supervision, Validation, Visualization, Writing – original draft.

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Conflict of interest

GA reports personal fees from Theramex, UCB, Lilly, Galapagos, Fresenius Kabi, Amgen. DG has received advisory board honoraria, consultancy fees and/or speaker fees from Abiogen, Celgene, Eli-Lilly, Neopharmed-Gentili, Pfizer, UCB. MR reports advisory board honoraria, consultancy fees and/or speaker fees from AbbVie, Amgen, BMS, Eli-Lilly, Galapagos, Menarini, Sandoz, Theramex, UCB, outside the submitted work. OV has received advisory board honoraria and speaker fees from Gilead, Fresenius Kabi, Biogen, Eli-Lilly, UCB, Abbvie, MSD, BMS. AF reports personal fees from Abiogen, Novartis, Neopharmed.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1291727/full#supplementary-material>

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The neutrophil-lymphocyte ratio predicts all-cause and cardiovascular mortality among U.S. adults with rheumatoid arthritis: results from NHANES 1999–2020

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Background: The neutrophil-to-lymphocyte ratio (NLR) is recognized as a biomarker for systemic inflammation and immune activation. However, its connection with the mortality risk in individuals with rheumatoid arthritis (RA) is not well understood. This study aimed to investigate the association between NLR and all-cause and cardiovascular mortality risk in U.S. adults with RA.

Methods: Data were gathered from the National Health and Nutrition Examination Survey (NHANES) cycles spanning 1999 to March 2020. We included adults aged ≥ 20 years. The NLR was computed by dividing the neutrophil count by the lymphocyte count from complete blood counts. The maximally selected rank statistics method helped identify the optimal NLR cutoff value associated with significant survival outcomes. Multivariable logistic regression models were performed to investigate the relationship between the NLR and the all-cause and cardiovascular mortality of RA. Restricted cubic spline (RCS) analyses were utilized to detect whether there were linear or non-linear relationships between NLR and mortality.

Results: In this study, 2002 adults with RA were included, with 339 having a higher NLR (≥ 3.28) and 1663 having a lower NLR (< 3.28). During a median follow-up of 84 months, 79 RA individuals died. Participants with higher NLR had a 2-fold increased risk of all-cause (HR = 2.02, 95% CI: 1.53–2.66) and cardiovascular mortality (HR = 2.48, 95% CI: 1.34–4.57) versus lower NLR, after adjusting for demographics, socioeconomic status, and lifestyle factors. Kaplan-Meier analysis revealed that the survival rate for the higher NLR group was significantly lower than the lower NLR group, in terms of both all-cause and cardiovascular mortality (both $P < 0.0001$). The RCS curve demonstrated a positive linear association between the NLR and all-cause and cardiovascular mortality.

Conclusion: A higher NLR was independently predictive of elevated long-term mortality risk in U.S. adults with RA. The NLR may serve as an inexpensive, widely available prognostic marker in RA.

KEYWORDS

neutrophil, lymphocyte, inflammation, mortality, rheumatoid arthritis

1 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by systemic inflammation that can lead to joint damage and disability (1). RA is a global public health concern, with prevalence estimates of 17.6 million cases worldwide in 2020, reflecting a 14.1% increase since 1990. Projections indicate that the RA burden will continue to rise, with forecasts predicting that the number of individuals affected globally will reach 31.7 million by 2050 (2). Patients with RA have a reduced life expectancy, with cardiovascular disease being the leading cause of premature mortality (3, 4). Understanding the factors influencing RA incidence and mortality is crucial for effective management and intervention strategies. Persistent inflammation in RA plays a crucial role in its pathogenesis and progression and is also linked to the increased cardiovascular risk and mortality observed in RA (4, 5). Therefore, markers of inflammation may have prognostic utility in this population.

A perfect prognostic scoring system should offer prognostic parameters that are easily discernible during diagnosis and be cost-effective in clinical practice, ensuring accessibility and affordability for widespread use. The neutrophil-to-lymphocyte ratio (NLR) stands as a burgeoning biomarker, offering insights into systemic inflammation and immune system activation (6). It provides a comprehensive perspective on both innate (neutrophils) and adaptive (lymphocytes) immune responses, showcasing its potential as an essential indicator in understanding the body's inflammatory and immune states (7). Additionally, the NLR can be easily calculated from routine complete blood counts, making it highly accessible for clinical use. Its association with unfavorable outcomes has been demonstrated across a spectrum of diseases (8, 9).

In RA, previous studies have touched upon the relationship between NLR and the presence of the disease (10, 11). Recently, there has been a surge in studies exploring the potential of these hematologically derived biomarkers to differentiate between RA patients with active disease and those in remission (12–14). However, these investigations were often limited by small sample sizes. Additionally, while these studies explored the connection between NLR and RA incidence or disease activity, there is a notable gap in the literature regarding the association of NLR with the survival status of RA patients.

In light of the necessity for affordable and accessible prognostic markers in RA, our objective was to investigate the association

between NLR and the risk of all-cause and cardiovascular mortality. We conducted these analyses using a vast, nationally representative sample of U.S. adults with RA obtained from the National Health and Nutrition Examination Survey (NHANES) dataset.

2 Methods

2.1 Study design and population

NHANES is an ongoing program conducted by the Centers for Disease Control and Prevention (CDC) of America to assess the health and nutritional status of US adults and children. The survey combines interviews, physical examinations, and laboratory tests. Detailed methods can be extracted from the official website (<http://www.cdc.gov/nchs/nhanes.htm>, accessed on 1 October 2023).

The original survey protocol underwent rigorous ethical scrutiny and received approval from the Institutional Review Board of the CDC. Prior to their participation, all individuals involved in the study willingly provided informed consent by signing consent forms. The present study, which utilized data derived from the approved survey, was reviewed by the Institutional Review Board of our center. Following careful evaluation, the Board determined that the present study fell under the category of exemption, signifying that it met the necessary ethical standards and regulations and therefore did not require additional ethical approval.

This prospective cohort study utilized data from the continuous NHANES conducted between 1999 and March 2020. Exclusion criteria encompassed individuals younger than 20 years, those with incomplete data regarding complete blood count parameters, participants lacking information on RA or other forms of arthritis, and individuals with missing data for other essential covariates. The flowchart of participants inclusion and exclusion is depicted in Figure 1.

2.2 Definition of primary variates

Participants were specifically asked “Has a doctor or other health professional ever told you that you had arthritis?” and they could respond with either “Yes” or “No.” To further differentiate between types of arthritis, individuals with a positive response were asked to specify the type of arthritis, with response options

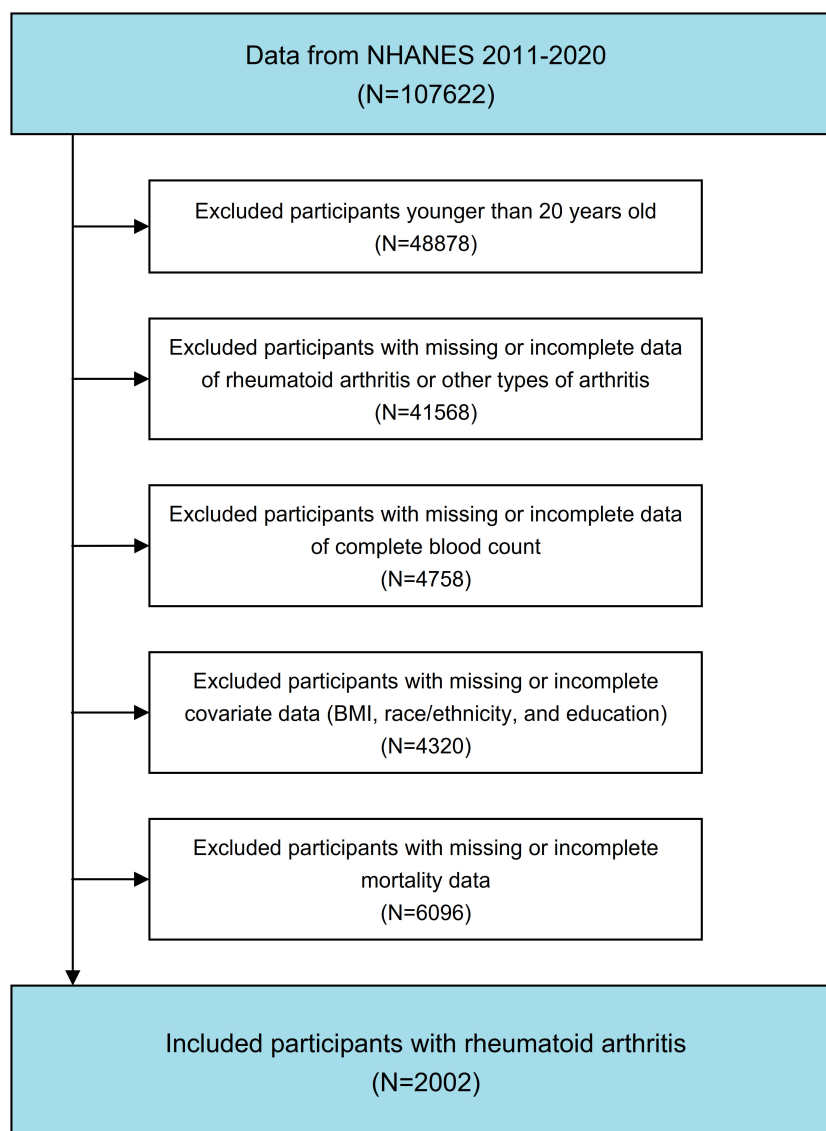


FIGURE 1
Flow chart portraying participant inclusion and exclusion in the current study.

including “Rheumatoid arthritis,” “Osteoarthritis,” “Psoriatic arthritis,” “Other,” “Refused,” and “Do not know.”

The NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count from the same automated complete blood count sample. The optimal NLR cutoff point of 3.28 for mortality prediction was determined using the maximally selected rank statistics method (Figure 2).

The primary outcome was all-cause mortality, ascertained using mortality-linked files (MLFs) documenting death from any cause. These mortality data were sourced from the National Death Index (NDI) database, accessible at <https://www.cdc.gov/nchs/data-linkage/mortality-public.htm>. Each participant’s follow-up duration was calculated from their date of participation until the date of death or until December 31, 2019, which marked the final update date of the NDI database.

2.3 Assessment of covariates

Demographic covariates included age (continuous), sex (male/female), and race/ethnicity (non-Hispanic white, non-Hispanic black, Mexican American, other Hispanic, and other/mixed race). Socioeconomic status was assessed using educational attainment (less than 9th grade, 9-11th grade, high school graduate, some college, college graduate and above) and poverty-income ratio (<1.29, 1.30-3.49, ≥3.50). Lifestyle and health-related factors comprised body mass index (BMI) categorized as underweight (<18.49 kg/m²), normal (18.50-24.99 kg/m²), overweight (25.00-29.99 kg/m²), or obese (≥30.00 kg/m²); smoking status (current smoker vs nonsmoker); and health insurance status (insured vs uninsured). All multivariable models sequentially adjusted for demographic factors, socioeconomics, lifestyle factors,

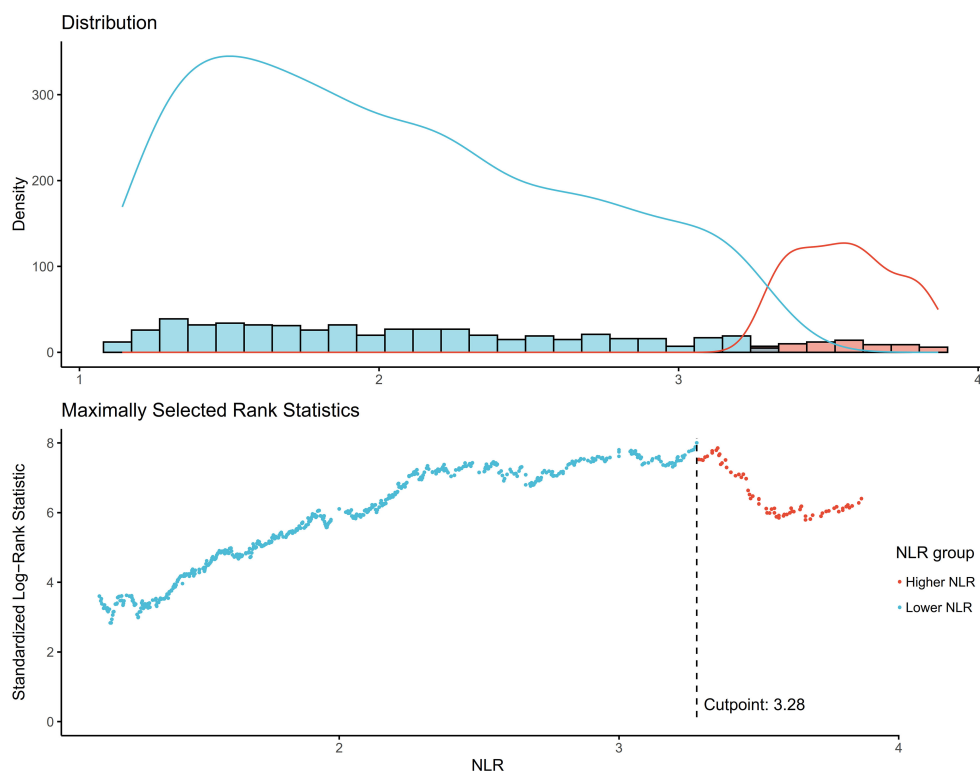


FIGURE 2
Determination of the NLR cutoff point using maximally selected rank statistics. Standardized Log-Rank Statistic was utilized in the calculation.

comorbidities, and medications where appropriate. Missing covariate data were addressed through listwise deletion.

2.4 Statistical analysis

Baseline characteristics were summarized using the mean (standard deviation) for continuous variables and the number (percentage) for categorical variables. Differences by the presence of RA or NLR groups were compared using chi-squared tests for categorical variables and *t* tests for continuous variables. Cox proportional hazards models were used to investigate the association between NLR and all-cause and cardiovascular mortality in individuals with RA. Three models were constructed with incremental adjustment for potential confounders. Model 1 was unadjusted. In model 2, age, sex, race/ethnicity, and educational attainment were additionally adjusted; Model 3 was additionally adjusted by age, sex, race/ethnicity, educational attainment, BMI, poverty income ratio, smoking status, and health insurance. Kaplan-Meier curves were created to illustrate the survival probabilities based on different NLR categories. Subgroup analyses were performed to assess effect modification by pertinent clinical confounders. Restricted cubic spline (RCS) model was constructed to visualize the relationship between continuous NLR

and outcomes. Analyses were performed using R version 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria). Two-sided $P < 0.05$ was considered statistically significant.

3 Results

3.1 Study population

Of 2002 participants with RA, 339 had higher NLR (≥ 3.28) while 1663 had lower NLR (< 3.28) (Table 1). Participants in the higher NLR group were older than those in the lower NLR group (mean age 62.19 vs 55.48 years, $P < 0.001$). The proportion of individuals aged 60–79 years was also higher in the higher NLR group compared to the lower NLR group (47.13% vs 33.73%, $P < 0.001$). The higher NLR group had a significantly greater proportion of non-Hispanic Whites compared to the lower NLR group (75.66% vs 67.57%, $P < 0.001$). There were no significant differences between the higher and lower NLR groups in terms of educational attainment, poverty income ratio, BMI, waist circumference, smoking status, and health insurance (all $P > 0.05$). The mean NLR was significantly higher in the higher NLR group compared to the lower NLR group (4.66 vs 1.91, $P < 0.001$). These results indicate that NLR levels are associated with age and race/

TABLE 1 Basic characteristics of participants with RA based on NLR category.

Characteristic	Overall (n=2002)	Higher NLR (n=339)	Lower NLR (n=1663)	P value
Weighted No.	44,689,857	7,531,516	37,158,342	
Age (year)	56.61 (14.43)	62.19 (14.72)	55.48 (14.11)	<0.001
Age group				<0.001
20-39 years	161 (11.56%)	20 (6.63%)	141 (12.56%)	
40-59 years	641 (41.06%)	73 (27.58%)	568 (43.79%)	
60-79 years	941 (35.99%)	174 (47.13%)	767 (33.73%)	
≥80 years	259 (11.39%)	72 (18.66%)	187 (9.91%)	
Sex				0.11
Female	1,173 (58.74%)	170 (53.05%)	1,003 (59.89%)	
Male	829 (41.26%)	169 (46.95%)	660 (40.11%)	
Race/ethnicity				<0.001
Mexican American	324 (5.89%)	49 (6.09%)	275 (5.85%)	
Other Hispanic	154 (4.85%)	25 (4.68%)	129 (4.88%)	
Non-Hispanic White	878 (68.93%)	192 (75.66%)	686 (67.57%)	
Non-Hispanic Black	569 (15.98%)	54 (7.48%)	515 (17.70%)	
Other/multiracial	77 (4.35%)	19 (6.09%)	58 (3.99%)	
Educational attainment				0.7
Less than 9th grade	349 (9.56%)	54 (9.98%)	295 (9.47%)	
9-11th grade	406 (17.41%)	61 (16.18%)	345 (17.66%)	
High school graduate	482 (28.10%)	91 (29.89%)	391 (27.74%)	
Some college	544 (31.21%)	92 (32.93%)	452 (30.86%)	
College graduate or above	221 (13.72%)	41 (11.02%)	180 (14.27%)	
Poverty income ratio	2.52 (1.58)	2.36 (1.51)	2.55 (1.60)	0.2
Poverty income ratio group				0.061
0-1.29	807 (31.03%)	128 (32.49%)	679 (30.73%)	
1.30-3.49	749 (37.62%)	140 (42.79%)	609 (36.57%)	
≥3.50	446 (31.35%)	71 (24.72%)	375 (32.70%)	
BMI (kg/m ²)	30.31 (7.37)	29.79 (7.38)	30.41 (7.37)	0.2
BMI group				0.3
Underweight	23 (1.24%)	4 (2.35%)	19 (1.01%)	
Normal	423 (22.80%)	90 (26.19%)	333 (22.12%)	
Overweight	613 (29.71%)	99 (29.71%)	514 (29.71%)	
Obese	943 (46.25%)	146 (41.75%)	797 (47.16%)	
Waist circumference (cm)	103.03 (16.96)	103.52 (17.49)	102.93 (16.85)	>0.9
Smoking status				0.3
Non-smoker	868 (40.04%)	148 (36.76%)	720 (40.71%)	
Smoker	1,134 (59.96%)	191 (63.24%)	943 (59.29%)	
Health insurance				0.7
Insured	1,751 (87.99%)	302 (87.23%)	1,449 (88.14%)	

(Continued)

TABLE 1 Continued

Characteristic	Overall (n=2002)	Higher NLR (n=339)	Lower NLR (n=1663)	P value
Uninsured	251 (12.01%)	37 (12.77%)	214 (11.86%)	
NLR	2.38 (1.41)	4.66 (1.89)	1.91 (0.63)	<0.001

Continuous variables are expressed as mean $\pm$ standard deviation (SD), while categorical variables are presented as number (%). All estimates were adjusted for sample weights and complex survey designs, with means and percentages further adjusted for the survey weights of NHANES. RA, rheumatoid arthritis; BMI, body mass index; NLR, neutrophil to lymphocyte ratio.

ethnicity, but not with socioeconomic factors, lifestyle factors, or other health-related variables. Participants with higher NLR were older and more likely to be non-Hispanic White.

3.2 Association between NLR and mortality

Over a median follow-up of 84 months, 79 RA individuals died. The higher NLR group had 24 observed deaths versus only 11.7 expected deaths. In contrast, the lower NLR group had fewer observed deaths (55) than expected (67.3). In the unadjusted model (Model 1), higher NLR as a continuous variable was significantly associated with increased risk of all-cause mortality (HR per 1 unit increase 1.24, 95% CI: 1.17-1.31, $P<0.001$) and cardiovascular mortality (HR = 1.29, 95% CI: 1.18-1.40, $P<0.001$). When NLR was evaluated as a categorical variable, participants in the higher NLR group had a 2-fold higher risk of all-cause mortality (HR = 2.11, 95% CI: 1.59-2.81, $P<0.001$) and a 3-fold higher risk of cardiovascular mortality (HR = 3.23, 95% CI: 1.82-5.72, $P<0.001$) compared to the lower NLR group. Model 2 was adjusted for demographic factors including age, sex, race/ethnicity, and education. The associations remained statistically significant. Model 3 was further adjusted for BMI, poverty income ratio, smoking, and insurance in addition to demographics. The positive associations between higher NLR and elevated mortality risks persisted. As a continuous variable, each 1-unit NLR increase was linked to 20% and 22% higher all-cause and cardiovascular mortality. Categorically, the higher NLR group had 2.02 and 2.48 times the risk of all-cause and cardiovascular mortality versus lower NLR participants (Table 2). Kaplan-Meier analysis revealed that the survival rate for the higher NLR group was significantly lower than the lower NLR group, in terms of both all-cause and cardiovascular mortality (both $P<0.0001$) (Figure 3). These findings suggest that

higher NLR, whether evaluated as a continuous or categorical variable, was consistently and independently associated with increased all-cause and cardiovascular mortality risk in US adults with RA.

3.3 Stratified analyses

In subgroup analyses stratified by sex, age, race/ethnicity, education, BMI, and smoking status, higher NLR was consistently associated with increased all-cause and cardiovascular mortality risk across all subgroups (Table 3). For all-cause mortality, no significant interaction was observed by sex ($P=0.659$), age ($P=0.536$), race/ethnicity ($P=0.863$), education ($P=0.735$), BMI ($P=0.873$), or smoking status ($P=0.277$). For cardiovascular mortality, a significant interaction was found by race/ethnicity ($P=0.014$). No other significant interactions were detected. These results indicate that the relationship between higher NLR and increased mortality risk persists across subgroups of potential confounders. The racial/ethnic difference in the association between NLR and cardiovascular mortality warrants further investigation.

3.4 Nonlinear relationship of NPR and mortality

Figure 4A displays the RCS curve for the association between continuous NLR and all-cause mortality risk in participants with RA. The overall association was statistically significant (P overall <0.0001). There was evidence of a nonlinear relationship, with the curve showing an initial steep increase in mortality risk at lower NLR values which plateaued at higher values (P non-linear = 0.0231). Figure 4B shows the RCS curve for the relationship

TABLE 2 Association of NLR with mortality risk in adults with RA.

Characteristic	Model 1		Model 2		Model 3	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
All-cause mortality						
NLR (continuous variable)	1.24 (1.17, 1.31)	<0.001	1.21 (1.14, 1.29)	<0.001	1.20 (1.14, 1.26)	<0.001
Higher NLR*	2.11 (1.59, 2.81)	<0.001	2.11 (1.59, 2.81)	<0.001	2.02 (1.53, 2.66)	<0.001
Cardiovascular mortality						
NLR (continuous variable)	1.29 (1.18, 1.40)	<0.001	1.26 (1.15, 1.38)	<0.001	1.22 (1.13, 1.31)	<0.001
Higher NLR*	3.23 (1.82, 5.72)	<0.001	2.66 (1.43, 4.96)	0.002	2.48 (1.34, 4.57)	0.004

In model 1, covariates were not adjusted; Model 2 was adjusted by age, sex, race/ethnicity, and educational attainment; Model 3 was adjusted by age, sex, race/ethnicity, educational attainment, BMI, poverty income ratio, smoking status, and health insurance. *Risk of mortality was analyzed compared to lower NLR group.

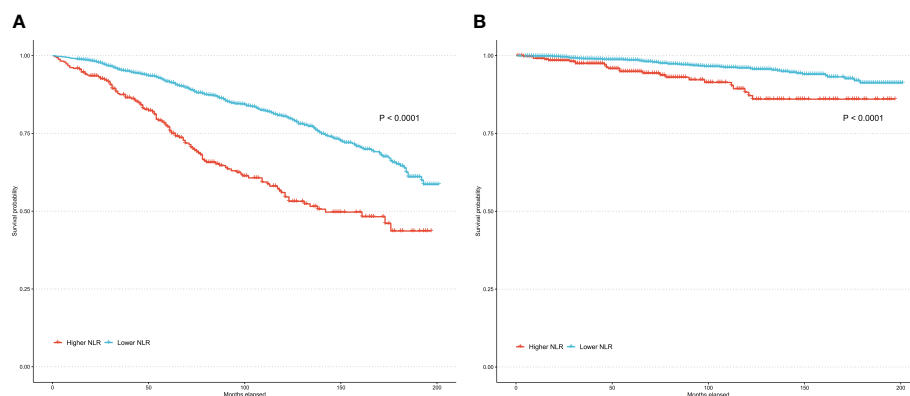


FIGURE 3
Kaplan–Meier curves of all-cause mortality (A) and cardiovascular mortality (B) among participates with RA.

between continuous NLR and cardiovascular mortality risk in participates with RA. The overall association was statistically significant (P overall = 0.0236). The curve depicts a linear relationship between increasing NLR and cardiovascular mortality without statistical evidence of nonlinearity (P non-linear = 0.5919). These findings highlight the differential relationships between this inflammatory biomarker and various mortality outcomes in individuals with RA.

4 Discussion

In this large nationally representative cohort of US adults, we found that a higher NLR was independently predictive of increased all-cause and cardiovascular mortality risk among adults with RA. Kaplan-Meier curves illustrated significantly lower survival probabilities for those with higher NLR. RCS analyses demonstrated linear positive associations between NLR and all-

TABLE 3 Subgroup analyses of NLR and mortality risk in RA.

Variable	All-cause mortality			Cardiovascular mortality		
	HR (95% CI)	P value	P for inter-action	HR (95% CI)	P value	P for inter-action
Overall	2.44 (1.97, 3.01)	<0.001		2.52 (1.56, 4.07)	<0.001	
Sex			0.659			0.684
Female	2.53 (1.87, 3.44)	<0.001		2.08 (0.94, 4.58)	0.07	
Male	2.29 (1.7, 3.09)	<0.001		2.62 (1.42, 4.85)	0.002	
Age group			0.536			0.927
<60 years	1.71 (0.83, 3.53)	0.144		1.85 (0.21, 16.55)	0.582	
≥60 years	2.15 (1.72, 2.69)	<0.001		2.13 (1.3, 3.49)	0.003	
Race/ethnicity			0.863			0.014
White	2.33 (1.8, 3.02)	<0.001		3.73 (2.14, 6.51)	<0.001	
Other	2.24 (1.53, 3.28)	<0.001		0.29 (0.04, 2.16)	0.229	
Educational attainment			0.735			0.683
Less than 9th grade	2.00 (1.24, 3.22)	0.004		1.63 (0.55, 4.86)	0.378	
9-11th grade	2.99 (1.86, 4.8)	<0.001		2.46 (0.8, 7.55)	0.116	
High school graduate	2.46 (1.67, 3.61)	<0.001		3.22 (1.44, 7.17)	0.004	
Some college	2.69 (1.63, 4.46)	<0.001		2.25 (0.58, 8.72)	0.239	
College graduate or above	2.58 (1.3, 5.12)	0.007		6.33 (1.05, 38.02)	0.044	
BMI group			0.873			0.753

(Continued)

TABLE 3 Continued

Variable	All-cause mortality			Cardiovascular mortality		
	HR (95% CI)	P value	P for inter-action	HR (95% CI)	P value	P for inter-action
Underweight	5.32 (0.74, 38.12)	0.096		4.87 (0.3, 77.93)	0.263	
Normal	2.29 (1.54, 3.39)	<0.001		3.21 (1.43, 7.25)	0.005	
Overweight	2.54 (1.77, 3.65)	<0.001		2.00 (0.65, 6.15)	0.226	
Obese	2.36 (1.64, 3.4)	<0.001		2.13 (1, 4.54)	0.05	
Smoking status			0.277			0.451
Smoker	2.20 (1.67, 2.9)	<0.001		2.15 (1.13, 4.09)	0.019	
Non-smoker	2.83 (2.02, 3.95)	<0.001		3.14 (1.52, 6.5)	0.002	

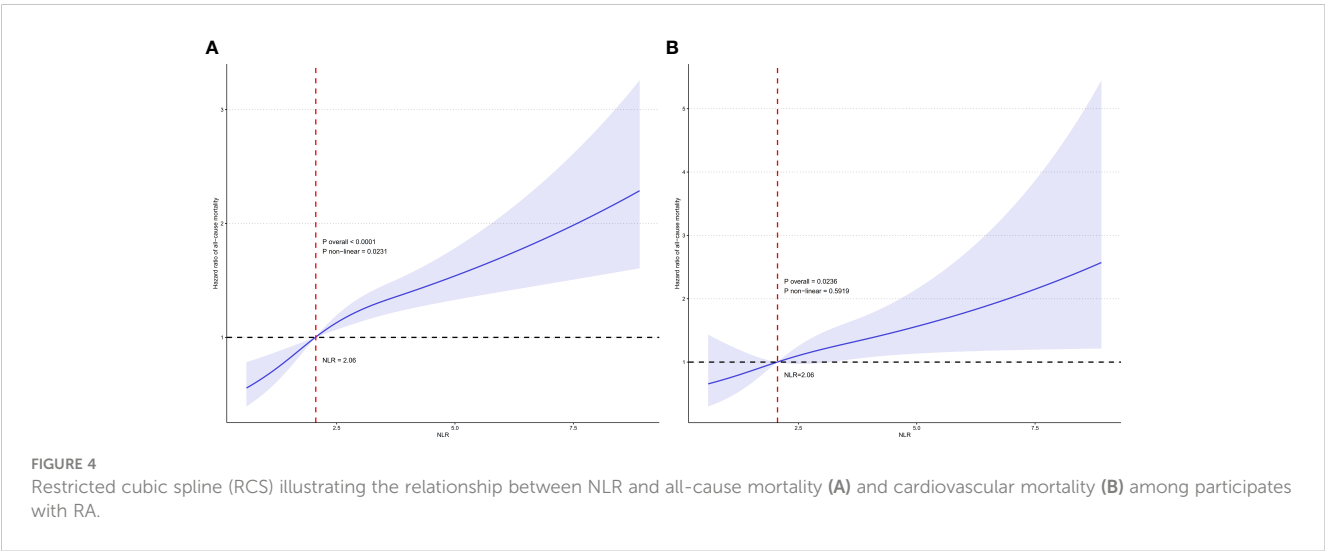
HRs were adjusted for age, sex, race/ethnicity, BMI, and smoking status. When analyzing age, adjustments were made for the other variables, and so on for subsequent analyses.

cause and cardiovascular mortality. These data highlight the potential utility of the NLR as an inexpensive and widely available marker for RA risk stratification and prognostication in clinical practice.

A number of prior studies have examined the association between NLR and RA with inconsistent findings. Several studies found significantly higher NLR values in RA patients than in healthy controls, suggesting that the NLR may be a useful biomarker for RA diagnosis (15–18). Other studies did not observe significant differences in NLR between RA and controls, implying limited diagnostic utility (18, 19). Regarding the relationship between the NLR and RA disease activity, most studies reported positive correlations, with a higher NLR associated with more active disease and an even lower response rate after TNF- α therapy or an increased risk of anti-TNF- α agent withdrawal due to a lack of efficacy (12, 14, 20–24). This indicates that the NLR could serve as an inflammatory marker reflecting RA disease activity. Compared to prior studies with small sample sizes, our study leveraged a large nationally representative cohort to provide robust evidence for the utility of the NLR as a biomarker for RA risk stratification and prognostication.

Prior studies have reported associations between higher NLR and increased mortality risk across the general population and

various conditions marked by significant inflammatory components (25, 26). In glomerulonephritis, a study established a connection between NLR and mortality prediction in patients with rapidly progressive glomerulonephritis (RPGN). It was found to be associated with systemic inflammation and exhibited a negative correlation with the percentage of fibro-cellular crescents (27). In asthma, a previous study concluded that the NLR and other complete blood cell count-derived inflammatory biomarkers are associated with a higher risk of all-cause and respiratory disease mortality in adult patients (28). In colon cancer, a study found that the NLR was an independent predictor of overall mortality, with the authors concluding that the NLR may serve as a useful prognostic marker (29). Similarly, in acute coronary syndrome (ACS), a meta-analysis of over 9,000 patients demonstrated that an elevated pretreatment NLR predicted higher medium- to long-term mortality and major adverse cardiac events (MACEs) (30, 31). More recently, in COVID-19, multiple studies have shown that the NLR correlates with disease severity and mortality (32, 33). A meta-analysis concluded that evaluating the NLR could help clinicians identify severe COVID-19 cases early and initiate timely management to potentially reduce mortality (26). However, the available evidence regarding the prognostic significance of NLR for survival in RA remains limited. Our study



effectively addresses this gap, providing new evidence that underscores the significance of the NLR as an independent predictor of all-cause mortality in RA.

Moreover, determining specific NLR cutoffs tailored to individual patient populations could improve the predictive accuracy of this marker in clinical settings. While a consensus on normal NLR values is lacking, there is a suggested range of 1–2 for normal values, 2–3 as a gray area indicating subclinical inflammation, and values above 3 indicating inflammation (34). Our study aligns with these recommendations, establishing an optimal NLR cutoff of 3.28, which correlated with the most significant survival outcomes. However, as noted in other conditions, the NLR alone without other inflammatory markers may have limited utility. Future research should examine combining NLR with other biomarkers to improve prognostic accuracy in RA.

The intricate pathological processes underlying autoimmune diseases have been meticulously explored, elucidating the interplay between innate and adaptive immunity. The NLR, comprising neutrophil and lymphocyte counts, provides valuable insights into the body's inflammatory status and immune response. A higher neutrophil count suggests an ongoing nonspecific inflammatory process, while a lower lymphocyte count indicates a relatively compromised immune system (35). Several lines of evidence suggest that dysregulated innate immunity and inflammation play key roles in RA pathogenesis (36, 37). Neutrophils are among the first responders during inflammation and immune activation in RA (37). Additionally, neutrophils may function as antigen-presenting cells (APCs) in presenting antigens and activating T cells to perpetuate chronic inflammation and autoimmunity (38). They also produce proteases such as elastase that contribute to cartilage and bone destruction in RA (39). Therefore, an elevated NLR likely implies higher activation of neutrophils and innate inflammatory pathways contributing to RA development and progression. The increased systemic inflammation marked by a high NLR could also accelerate atherosclerosis, conferring greater cardiovascular risk and mortality (40). Conversely, lymphocytes, such as regulatory T cells, normally act to suppress inflammatory responses and restore immune homeostasis (41). A lower lymphocyte count signified by a higher NLR may indicate impaired anti-inflammatory immunity. In summary, an elevated NLR represents enhanced proinflammatory innate responses along with potential deficiencies in anti-inflammatory lymphocyte activity. This imbalance could promote the inflammatory and immunopathogenic mechanisms underlying RA onset and severity. The NLR provides a simple readout of the skewing between pathological innate immunity and protective adaptive responses in RA.

Several limitations should be noted. First, the observational design of this study limit causal inferential ability. We identified associations between NLR and outcomes but cannot confirm a definitive causal relationship. Second, residual confounding is possible despite adjustments for an extensive set of potential confounders. Unmeasured factors such as comorbidities, smoking intensity, medications, and RA characteristics could influence the observed relationships. Third, the NLR was measured at a single timepoint and may not reflect variability over time or with interventions. Serial NLR measurements could provide a more accurate representation of inflammatory status. Finally, the study

population comprised U.S. adults, which may restrict generalizability to other populations. Further studies in diverse cohorts are warranted to validate our findings. However, the strengths of a large nationally representative sample, long follow-up, and robust adjustment for confounders enhance the reliability of the results.

5 Conclusion

In summary, the NLR provides a simple readout of the balance between pathological innate inflammation and protective adaptive immunity. Our study of a large nationally representative cohort demonstrates that a higher NLR is independently predictive of increased risks of both all-cause and cardiovascular mortality among adults with RA. These findings highlight the potential clinical utility of the NLR as an inexpensive and widely available biomarker that can be integrated into routine care to improve risk stratification and prognostication in RA patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Ethics statement

The original survey protocol underwent rigorous ethical scrutiny and received approval from the Institutional Review Board of CDC. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JW: Conceptualization, Writing – review & editing. EZ: Data curation, Writing – original draft. XZ: Methodology, Writing – review & editing. YY: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Efficacy and safety of anakinra and canakinumab in PSTPIP1-associated inflammatory diseases: a comprehensive scoping review

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Introduction: This scoping review explores the effectiveness of IL-1 pathway inhibitors in managing PSTPIP1-associated inflammatory diseases (PAID). These diseases are marked by abnormal IL-1 pathway activation due to genetic mutations.

Methods: Our methodology adhered to a pre-published protocol and involved a thorough search of MEDLINE and EMBASE databases up to February 2022, following the Joanna Briggs Institute Reviewer's Manual and the PRISMA Extension for Scoping Reviews. The review included studies reporting on IL-1 pathway inhibitor use in PAID patients.

Results: From an initial pool of 5,225 articles, 36 studies involving 43 patients were selected. The studies predominantly used observational designs and exhibited diversity in patient demographics, treatment approaches, and outcomes. Anakinra and canakinumab demonstrated promise in treating sterile pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) and PSTPIP1-associated myeloid-related-proteinemia inflammatory (PAMI) syndromes, with scant data on other syndromes. Notably, there was a paucity of information on the adverse effects of these treatments, necessitating cautious interpretation of their safety profile.

Conclusion: Current evidence on IL-1 pathway inhibitors for PAID is primarily from observational studies and remains limited. Rigorous research with larger patient cohorts is imperative for more definitive conclusions. Collaborative efforts among specialized research centers and international health initiatives are key to advancing this field.

KEYWORDS

autoinflammatory diseases, PSTPIP1 gene mutations, PAID, interleukin 1 pathway, IL-1 inhibitors, anakinra, canakinumab, scoping review

Introduction

Autoinflammatory syndromes are marked by an innate immune response dysregulation, leading to recurrent systemic inflammation episodes (1). Central to this process are mutations in the PSTPIP1 gene, which lead to the accumulation of intracellular triggers that exacerbate cellular stress and amplify the activation of inflammasome sensors (2, 3). Inflammasomes are cytosolic protein complexes that play a crucial role in producing key proinflammatory cytokines like interleukin-1 β (IL-1 β) and IL-18 through caspase 1 activation (4, 5).

The PSTPIP1 gene encodes the proline-serine-threonine phosphatase-interacting protein 1, predominantly expressed in hematopoietic tissues. This protein is essential in cytoskeletal organization and inflammatory responses. It consists of four distinct domains: the N-terminal FER-CIP4 homology domain, the cdc15-like segment, a PEST-rich region, and the C-terminal SH3 domain. Notably, the coiled-coil region within the cdc15-like segment and the C-terminal SH3 domain are critical for binding to pyrin, an inflammasome sensor (6, 7).

One of the earliest identified PAID was sterile pyogenic arthritis (PA), pyoderma gangrenosum (PG), and acne (PAPA) syndrome (Orphan ID: 69126), first described in 1997 and linked to chromosome 15 (5). PAPA syndrome is characterized by its namesake symptoms and shows high variability in its manifestation and genetic penetrance, often associated with various heterozygous mutations in the PSTPIP1 gene (8, 9).

In 2012, Braun-Falco et al. reported a related condition, PASH syndrome (Orphan ID: 289478), involving PG, acne, and hidradenitis suppurativa (HS) (10). This syndrome is linked to alleles with a high number of CCTG motif repeats near the PSTPIP1 promoter and other mutations in the gene (11). Moreover, variations in PSTPIP1 have been connected to other syndromes, including PAPASH syndrome (Orphan ID: 641380), PsAPASH syndrome, and PSTPIP1-associated myeloid-related-proteinemia inflammatory (PAMI) syndrome (Orphan ID: 251523) (12, 13), as outlined in Table 1. PG is a common clinical manifestation among all these syndromes, Table 1. The mutations causing PAPA, as well as in most of the other PAID syndromes, lead to a gain of function of the PSTPIP1 protein. Interestingly, there are mutations in this same gene, caused by aberrant splicing, that result in a partial, if not any, lack of function of the protein and leads to sporadic PG alone (12). However, IL-1b is upregulated in both, sporadic and syndromic PG (13).

Treating PAID syndromes is complex and typically necessitates a multidisciplinary strategy (14). A variety of treatment options have been explored, ranging from monotherapy to combination therapies. These include isotretinoin, various anti-inflammatory agents (such as non-steroidal anti-inflammatory drugs, corticosteroids, colchicine, and thalidomide), immunosuppressive agents (like methotrexate, cyclosporine, mycophenolate mofetil, tacrolimus, azathioprine, and leflunomide), tumor necrosis factor- α inhibitors (infliximab, etanercept, and adalimumab), as well as plasmapheresis, intravenous immunoglobulins, and allogeneic hematopoietic stem cell transplantation (15). The efficacy of these

treatments varies from patient to patient, often requiring a personalized, trial-and-error approach to identify the most effective regimen for each individual.

With advancing knowledge of the pathogenesis of autoinflammatory diseases, targeted treatments have become more prevalent. IL-1 β , in particular, has garnered significant attention within the IL-1 family for its crucial role in several autoinflammatory diseases (16). In a non-sensitive genetic background, the inflammasome typically activates only in response to infections, playing essential roles in pathogen defense, damaged cell removal, and adaptive immune response stimulation (17). However, in various inflammatory disorders, infectious diseases, and cancers, this regulatory balance can be disrupted (18).

PSTPIP1 interacts with several proteins, including protein-tyrosine phosphatase (PTP-PEST), the Wiskott-Aldrich syndrome protein (WASP), the c-Abl kinase, CD2, the Fas ligand (FASL), and Pyrin/TRIM20 (19, 20). The activation of Pyrin triggers the inflammasome and consequently leads to the production of inflammatory molecules, notably IL-1 β and IL-18 (21). Both IL-18 and IL-1 β , members of the IL-1 family, play key roles in innate immunity and are closely linked to autoinflammatory diseases (5). Specifically, IL-1 β is known as a potent endogenous pyrogen and an effective recruiter and activator of neutrophils and macrophages (20).

The development of drugs specifically targeting IL-1 β offers promising treatment options for autoinflammatory diseases (22, 23). These include monoclonal antibodies (mAbs) and recombinant receptor proteins fused to human immunoglobulin G (IgG) fragments. Currently, five drugs are specifically designed to target the IL-1 pathway:

a) Anakinra, a recombinant IL-1 receptor antagonist (IL-1RA), which competes with IL-1 receptor agonists for receptor binding. b) Rilonacept, acting as a soluble decoy receptor, inhibits the activation of IL-1 receptor I. c) Canakinumab, a human monoclonal antibody, selectively targets IL-1 β . d) Bermekimab (MABp1), an anti-IL-1 α monoclonal antibody. e) Gevokizumab, another anti-IL-1 β monoclonal antibody.

The safety and efficacy of these drugs in treating immune-mediated disorders were recently reviewed by Arnold et al. (22).

Given the rarity and recent identification of PAID syndromes, there's a notable scarcity of evidence and secondary research, like systematic reviews, on IL-1 pathway-modulating agents for their treatment (24, 25). This gap underscores the necessity of synthesizing evidence from primary studies on the use of anti-IL-1 drugs in treating PAPA, PASH, and other PAID syndromes. Such a synthesis should include mapping published articles, examining the epidemiology and genetic characteristics, and evaluating the efficacy and safety of anti-IL-1 drugs. This analysis will be crucial in identifying knowledge gaps and formulating research questions for future systematic reviews.

We aim to present and analyze the current evidence on the use of drugs targeting the Interleukin 1 Pathway in PSTPIP1-associated inflammatory diseases, specifically focusing on PAPA, PASH, PAPASH, PASS, PAMI, PAC, and PsAPASH syndromes.

TABLE 1 Genetics and clinical manifestations of the different PAID syndromes.

Full name	Syndrome (ORPHAN ID)	First Reported Case	Clinical Manifestations								Syndrome-Associated PSPTIP1 Mutations	Affected PSPTIP1 Protein Domain
			PA	A	PG	SH	SA	PsA	UC	Additional specific signals or symptoms		
Sterile pyogenic arthritis, pyoderma gangrenosum and acne syndrome	PAPA (69126)	1997								Arthralgia, tendinitis, periostitis with osteolytic lesions, ulcers, pustulosis, abscesses, rosacea, urticaria, edema, thrombophlebitis; anemia, elevated acute phase reactants, otitis, renal involvement, and diabetes mellitus.	p.A230T p.E250Q p.E250K p.D246N p.E256G p.D266N p.G258A c.G904A	2
PSTPIP1-associated myeloid-related-proteinemia inflammatory syndrome	PAMI (251523)	2015								Hyperzincemia, hypercalprotectinemia, neutropenia, arthralgia, morning stiffness, pustular rash, abscesses, erythema multiform; splenomegaly, anemia, thrombocytopenia, growth failure, lymphadenopathy, renal involvement, liver involvement, osteomyelitis, colitis, bleeding diathesis, muscular atrophy, granular T-lymphocyte clonal proliferation, and cerebral arterial vasculopathy/vasculitis with dissecting aneurysm.	p.E250K p.E257K	2
Sterile pyogenic arthritis, pyoderma gangrenosum, acne and hidradenitis suppurativa syndrome	PAPASH (641380)	2013								Abscesses	p.E277D	2
Pyoderma gangrenosum, acne and hidradenitis suppurativa syndrome	PASH (289478)	2012								High BMI, depression, diabetes mellitus, hypertension, hepatopathy; increased leukocytes and thrombocytes count, and CRP, GGT, and serum amyloid A levels.	p.Y345C p.A405C Increased number of CCTG microsatellite repeats in the 5'UTR of PSPTIP1 gene	4
Pyoderma gangrenosum, acne vulgaris, hidradenitis suppurativa and ankylosing spondylitis syndrome	PASS (641385)	2012								Hepatitis B infection, α -thalassemia, elevated CRP, and intermittent fever.	–	–
Psoriatic Arthritis, pyoderma gangrenosum, acne, suppurative hidradenitis	PsAPASH (641390)	2015								Psoriasis, pseudotinea amiantacea, and purulent painful nodules.	–	–
Pyoderma gangrenosum, acne and ulcerative colitis syndrome	PAC (-)	2015								Recalcitrant pustular rash	p.G403R	4

PA, pyogenic arthritis; A, acne; PG, pyoderma gangrenosum; SH, suppurative hidradenitis; SA, ankylosing spondylitis; PsA, psoriatic arthritis; UC, ulcerative Colitis; p, protein; c, cDNA; blue light colour: symptom showed by the syndrome (blue light colour).

Methods

We have pre-published a scoping review protocol to guide our study (26). The conduct and reporting of our research adhered to the methodologies outlined in the Joanna Briggs Institute Reviewer's Manual (27) and followed the PRISMA Extension for Scoping Reviews (PRISMA-ScR) guidelines (28).

Literature searches

Strategies for literature search and eligibility criteria are described in [Supplementary Methods \(Supplementary Table S1\)](#).

Eligibility criteria

For inclusion in our review, papers needed to present evidence of using drugs that target the interleukin 1 pathway in PAID. Criteria for inclusion were: studies written in English, involving human participants, and addressing the conditions specified in our research question, with no restrictions on publication date or format. Exclusion criteria encompassed studies outside our conceptual framework, such as those not focused on IL-1 pathway inhibitors in the target population, animal or *in vitro* studies, articles not in English, and non-scientific reviews.

Data charting

A data charting form was collaboratively developed by two researchers to identify the variables for extraction. This form was

initially tested on five studies, with the selected variables being recorded in a.csv file. Both reviewers independently charted data from the studies, regularly discussed their findings, and iteratively updated the form. The final report includes variables related to study design and metadata extracted from primary sources. Data were primarily gathered from clinical trial webpages, supplemented by congress abstracts and full-text articles when necessary.

Collation, summarization and reporting of results

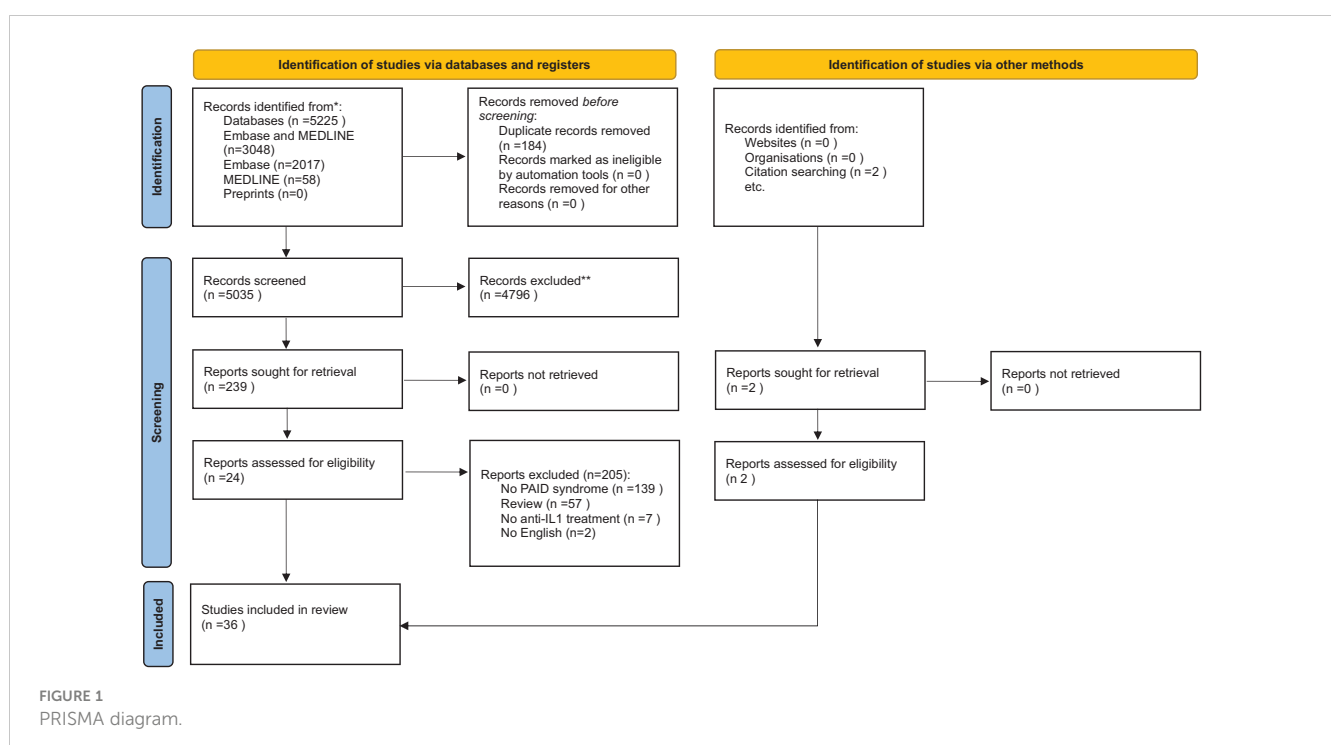
The results of the comprehensive research are presented using a PRISMA flow diagram ([Figure 1](#)). We first grouped the references and primary studies, syndrome-wise. Second, a narrative and qualitative synthesis of PAID mapping references, studies, and efficacy and safety data findings were elaborated using tables.

Deviations from the original scoping review protocol

In conducting this scoping review, we adhered to the protocol as outlined in our published methodology in *BMJ Open* (26). No deviations from the original protocol were made during the review process. The data charting, collation, summarization, and reporting followed the predetermined methods and variables specified in the protocol.

Compliance with ethics guidelines

This article is a review based on previously published studies and does not include any original research involving human



participants or animals. Therefore, no ethical approval was required for this study.

Results

Searches

In February 2022, we conducted an extensive literature search on the use of anti-IL1-related drugs in autoinflammatory diseases associated with PSTPIP1 mutations, focusing on EMBASE and MEDLINE databases. This search yielded a total of 5,525 articles. After removing duplicates and conducting title, abstract, and keyword screenings, 239 studies were identified for full-text review. Ultimately, 34 articles met our inclusion criteria, and an additional two were added after reference checks of these studies (see [Figure 1](#) PRISMA diagram). [Supplementary Tables S2, S3](#) in the [Supplementary Material](#) detail the reference lists of all reviewed articles, including reasons for their inclusion or exclusion.

Mapping studies

PAPA syndrome

From 2005 to 2019, 16 observational studies were published on this topic, including 11 case reports and 5 case series. These were presented as full papers (7), congress abstracts (6), or letters (3), as detailed in [Supplementary Table S4](#). Notably, none of these studies followed a pre-established protocol or were registered in any public registry.

Of these studies, 11 (68.7%) were multicentric collaborations, involving an average of 7.3 authors per article (range: 3 to 14). They spanned multiple medical specialties: pediatrics and rheumatology (11 each), allergy-immunology (8), dermatology (6), internal medicine-infectious diseases (5), and others including pathology, radiology, oncology genome, laboratory medicine, and genetics (one each).

Geographically, most studies (7) originated from Italy, with others conducted in the United States, The Netherlands, Canada, Germany, Austria, and Bosnia and Herzegovina. The studies were published across various specialized journals in fields like rheumatology (11), dermatology (3), pediatrics (1), and infectious diseases (1).

Regarding funding, only two studies (12.5%) declared their sources, with one citing public funding and another disclosing both public and pharmaceutical funding. Conflicts of interest (CoIs) were declared in four studies (25%), with the most frequently cited pharmaceutical companies being Novartis, Sobi, Pfizer, Abbvie, Novimmune, Roche, and Sanofi. These disclosures are crucial for maintaining transparency about potential influences on research findings.

PAMI syndrome

From 2017 to 2021, we identified seven studies focusing on PAMI syndrome, all observational in nature, comprising four case reports and three case series. Notably, none adhered to a pre-

established protocol or were registered in any public registry, underscoring a lack of pre-defined design and registration. Of these, five were multicenter and two unicenter, with a broad geographic distribution across Switzerland (1 study), Russia (2), Italy (3), and Brazil (1), as listed in [Supplementary Table S5](#).

In terms of journal publications, the studies were diverse: one in a general medicine journal, one in allergy/immunology, two in dermatology, one in rheumatology, and two in pediatrics. The average author count was 9.14, ranging from 6 to 14. These included one full paper, one letter, three case reports, one brief report, and one abstract. The represented medical specialties were Pediatrics and Immunology (in three papers each), Dermatology, Genetics, and Rheumatology (two each), and Molecular Biology, Allergy, Nephrology, and Hematology (one each).

Funding-wise, two studies reported public funding, four declared no funding, and one did not specify. Furthermore, one study disclosed CoIs with Novartis.

PASH syndrome

From 2012 to 2020, seven observational case report studies on the use of anti-IL1 drugs for PASH syndrome were published, none of which followed a pre-established protocol or were registered in a public registry. Of these, five (71.4%) were unicenter studies, with two conducted in Germany and one each in Spain, France, the USA, Ireland, Australia, and Austria. All studies were published in dermatology journals, as detailed in [Supplementary Table S6](#).

The publication formats varied, with three being full papers, two letters, one a case report, and one an abstract from a conference. The average number of authors was 5.29, ranging from 4 to 7. Dermatology was the most common specialty, featured in all seven papers, while rheumatology and allergy were each mentioned in one paper.

Regarding funding, most studies did not declare any, and one study provided no information on this aspect. One study disclosed a CoI with Sun Pharma, another provided no CoI information, and the remaining five declared no CoIs.

PASS, PAPASH and PAC syndromes

Between 2013 and 2020, we identified six studies focusing on different PAID syndromes: three investigating PASS syndrome, two examining PAPASH syndrome, and one on PAC syndrome. All studies were observational: one was a case series and the rest were case reports, as listed in [Supplementary Table S7](#). Similar to previous sections, none followed a pre-established protocol or were registered in a public registry.

Geographically, two unicenter studies focused on PAC and PASS syndromes in Israel and Switzerland, respectively, while four multicenter studies on PASS and PAPASH syndromes were conducted in France and Italy. Journal-wise, five studies appeared in dermatology journals and one in a general medicine journal. The formats included three full papers (one each for PAC, PASS, and PAPASH) and three letters (one PAPASH and two PASS).

The average author count was 9, ranging from 6 to 14. Dermatology was the most common specialty, represented in all six papers, followed by Rheumatology in three, and Genetics and

Pathology in two each. Other represented fields included Pediatrics, Gastroenterology, and General Internal Medicine.

Regarding funding, three studies disclosed their sources: two cited public funding, and one indicated no funding. Two studies omitted funding details. Concerning CoIs, one study disclosed multiple affiliations with pharmaceutical companies such as AbbVie, Amgen, Boehringer-Ingelheim, Celgene, Janssen, Leo Pharma, Lilly, Mylan, Novartis, Pfizer, Sun Pharmaceuticals, Bristol-Myers Squibb, MSD, Roche-Chugai, AstraZeneca, Grunenthal, Ipsen/Menarini, Savient, Sanofi Aventis, UCB, and also reported grant support from Pfizer and L'Oreal. One paper did not provide CoI information.

Epidemiology

PAPA syndrome

Data were gathered from 22 patients treated with anakinra (19 patients) or anakinra followed by canakinumab (3 patients). Aggregate data from three studies (#12, #25, and #26) on patients treated with these regimens were also included in the analysis. Out of these patients, gender information was available for 20, with 30% (six patients) being female. Geographically, the patient cohort was diverse, including seven from Italy, five from the USA, four from Canada, two each from Germany and the Netherlands, and one each from Austria and Bosnia and Herzegovina.

Arthritis emerged as the most prevalent symptom, affecting 90.1% (20 out of 22 patients). Commonly affected joints were the knees and elbows. Other symptoms observed included acne in half of the patients, with five cases being severe nodulocystic acne; PG in 45.4% (10 patients); anemia in 22.7% (5 patients); fever in 18.1% (4 patients); and leukocytosis in 13.6% (3 patients). Additional reported symptoms included lymphadenopathy, splenomegaly, thrombocytopenia, pharyngeal papillomatosis, sterile osteomyelitis, skin abscesses, recurrent otitis, pathergy, pustules, elevated CRP and ESR, abdominal pain, spontaneous abortion, oral mucosa aphthous lesions, pyogenic muscular abscess, dactylitis/tendinitis, palpebral edema, growth delay, and synovitis.

The onset age, available for 14 patients, had a median of 5 years (range: <1 to 18 years), with a median diagnosis age of 17 years (range: 2–51 years). Notably, 95.5% (21 out of 22) had received prior treatments before anti-IL1 drugs, including systemic and intralesional corticosteroids, NSAIDs, methotrexate, infliximab, tacrolimus ointment, adalimumab, cyclosporine A, isotretinoin, dapsone, antibiotics, and in some cases, gold salts, hydroxychloroquine, etanercept, colchicine, mycophenolate mofetil, plasmapheresis, and thalidomide. This diversity highlights the range of treatments attempted before IL-1 targeted therapy.

PAMI syndrome

Data were obtained from nine patients treated with anakinra (5 patients), anakinra followed by canakinumab (2 patients), or solely canakinumab (2 patients). Of these, three were female, five were male, and data were unavailable for one patient. Geographically, two patients each hailed from Italy and Russia, with one each from

Brazil, Germany, and the United Kingdom, and data missing for one patient.

Regarding symptoms, arthritis was observed in 55.5% (5 out of 9 patients), arthralgia in 11.1% (1 patient), with data missing for two patients. Acne was present in 22.2% (2 patients), absent in 44.4% (4 patients), and data were unavailable for three patients. PG was noted in 22.2% (2 patients), absent in 55.5% (5 patients), with missing data for two. Other symptoms included elevated levels of zinc and calprotectin MRP8/14, anemia, hepatosplenomegaly, leukopenia with neutropenia, mild thrombocytopenia, osteomyelitis, trilineage dysplasia, and increased CRP and ESR.

The onset age, available for five patients, had a median of 1.2 months (range: birth to 7 years). The median diagnosis age was 4.7 years (range: 6 months to 23 years). Patients had previously received treatments including adalimumab, various antibiotics, azithromycin, ceftriaxone, colchicine, corticosteroids, cyclophosphamide, dapsone, etanercept, IVIG or HDIVIG, infliximab, intra-articular steroid injections, methylprednisolone, NSAIDs, prednisone, isotretinoin, rituximab, ruxolitinib, secukinumab, tacrolimus, tocilizumab, and topical tacrolimus.

PASH syndrome

Data from seven patients (three females) treated with anakinra (six patients) and canakinumab (one patient) were analyzed. These patients were from diverse locations including Russia, Spain, Turkey, Ireland, the USA, Australia, and France. All patients exhibited HS, while acne and PG were also prevalent symptoms. A high BMI was noted in 42.9% (three out of seven) of the patients. Other observed comorbidities and laboratory abnormalities included depression, diabetes mellitus, hypertension, hepatopathy, and elevated levels of leukocytes, thrombocytes, CRP, gamma-glutamyltransferase (GGT), and serum amyloid A.

The age at onset, available for five patients, had a median of 24.5 years (range: 15 to 37 years). The median age at diagnosis was 34 years (range: 22 to 59 years). Prior treatments for these patients were varied and included corticosteroids, ciclosporin, methotrexate, sulfone, antibiotics, finasteride, surgery, infliximab, adalimumab, ustekinumab, etanercept, fumaric acid, dapsone, morphine, isotretinoin, and tildrakizumab.

PASS, PAPASH and PAC syndromes

We collected data from five patients treated with anakinra, encompassing various syndromes. Three patients, two females from France and one male from Congo, had PASS syndrome; one female from Moldavia had PAPASH syndrome; and one male from Israel had PAC syndrome. HS was noted in all patients with PAPASH or PASS syndromes. Acne was present in four patients: one each with PAPASH and PAC, and two with PASS syndrome. All five patients exhibited PG, and all except the PAC syndrome patient had arthritis. The three PASS syndrome patients also had spondylitis (two ankylosing spondylitis, one undifferentiated spondylitis), along with other symptoms including hepatitis B infection, α -thalassemia, elevated CRP, and intermittent fever. The PAC syndrome patient showed symptoms of ulcerative colitis and a recalcitrant pustular rash.

Onset age data, available for three patients, had a median of 30 years (range: 14 to 32 years). The median age at diagnosis, obtained from five patients, was 32 years (range: 16 to 33 years). These patients had received a variety of prior treatments, including azithromycin, dapson, etanercept, infliximab, isotretinoin, methylprednisolone, prednisolone, secukinumab, tocilizumab, topical tacrolimus, and ustekinumab.

Genotypic variations

PAID syndromes are rare, autosomal-dominant autoinflammatory diseases characterized by incomplete penetrance and variable expression (29, 30). These genetic traits result in a spectrum of clinical manifestations linked to each mutation in the PSTPIP1 gene, complicating both the diagnosis and treatment of the disease (31).

PAPA syndrome

In our review, various mutations in the PSTPIP1 gene were identified among PAPA patients treated with anti-IL-1 drugs, as outlined in [Supplementary Table S8](#). All cases exhibited these mutations in heterozygosity. A notable mutation was the A230T missense mutation, an alanine to threonine substitution at amino acid position 230, found in seven patients (IDs 1, 3-4, 16, 19, 29 in [Supplementary Table S2](#)). At amino acid position 250, two distinct substitution mutations were observed: E250Q in five patients (IDs 8, 13, 17, 19 in [Supplementary Table S2](#)) and E250K in two patients (IDs 17, 29 in [Supplementary Table S2](#)). The E256G mutation occurred in two patients (ID 17 in [Supplementary Table S2](#)). Additionally, a c.G904A nucleotide substitution mutation was noted in patient #8 (ID 16), while patient #10 had a wild-type allele of PSTPIP1 (ID 11 in [Supplementary Table S2](#)), and patient #12 had the G258A mutation (ID 6 in [Supplementary Table S2](#)). Unspecified mutations in PSTPIP1 were found in patients #15 (ID 2 in [Supplementary Table S2](#)) and patients #21 and #22 (ID 22 in [Supplementary Table S2](#)).

PAMI syndrome

For PAMI syndrome, all eight patients featured in this review shared the E250K mutation in the PSTPIP1 gene, as indicated by IDs 21 and 23 in [Supplementary Table S2](#).

PASH syndrome

In patients with PASH syndrome, only one type of mutation in the PSTPIP1 gene was reported. This involved an increased number of CCTG microsatellite repeats in the PSTPIP1 promoter region, specifically 5 repeats on one allele and 8 on the other, indicating allelic heterogeneity (ID 5 in [Supplementary Table S2](#)). No additional mutations in the PSTPIP1 gene were identified for this patient.

PASS, PAPASH and PAC syndrome

For PASS syndrome, genetic information was unavailable for two patients (IDs 15 and 24 in [Supplementary Table S2](#)). The third patient (ID#4) exhibited a Q703K amino acid substitution variant in

the NLRP3 gene (ID 28 in [Supplementary Table S2](#)). However, this NLRP3 variant is common in the general population and may also occur in asymptomatic individuals, suggesting it may be a genetic risk factor rather than the definitive cause of the syndrome. In the case of PAPASH syndrome, the single patient identified had a missense mutation in the PSTPIP1 gene, specifically E277D (ID 7 in [Supplementary Table S2](#)). For the patient with PAC syndrome, a G403R mutation in the PSTPIP1 gene was noted (ID 9 in [Supplementary Table S2](#)).

Efficacy and Safety of IL-1 Based Agents Drugs in Treatment of PAID Syndromes

PAPA syndrome

We collected data for 22 patients treated with anakinra (IDs 1-4, 6, 8, 11, 13, 16-17, 19, 22, 29 in [Supplementary Table S2](#)) and three additional patients treated with canakinumab (IDs 6, 17, 19 in [Supplementary Table S2](#)), [Table 2](#). Anakinra treatment duration varied from five days to 38 months, while canakinumab treatment lasted eight to nine months. The most common anakinra dosage was 100 mg/day, used in 58.3% of patients with available data (seven out of 12), with no dosage reductions required. Corticosteroids were concomitantly used in five anakinra-treated patients (IDs 2, 17, 22 in [Supplementary Table S2](#)), with two requiring dosage reductions.

Short-term (<12 weeks) clinical response data available for seven patients showed complete improvement in six treated with anakinra and one with canakinumab. One anakinra-treated patient did not respond well in the short term. In the medium to long term (>12 to >24 weeks), 10 patients (83.3% with available data) responded well to treatment. Regarding specific symptoms, PA improved in 82.3% (14 out of 17) of patients, acne in 62.5%, and PG in 50%, with variations in response completeness.

Among the three canakinumab-treated patients, one showed complete improvement in acne, PG, and arthritis. The second patient had a long-term response improving PG but not acne or arthritis, while the third improved in arthritis without acne development or reported PG.

For anakinra safety, reported AEs included transient injection-site reactions (2 patients), infections like hepatitis B reactivation, MRSA infection, and multiple infections, plus one sickness episode. Anakinra was discontinued in four patients due to disease flare-ups. No AEs leading to discontinuation were reported for canakinumab.

PAMI syndrome

Efficacy and safety data were analyzed for eight patients with PAMI syndrome, as shown in [Table 3](#). Four patients received anakinra treatment (IDs 31-32, 35 in [Supplementary Table S2](#)), three were treated with canakinumab (IDs 21, 33-34 in [Supplementary Table S2](#)), and two underwent sequential treatment with anakinra followed by canakinumab (IDs 31, 34 in [Supplementary Table S2](#)). Data were unavailable for one patient (ID 23 in [Supplementary Table S2](#)).

Anakinra treatment duration ranged from 6 months to 2 years, and canakinumab treatment lasted from 3 months to 1 year. Corticosteroids were used alongside anakinra in one patient (ID

TABLE 2 Treatment response to anti-IL1 drugs in PAPA syndrome.

ID article (IDPatient)/ Cycles	DRUG/ DOSE- duration	Previous treatment	Concomitant treatment	Clinical efficacy			Efficacy 6-12 weeks/ 12-24 weeks/ >24 weeks/	Adverse events/ recurrence
				PA	Acne	PG		
19 (1)/1	Anakinra 100 mg/day-180 days	Corticosteroids gold salts, NSAIDs, isotretinoin	Isotretinoin	Yes (c)	No	–	NA NA NA	NA NA
19 (1)/2	Canakinumab 150mg/8weeks- NA-	Corticosteroids gold salts, NSAIDs, isotretinoin	Isotretinoin	Yes (c)	No	–	NA NA NA	NA NA
17 (2)/1	Anakinra 1,5/kg/day- 38 months	Corticosteroid metotrexate	Corticosteroid no	Yes (p)	–	–	NA NA 152 weeks	NA NA
17 (3)/1	Anakinra 2mg/kg/ day 31 months	no	no	–	–	Yes (p)	NA NA 124 weeks	NA NA
17 (3)/2	Canakinumab 2- 4mg/8weeks 8 months	no	no	–	–	Yes (c)	NA NA 32 weeks	NA NA
17 (4)/1	Anakinra 100mg/day 36 months	no	no	Yes (c)	Yes (c)	Yes (p)	NA NA 144 weeks	NA No
17 (5)/1	Anakinra 2mg/kg/ day 21 months	Intrarticular corticosteroids	no	Yes (c)	–	–	NA NA 84 weeks	NA No
17 (6)/1	Anakinra 100mg/day 8 months	Corticosteroid metotrexate	metotrexate	Yes (c)	NA	–	NA NA 32 weeks	NA Articular flares
16 (7)/1	Anakinra 12 weeks	Corticosteroid	NA	Yes (c)	–	–	2 weeks 12 weeks NA	NA NA
16 (8)/1	Anakinra 12 weeks	Corticosteroids	NA	Yes (c)	–	–	2 weeks NA NA	NA NA
13 (9)/1	Anakinra 1 month	NSAID Corticosteroids Metotrexate Infliximab Adalimumab	NA	NA	NA	NA	NA NA NA	Hepatitis B reactivation NA
11 (10)/1	Anakinra 100mg/day 26 months	NA	NA	–	–	Yes (c)	NA NA 104 months	NA No
8 (11)/1	Anakinra 6 months	NASAID Corticosteroids	NA	Yes (c)	–	–	NA 24 weeks NA	NA NA
6 (12)/1	Anakinra 100mg/day Few days	Antibiotics Corticosteroids	NA	No	No	No	NA NA NA	Adverse reaction at injection site and sickness NA
6 (12)/2	Canakinumab 150mg/8 weeks 9 months	Antibiotics Corticosteroids	NA	Yes (c)	Yes (c)	Yes (c)	8 weeks Yes 36 weeks	NA NA

(Continued)

TABLE 2 Continued

ID article (IDPatient)/ Cycles	DRUG/ DOSE- duration	Previous treatment	Concomitant treatment	Clinical efficacy			Efficacy 6-12 weeks/ 12-24 weeks/ >24 weeks/	Adverse events/ recurrence
				PA	Acne	PG		
4 (13)/1	Anakinra 100mg/day 5 days	NSAID Corticosteroids	NA	Yes (c)	NA	–	5 days NA NA	Pain at injection site NA
3 (14)/1	Anakinra 100mg/day 8 months	Corticosteroids Tacrolimus ointment	NA	Yes (c)	Yes (c)	Yes (c)	5 days Yes 36 weeks	NA No
2 (15)/1	Anakinra 0,3 to 1mg/kg/day 6 months	Corticosteroids Hydroxychloroquine Methotrexate Etanercept Colchicine Tacrolimus ointment Dapsone Mycophenolate mofetil Cyclosporina A Infliximab	Corticosteroids	NA	NA	No	No No NA	Infection with MRSA No
1 (16)/1	Anakinra 1mg/kg/ day 7 days	Intrarticular corticosteroids	NA	Yes (c)	NA	–	2 days NA NA	NA Yes
29 (17)/1	Anakinra	Plasmapheresis Thalidomide Dapsone Infliximab Tacrolimus	NA	No	No	No	NA NA NA	NA NA
29 (18)/1	Anakinra	Corticosteroids Etanercept Adalimumab Infliximab	NA	NA	–	NA	NA NA NA	Multiple infections No
29 (19)/1	Anakinra	Isotretinoin	NA	Yes (c)	Yes (c)	–	NA NA NA	NA NA
29 (20)/1	Anakinra	Corticosteroids Cyclosporine Tacrolimus	NA	No	–	No	NA NA NA	NA NA
22 (21)/1	Anakinra 6 weeks	NSAID Corticosteroids	Corticosteroids NSAID	Yes (c)	–	–	6 weeks NA NA	NA No
22 (22)/1	Anakinra	NSAID Corticosteroids	Corticosteroid NSAID	Yes (c)	Yes (c)	NA	NA NA NA	NA NA

PA, pyogenic arthritis; A, acne; PG, pyoderma gangrenosum; NA, not available; –, not applicable; NSAID, Non-steroidal anti-inflammatory drugs; c, complete (dark green colour); p, partial (green light colour); No, not response (red light colour). Partial (p) and Complete (c). Response to treatment are terms defined by the original studies.

28 in [Supplementary Table S2](#)) and with canakinumab in two patients (IDs 21, 34 in [Supplementary Table S2](#)), with one also receiving tacrolimus (ID 34 in [Supplementary Table S2](#)).

Two anakinra-treated patients showed good clinical responses in both short-term (<12 weeks) and long-term (>24 weeks) periods (IDs 32, 35 in [Supplementary Table S2](#)), with complete arthritis recovery reported for one (ID 32). Data on acne and PG responses were not available for these patients.

For canakinumab-treated patients, one exhibited a good response in both short-term and medium/long-term, with complete arthritis recovery (ID 33 in [Supplementary Table S2](#)), but no information on acne or PG responses was provided. Another patient showed no clinical efficacy with canakinumab alone but responded well to subsequent cyclosporine treatment, particularly in PG healing (ID 21 in [Supplementary Table S2](#)). AE data for canakinumab-treated patients were not fully reported.

TABLE 3 Treatment response to anti-IL1 drugs in PAMI syndrome.

ID article (IDPatient)/ Cycles	Syndrome	DRUG/ DOSE- duration	Previous treatment	Concomitant treatment	Clinical efficacy			Efficacy 6-12 weeks/ 12-24 weeks/ >24 weeks/	Adverse events/ Recurrence
					Acne	PG	PA		
21 (5)/1	PAMI	Canakinumab 300mg every 4 weeks	Prednisolone, topical tacrolimus, infliximab, Secukinumab, prednisone and isotretinoin	Corticosteroids, cyclosporine	NA	NA	NA	No	NA
		3 months						No	NA
								NA	
23 (6)/1	PAMI	NA	NA	NA	NA	NA	NA	NA	NA
31 (8)/1	PAMI	Anakinra	Infliximab, tocilizumab, HD IVIG, corticosteroids, ruxolitinib, rituximab, cyclophosphamide	NA	-	-	NA	NA	NA
31 (9)/1	PAMI	Anakinra	NA	NA	-	-	-	NA	NA
31 (9)/1		Canakinumab	NA	NA	-	-	-	NA	NA
31 (10)/1	PAMI	Anakinra	Etanercept, infliximab, corticosteroids	NA	-	-	NA	NA	NA
33 (11)/1	PAMI	Canakinumab 2mg/kg/ month and 4mg/ kg/month	Antibiotics, corticosteroids, IVIG, colchicine,	Cyclosporine	NA	-	Yes (c)	Yes	No
								Yes	No
								Yes (2 years)	
34 (12)/1	PAMI	Anakinra	Intra-articular steroids injections, antibiotics, adalimumab, colchicine	NA	Yes (c)	Yes (c)	NA	Yes	NA
		2 year						NA	NA
								NA	Poor compliance
34 (12)/2		Canakinumab	Intra-articular steroids injections, antibiotics, adalimumab, colchicine, anakinra	Corticosteroids	Yes (p)	Yes (p)	NA	Yes	Proteinuria
								Yes	Flares of acne and PG
								Yes	
34 (12)/3		Canakinumab 150mg monthly	Intra-articular steroids injections, antibiotics, adalimumab, colchicine, anakinra, corticosteroids, tacrolimus	Tacrolimus	Yes (p)	Yes (p)	NA	Yes	NA
				Corticosteroids				Yes	NA
								Yes	
35 (13)/1	PAMI	Anakinra 3mg/kg once daily	NA	NA	NA	NA	NA	Yes	Mild transient urticarial skin reaction at the injection site
		6 months						Yes	No
								Yes (6 months)	

PA, pyogenic arthritis; A, acne; PG, pyoderma gangrenosum; NA, not available; -, not applicable; NSAID, Non-steroidal anti-inflammatory drugs; c, complete (dark green colour); p, partial (green light colour). Partial (p) and Complete (c). Response to treatment are terms defined by the original studies.

Among patients receiving both anakinra and canakinumab, one demonstrated a good short-term response with improvements in acne and PG, but no medium/long-term response data were available (ID 34 in [Supplementary Table S2](#)). Information on arthritis and AEs, apart from reported proteinuria, was not provided. No efficacy or safety data were available for the other patient (ID 31 in [Supplementary Table S2](#)).

PASH syndrome

Efficacy and safety data were analyzed for seven patients with PASH syndrome, as detailed in [Table 4](#). Six patients received anakinra at dosages ranging from 100 to 200 mg/day for 12 to 52 weeks, and one patient was treated with canakinumab at 150 mg/week for four weeks.

Among the anakinra-treated patients, two (33.3%) showed partial improvement in PG, acne, and HS. One patient improved at week 24 (ID 5 in [Supplementary Table S2](#)), and the second improved at week 52 after increasing the anakinra dosage to 200 mg/day (ID 18 in [Supplementary Table S2](#)). Both continued anakinra treatment, with the first also receiving cyclosporine.

However, three other patients on a 100 mg/day anakinra dosage did not respond and discontinued the drug (IDs 5, 10, 36 in [Supplementary Table S2](#)), subsequently improving with alternative treatments such as infliximab (plus dapsone and cyclosporine), corticosteroids, and tildrakizumab. One patient discontinued anakinra after 15 days due to sigmoid diverticulitis and a benign exanthematous drug reaction. No additional AEs were reported for anakinra.

The patient treated with canakinumab did not respond effectively in either short or medium to long-term analysis and later showed improvement with infliximab. No AEs were reported with canakinumab.

PASS, PAPASH and PAC syndromes

Efficacy and safety data were compiled for patients with PASS, PAPASH, and PAC syndromes, as outlined in [Table 5](#). In PASS syndrome, three patients were treated with anakinra at 100 mg/day. Two of these patients (66.67%) responded partially or completely to the treatment (IDs 15, 28 in [Supplementary Table S2](#)). One patient experienced total relief from PG and partial relief from arthritis, HS,

TABLE 4 Treatment response to anti-IL1 drugs in PASH syndrome.

ID article (ID Patient)/Cycles	DRUG/DOSIS-duration	Previous treatment	Concomitant treatment	Clinical efficacy			Efficacy 6-12 weeks/12-24 weeks/>24 weeks/	Adverse events /recurrence
				SH	Acne	PG		
5 (1)/1	Anakinra 100mg/day 24 weeks	Isotretinoin 40mg	Ciclosporine	Yes (p)	Yes (c)	Yes (c)	NA NA 24 weeks	NA NA
10 (2)/1	Anakinra 100mg/day 12 weeks	Corticosteroids, antibiotics, methotrexate, sulfone, ciclosporin, finasteride, surgery, infliximab, adalimumab, ustekinumab	NA	No	–	–	No NA NA	NA NA
14 (3)/1	Anakinra NA NA	Corticosteroids, etanercept, adalimumab, fumaric acid, cyclosporine, dapsone, antibiotics, infliximab	NA	No	No	No	NA NA NA	NA NA
18 (4)/1	Anakinra 100-200mg/day 52 weeks	Morphine, antibiotics, cyclosporine, entecavir	Prednisone, NSAID	Yes (200mg/day)	Yes (c)	Yes (c)	6 weeks NA 52 weeks	NA NA
20 (5)/1	Canakinumab 150mg weekly 4 weeks	Prednisone, adalimumab, infliximab, minocycline, dapsone, methotrexate	NA	No	No	No	No NA NA	NA NA
27 (6)/1	Anakinra NA NA	Prednisolone 15mg, methotrexate, infliximab, isotretinoin, vancomycin, clindamycin, ertapenem, tildrakizumab	NA	No	No	No	NA NA NA	NA NA
36 (7)/1	Anakinra 100mg/day 15 days	Prednisone Etanercept	NA	NA	NA	NA	NA NA NA	Sigmoid diverticulitis, exanthematous drug reaction Yes

A, acne; PG, pyoderma gangrenosum; SH, suppurative hidradenitis; NA, not available; –, not applicable; NSAID, Non-steroidal anti-inflammatory drugs; c, complete (dark green colour); p, partial (green light colour); No, not response (red light colour). Partial (p) and Complete (c). Response to treatment are terms defined by the original studies.

TABLE 5 Treatment response to anti-IL1 drugs in PASS, PAPASH, and PAC syndromes.

ID article (IDPatient)/ Cycles	Syndrome	DRUG/ DOSE- duration	Previous treatment	Concomitant treatment	Clinical efficacy						Efficacy 6-12 weeks/ 12-24 weeks/ >24 weeks/	Adverse events /recurrence
					SH	Acne	PG	PA	UC	AS		
15 (2)/1	PASS	Anakinra 100mg/ day 4 weeks	Etanercept	NA	Yes (p)	Yes (p)	Yes	–	–	Yes (p)	Yes NA NA	NA Yes
24 (3)/1	PASS	Anakinra 100mg/day NA	Ustekinumab, tocilizumab	NA	NA	–	NA	–	–	NA	No NA NA	NA NA
28 (4)/1	PASS	Anakinra 100- 200mg/day 8 months	Prednisone	Corticosteroids	No	Yes	Yes	–	–	Yes	Yes Yes No	NA NA
7 (1)/1	PAPASH	Anakinra 100mg/ day NA	Azithromycin, dapsone, methylprednisolone, topical tacrolimus,	NA	Yes (p)	NA	NA	NA	–	–	Partial	Partial
9 (7)/1	PAC	Anakinra 100mg/ day 24 months	Prednisolone, topical tacrolimus, infliximab, Secukinumab, prednisone and isotretinoin	Corticosteroids, Isotretinoin	–	NA	NA	–	NA	–	Partial	Total

PA, pyogenic arthritis; A, acne; PG, pyoderma gangrenosum; SH, suppurative hidradenitis; SA, ankylosing spondylitis; PsA, psoriatic arthritis; UC, ulcerative Colitis; NA, not available; –, not applicable, HDIVIG, high-dose intravenous immunoglobulin; c, complete (dark green colour); p, partial (green light colour); No, not response (red light colour). Partial (p) and Complete (c). Response to treatment are terms defined by the original studies.

and acne but had a recurrence after stopping treatment and switched to infliximab (ID 15 in [Supplementary Table S2](#)). Another patient showed improvement in arthritis, PG, and acne initially but not in the long-term, also switching to infliximab (ID 28 in [Supplementary Table S2](#)). The third patient did not respond to anakinra (ID 24 in [Supplementary Table S2](#)), and no AEs were reported for these patients.

For PAPASH syndrome, one patient (ID 7 in [Supplementary Table S2](#)) treated with 100 mg/day of anakinra showed partial improvement in HS, acne, PG, and arthritis in the short term, but medium/long-term efficacy data were not available. No AEs were reported during treatment.

In the case of PAC syndrome, one patient (ID 9 in [Supplementary Table S2](#)) received 100 mg/day of anakinra alongside prednisone and isotretinoin. This regimen led to total efficacy in the short and long term, fully controlling PG and improving acne, with no reported AEs.

Discussion

Summary of findings

To the best of our knowledge, this review represents the first extensive evaluation of the therapeutic efficacy of IL-1 pathway inhibitors, particularly anakinra and canakinumab, in treating autoinflammatory diseases linked to PSTPIP1 gene mutations. The findings suggest promising results for these agents in managing various PAID syndromes, notably in improving skin manifestations and arthritis.

Anakinra and canakinumab have been used to treat PAPA, PASH, and PAMI syndromes, while only anakinra has been utilized for PASS, PAC, and PAPASH syndromes. No evidence was found for their use in PsAPASH syndrome. PAPA syndrome, being the first reported, showed the most extensive drug use evidence. The efficacy of these treatments varies based on the specific IL-1 inhibitor, the treated syndrome, and sometimes the patient's genetic mutation. Overall, better responses were observed in patients with PAPA and PAMI syndromes compared to those with PASH syndrome.

Anakinra's treatment duration varied, with some patients also receiving corticosteroids. While short-term response data were inconsistent, medium to long-term results indicated good responses in 91% of patients with available data. Patients non-responsive to anakinra initially showed improvement with infliximab. Anakinra demonstrated varying degrees of clinical improvement across PASH, PAPASH, and PAC syndromes, with dosage adjustments necessary in some cases.

Canakinumab exhibited mixed results, showing good response in a patient with PAMI syndrome, but data were insufficient for other patients. Safety-wise, anakinra and canakinumab generally showed favorable profiles, with similar AEs across syndromes and rare drug withdrawals due to AEs. Anakinra's common AE was transient injection-site reactions, while infections like hepatitis B reactivation and MRSA were also reported, highlighting the

potential risk associated with immunosuppressive therapies (32). Canakinumab's safety profile was favorable, with no discontinuation-required AEs reported. However, the limited number of patients treated with canakinumab restricts the certainty of these safety conclusions. The small sample size and retrospective nature of the analysis, along with a high degree of underreporting or absence of reporting, should be considered when interpreting these findings.

Research gaps

Enhancing the evidence base and collaboration for PAID syndromes

To strengthen the treatment evidence for PAID syndromes, it is crucial to prioritize well-designed, prospective studies and adopt a coordinated, multidisciplinary approach involving specialized centers and collaborative initiatives. This approach is expected to yield improved patient care, more robust research outcomes, and better-informed treatment decisions for these rare diseases.

Currently, much of the evidence in this field comes from observational studies, case reports, and retrospective analyses, which inherently limit the study design and introduce potential biases. The lack of *a priori* experimental designs has led to lower-quality evidence, affecting the strength and reliability of the evidence base for IL-1 inhibitors in treating PAID syndromes.

Future research should focus on well-structured, prospective studies with appropriate control groups to draw more definitive conclusions about the effectiveness and safety of IL-1 inhibitors. Rigorous study designs will enhance evidence quality and increase confidence in treatment recommendations.

Enhancing drug treatment evidence for PAID syndromes requires a collaborative effort involving specialized centers for rare diseases, such as NIAMS, CNDR, CIEMA, and the National Reference Center for Autoinflammatory Diseases and Periodicity Syndromes. Platforms like the Autoinflammatory Alliance and the Eurofever registry are vital for advancing research and understanding these conditions, facilitating collaboration, data sharing, and resource pooling.

Given the high costs of treating these rare diseases, fostering public-private partnerships in research and treatment is essential. Initiatives like the International Rare Diseases Consortium (IRDiRC, www.irdirc.org) and the European Alliance for Personalized Medicine (EAPM, <https://www.eapm.eu.com>) highlight the benefits of collaborative efforts in managing rare diseases, providing necessary resources and support for research, treatment, and access to therapies, ultimately aiding patients with PAID syndromes.

Addressing disparities in healthcare for PAID syndromes in non-developed countries: challenges and opportunities

Our review reveals an underrepresentation of PAID patients from non-developed countries, highlighting healthcare disparities (33). Contributing factors include limited awareness and knowledge of PAID among healthcare professionals in these regions. Given the

rarity of these diseases, clinicians may lack familiarity with their clinical presentations, diagnostic criteria, and management strategies.

A critical issue is the inadequate access to specialized care. Specialized centers and experts in PAID are predominantly located in developed countries or major urban areas, limiting access for patients in non-developed countries. This is compounded by the limited availability of specialized diagnostic tools, genetic testing, and advanced laboratory facilities.

Economic constraints further exacerbate these disparities. The affordability of diagnostic tests, medications, and long-term care poses a significant challenge, often placing these healthcare services beyond the reach of many individuals, thus impacting health outcomes.

Addressing these disparities requires further research to understand the unique challenges in diagnosing and treating rare diseases like PAID syndromes in developing countries. Efforts to improve awareness, access to diagnostic resources, and treatment availability are vital in these regions.

Understanding the genotype-phenotype associations in PAID syndromes: challenges and insights

In diagnosing PAID syndromes, PSTPIP1 gene sequencing has been conducted in all studied cases, though less frequently in PASH syndrome, as detailed in [Tables 1](#); [Supplementary Tables S8-11](#). The rarity of mutations in PASH syndrome cases might be incidental, given the small patient sample. Variants in the PSTPIP1 gene have varied interpretations in autoinflammatory diseases, from pathogenic to uncertain clinical significance, due to their incomplete penetrance and variable expression, complicating genotype-phenotype correlations.

Among the phenotypes associated with PSTPIP1 mutations, three main syndromes emerge: PAMI, PAPA, and PASH ([34](#)). In PAPA syndrome, the most commonly found variants were p.A230T and p.E250Q, previously deemed pathogenic ([25](#)). All PAMI syndrome patients reviewed exhibited the p.E250K variant, a known mutation associated with the syndrome. In the PASH case reviewed, the identified variant's functional effect was unknown, and the patient showed a partial response to anakinra ([35](#)).

These findings underscore the complexity and variability in genotype-phenotype associations in PSTPIP1-related autoinflammatory diseases, highlighting the need for more research to clarify their implications in diagnosis and treatment. Moreover, given PSTPIP1's potential interactions with proteins other than pyrin, alternative mechanisms beyond IL-1 signaling activation might contribute to disease pathogenesis, as suggested by Omenetti et al. ([36](#)). This is supported by Klötgen et al.'s report of canakinumab's failure and the partial positive response to secukinumab, an IL-17A inhibitor, in a PAMI case ([37](#)).

Improving measurement of response in PAID syndromes

The current approach to evaluating PAID syndromes relies heavily on clinical evolution and analytical parameters, yet it lacks standardized assessment criteria or consensus protocols. This can

hinder the comparability and reliability of results. Implementing tools tailored to PAID syndromes, like the Auto-Inflammatory Diseases Activity Index (AIDAI), could significantly enhance the measurement of treatment response ([38](#)). AIDAI offers a standardized framework that combines various clinical and laboratory parameters, providing a consistent and objective method to assess disease activity.

Adopting a similar approach for PAID syndromes would enable more consistent and objective measurements, improving comparisons across studies and the overall quality of evidence. Studies like Omenetti et al.'s, which utilized objective measurements to assess treatment response, underscore this point ([36](#)). Their research showed reduced IL-1 β secretion by monocytes and clinical improvements following anti-IL-1 treatment, demonstrating the importance of integrating biomarkers and objective assessments in response evaluation. Long-term follow-up data also affirms the efficacy of IL-1 blockers in reducing flare frequency and normalizing acute phase reactants.

By incorporating standardized assessment tools and objective measurements, such as biomarkers, into treatment evaluations for PAID syndromes, we can improve the accuracy and reliability of outcome assessments. This advancement will not only enhance patient care but also contribute to the generation of more robust evidence regarding treatment efficacy for these conditions.

Evaluating the efficacy of anti-IL-1 treatment in cases with pathogenic PSTPIP1 mutations

The effectiveness of anti-IL-1 treatment in cases with pathogenic PSTPIP1 mutations requires further exploration. Notably, in PAPA and PAMI syndromes, most patients with such mutations showed either complete or partial responses to anakinra or canakinumab, particularly regarding PG, acne, and PA, as seen in long-term follow-up. However, these treatments did not resolve neutropenia in reported PAMI cases (IDs 32-34 in [Supplementary Table S2](#)). This finding is consistent with prior research suggesting that inhibiting the IL-1 pathway may not effectively address neutropenia in PAMI patients, though it can mitigate inflammation related to pyrin in other autoinflammatory disorders ([39](#)).

In contrast, PASH syndrome cases mostly did not respond to these treatments, with those responding showing only partial improvement at higher doses or within shorter follow-up periods. The data on PAPASH, PASS, and PAC syndromes are limited and anecdotal, with anakinra being the sole treatment used to date.

Comprehensive understanding of anti-IL-1 treatment efficacy in cases with PSTPIP1 mutations necessitates more extensive research. This will enable the collection of stronger evidence and provide insights into treatment responses across various syndromes associated with these mutations.

Addressing underreporting and improving safety data in drug studies

The substantial underreporting and lack of detailed safety data, including serious adverse reactions, in the analyzed studies pose significant concerns. Inadequate reporting can obscure the true

safety profile of drugs, leading to their potentially inappropriate use or a failure to recognize and address critical adverse reactions (40). Addressing this challenge necessitates concerted efforts from researchers, journals, and regulatory bodies.

Implementing improved reporting practices is vital for enabling informed medical decisions about the benefits and risks of IL-1 based agents. Adhering to established case publication protocols like The CARE guidelines, which offer standardized reporting guidelines for clinical case reports, could significantly enhance the completeness and quality of safety data (41). By tackling underreporting and enhancing safety data quality, we can better understand the risks associated with IL-1 based agents and other medications, ultimately improving patient care and safety.

Strengths and limitations

This study boasts several strengths that bolster its rigor and credibility. Key among these is the adherence to a pre-established protocol and the employment of a scoping review-specific methodology, which significantly enhance the study's transparency and reproducibility. The research team's prior experience in conducting similar reviews further adds to the study's rigor, bringing valuable expertise and knowledge to the table. Notably, the independence of the working groups, indicated by the absence of external funding, implies that data collection and analysis were conducted without significant external influences, thereby enhancing the objectivity and reliability of the findings. The low incidence of reported conflicts of interest (CoIs) among the authors also strengthens the study's credibility, suggesting minimal financial or personal biases that could influence the results' interpretation. Collectively, these elements contribute to the robustness of the research, providing confidence in the validity of its findings and conclusions.

This study faces several notable limitations. Primarily, its reliance on observational studies, which are inherently susceptible to bias and confounding variables, makes it difficult to establish causality. Consequently, the results from these studies should be interpreted with caution. The absence of randomized controlled trials (RCTs) further limits the ability to conclusively determine the drugs' effectiveness and safety. Another significant drawback is the inclusion of a considerable number of abstracts and letters, rather than full-length articles, which often lack detailed methodology and comprehensive results. This can hinder a thorough analysis and understanding of the data.

A critical concern is the underreporting or absence of reporting on AEs. Since AEs are essential in assessing a drug's safety profile, their incomplete documentation can obscure a complete understanding of the potential risks. While efforts were made to obtain additional information from researchers, the study still faces the challenge of a lack of comprehensive adverse event data.

Additionally, the time constraints on the literature review process may have led to the exclusion of more recent studies, potentially omitting relevant and up-to-date research. This could affect the overall comprehensiveness and relevance of the findings. Given these limitations, it's crucial to approach the study's safety

findings with caution and acknowledge the need for further, more robust research to provide clearer evidence regarding the safety of IL-1 based agents in treating the studied syndromes.

Considering these limitations, it is important to approach the safety findings with caution and recognize the need for further research, including well-designed RCTs and studies with robust methodologies, to provide more definitive and reliable evidence regarding the safety of IL-1 based agents in the treatment of the studied syndromes.

Conclusions

In conclusion, while IL-1 based blockers like anakinra and canakinumab demonstrate potential in treating PAID syndromes, the current evidence has notable limitations. The predominance of observational studies in this research limits the ability to establish causality and draw firm conclusions. Additionally, the study underscores issues such as potential biases, limited data availability, and the underreporting of AEs. To overcome these challenges and provide more conclusive evidence, there is a pressing need for well-designed studies, particularly RCTs. Future research should aim to ascertain the efficacy, determine optimal dosages and treatment durations, and evaluate the long-term safety profiles of IL-1 based agents in managing PAID syndromes.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Author contributions

JS: Data curation, Formal analysis, Writing – original draft. FG: Data curation, Investigation, Supervision, Writing – original draft, Methodology. PA: Writing – review & editing. AM: Writing – review & editing. JG: Writing – review & editing. JR: Investigation, Supervision, Writing – original draft, Writing – review & editing, Methodology, Resources. BI: Writing – original draft, Writing – review & editing. EP: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1339337/full#supplementary-material>

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Causal role of immune cells in generalized anxiety disorder: Mendelian randomization study

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Background: Generalized anxiety disorder (GAD) is a prevalent emotional disorder that has received relatively little attention regarding its immunological basis. Recent years have seen the widespread use of high-density genetic markers such as SNPs or CNVs for genotyping, as well as the advancement of genome-wide association studies (GWAS) technologies, which have facilitated the understanding of immunological mechanisms underlying several major psychiatric disorders. Despite these advancements, the immunological basis of GAD remains poorly understood. In light of this, we aimed to explore the causal relationship between immune cells and the disease through a Mendelian randomization study.

Methods: The summary information for GAD (Ncase=4,666, Ncontrol=337,577) was obtained from the FinnGen dataset. Summary statistics for the characterization of 731 immune cells, including morphological parameters (MP=32), median fluorescence intensity (MFI=389), absolute cells (AC=118), and relative cells (RC=192), were derived from the GWAS catalog. The study involved both forward MR analysis, with immune cell traits as the exposure and GAD as the outcome, and reverse MR analysis, with GAD as the exposure and immune cell traits as the outcome. We performed extensive sensitivity analyses to confirm the robustness, heterogeneity, and potential multi-biological effects of the study results. Also, to control for false positive results during multiple hypothesis testing, we adopted a false discovery rate (FDR) to control for statistical bias due to multiple comparisons.

Results: After FDR correction, GAD had no statistically significant effect on immunophenotypes. Several phenotypes with unadjusted low P-values are worth mentioning, including decreased PB/PC levels on B cells ($\beta = -0.289$, 95% CI=0.044~0.194, $P = 0.002$), reduced PB/PC AC in GAD patients ($\beta = -0.270$, 95% CI=0.77~0.92, $P = 0.000$), and diminished PB/PC on lymphocytes ($\beta = -0.315$, 95% CI=0.77~0.93, $P = 0.001$). GAD also exerted a causal effect on CD27 on IgD-CD38br ($\beta = -0.155$, 95% CI=0.78~0.94, $P = 0.002$), CD20-%B cell ($\beta = -0.105$, 95% CI=0.77~0.94, $P = 0.002$), IgD-CD38br%lymphocyte ($\beta = -0.305$, 95% CI=0.79~0.95, $P = 0.002$), FSC-A level on granulocytes ($\beta = 0.200$, 95% CI=0.75~0.91, $P = 8.35 \times 10^{-5}$), and CD4RA on TD CD4+ ($\beta = -0.150$, 95% CI=0.82~1.02, $P = 0.099$). Furthermore, Two lymphocyte subsets were identified to be significantly associated with GAD risk: CD24+ CD27+ B cell (OR=1.066, 95% CI=1.04~1.10, $P = 1.237 \times 10^{-5}$), CD28+CD4+T cell (OR=0.927, 95% CI=0.89~0.96, $P = 8.085 \times 10^{-5}$).

Conclusion: The study has shown the close association between immune cells and GAD through genetic methods, thereby offering direction for future clinical research.

KEYWORDS

generalized anxiety disorder, immunity, causal inference, MR analysis, sensitivity

Introduction

Generalized Anxiety Disorder (GAD) is a pervasive mental health issue characterized by persistent, uncontrollable worry about events or activities (1, 2). Typically emerging in childhood or adolescence, GAD tends to persist if left untreated (3). It is often linked with other mental illnesses, such as depression, resulting in social, occupational, and educational challenges, increased healthcare utilization, and a heightened risk of substance abuse (4, 5). Consequently, it significantly impacts an individual's overall health and daily functioning.

Current research has revealed the intricate relationship between anxiety disorders and the immune system. Laboratory findings have shown that abnormal secretion of inflammatory markers such as TNF- α and interleukin-6 (IL-6) can lead to elevated anxiety levels (6). Additionally, the activation and functional changes of immune cells, such as monocytes and T cells, can impact the neurotransmitter system, thereby influencing emotions and anxiety states (7). These studies suggest significant potential in exploring immune regulation for controlling anxiety disorders (8). However, for individuals with GAD, it remains a worthwhile area of research to investigate whether the persistent presence of anxiety mood and state is influenced by deeper genetic variations. Some scholars propose the involvement of acquired genetic and epigenetic risks in explaining the differential susceptibility to anxiety disorders, considering their highly complex and polygenic nature (9). However, only a few risk loci for anxiety disorders have been identified thus far. Furthermore, studies have found evidence of familial heritability in GAD and have focused on candidate genes such as 5-HTT, 5-HT1A, MAOA, and BDNF to explore the clinical

genetics of GAD (10). Research also aims to address the high non-response or partial response of GAD patients to existing pharmacological treatments by seeking safer and more effective approaches through pharmacogenetics (PGx) (11). Genome-wide association studies (GWAS) play a crucial role in analyzing genetic variations across the entire genome in large cohorts, identifying expected genetic loci and pathways, and enhancing our understanding of the complex genetic factors underlying diseases (12). This will also contribute to the in-depth exploration of the relationship between susceptibility, immune inflammation, and genetic background in GAD patients in our study.

Mendelian randomization (MR) is a statistical mean primarily used to infer epidemiological causality based on Mendelian genetic principles (13). In the Mendelian randomization method, it is crucial to ensure the logical order of causality (14). Previous observational research have revealed certain links between immune cell traits and anxiety disorders, supporting the hypothesis of their association (15, 16). The study conducted a thorough two-sample MR analysis to explore the causal relationship between immune cell traits and GAD.

Materials and methods

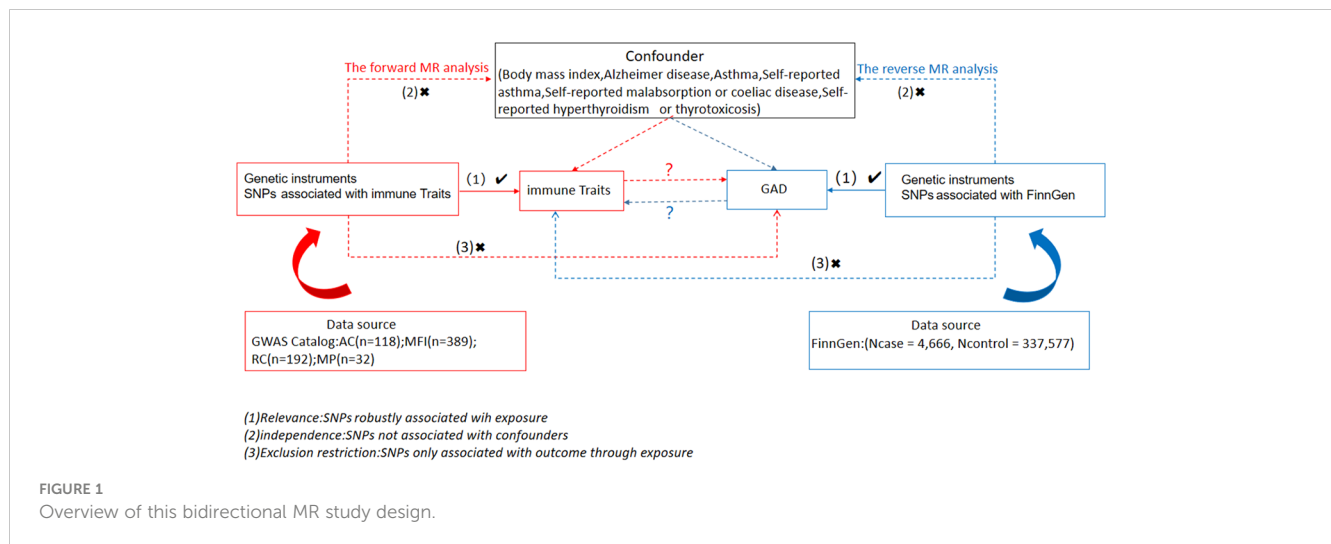
Study design

We examined causal associations between 731 immune cell traits and GAD using two-sample MR analyses. MR utilizes genetic variation as a surrogate for risk varies and requires validated instrumental variables (IVs) to satisfy three key assumptions in causal inference (1): Exposure is directly linked to genetic variation (2); there is no genetic link between exposure and outcome that is a potential confounder and (3) there is no genetic influence on outcome through channels unrelated to exposure (Figure 1). Our study was authorized by the appropriate institutional review board and informed permission was obtained from participants.

Sources of immunity-spanning GWAS data

Summary statistics for all immunologic characteristics in the GWAS catalog (from accession numbers GCST0001391 to

Abbreviations: GAD, generalized anxiety disorder; GWAS, genome-wide association studies; FDR, false discovery rate; MP, morphological parameters; AC, absolute cells; RC, relative cells; MFI, median fluorescence intensity; PB/PC AC, Peripheral Blood/Plasma Cell Antibody Count; PB/PC 5B Cell, Peripheral Blood/Plasma Cell Percentage of B Cell; CD28+CD4+T cell, CD28 positive CD4 positive T cell; CD24+ CD27 + B cell, CD24 positive CD27 positive B cell; FSC-A on granulocyte, Forward Scatter-Area on Granulocyte; IgD-CD38br% Lymphocyte, Immunoglobulin D negative CD38 bright percentage of lymphocyte; HLA DR on HLA DR+ T cell, Human Leukocyte Antigen DR on Human leukocyte Antigen DR Positive T Cell.



GCST0002121) are readily available (17). GWAS involves 3,757 nonoverlapping European individuals. A high-density array based on a reference panel of Sardinian sequences (18) was estimated for approximately 22 million SNPs and tested for correlation after controlling for covariates such as age, age², and sex. A number of 731 immunophenotypes were examined, including relative cell counts (RC) (192), morphologic parameters (MP) (32), absolute cell counts (AC) (118), and median fluorescence intensities (MFI) representing surface antigen levels (389). Of these, MP features included CDC and TBNK panels, whereas MFI, RC, and AC features included B cells, CDC, T cell maturation stage, myeloid cells, monocytes, and TBNK (T cells, B cells, natural killer proteins).

Data sources from the genome-wide association study for GAD

The summary of GWAS statistics for GAD was obtained from FinnGen (<https://www.finnngen.fi/en>). This summary was derived from a study involving 55,293 European individuals (Females=36,531, Males=18,494) with a median age (years) at first onset of GAD of 37.54 (Females=36.46, Males=40.62). The study encompassed a total of 342,243 samples (Ncase=4,666, Ncontrol=337,577), and the GWAS analysis incorporated over 500,000 phenotypic data points associated with GAD, identifying more than 16 million independent single nucleotide polymorphisms (SNPs).

Selection of instrumental variables

A selection of instrumental variables (IVs) (version v1.90) (chain disequilibrium [LD] r^2 threshold < 0.1 at a distance of 500 kb) (19) was used to modify these SNPs. 1000 Genomes Project was used as a reference panel for the calculation of the LD r^2 . 5×10^{-8} is the new GAD significance threshold. To assess IV strength and mitigate weak instrumental biases. The F statistic was calculated. The length of the independent variable (IV) for immunophenotypes

ranged from 3 to 1,643 and explained on average 0.137% (0.009–0.995%) of the variance in the relevant immune characteristics.

Statistical analysis

The R version 4.3.1 program (<http://www.Rproject.org>) was used in all studies. To specifically assess the causal relationship between the 731 immunophenotypes and GAD, we performed median-based weighted analyses (20), pattern-based weighted analyses (21), and inverse variance weighted analyses (IVW) (22) using the “Mendelian Randomization” software (version 0.4.3) (23). Instrumental heterogeneity between variables was assessed using Cochran’s Q statistic and its p-value (IV) and combined with the MR-Egger method for horizontal multidimensionality, which was recognized if the intercept term was large (24). Meanwhile, in the MR-PRESSO package, we used the technique of robust MR multidirectional entropy residuals and outliers (MR-PRESSO) (25) to find and remove horizontal multidirectional entropy outliers that may seriously affect the estimation results. After removing these SNPs, we ran the IVW analysis again. In addition, we looked for SNPs with suggestive associations ($P < 10^{-5}$) with these risk factors on the Phenoscanner V2 Web site (<http://www.phenoscanner.medschl.cam.ac.uk/>). Lastly, we used funnel plots and scatter plots. Scatter plots showed that outliers had minimal effect on the data, whereas funnel plots showed a high degree of association and a lack of heterogeneity.

Results

Examination of the causal relation of GAD onset on immunophenotypes

In our exploration of GAD’s causal effects on immunophenotypes, we utilized the IVW method as the principal analysis in a two-sample MR analysis. Despite conducting multiple test adjustments via the FDR method, we did not identify any immune traits at a significance level of

0.05. Nonetheless, we detected nine suggestive immunophenotypes at a significance level of 0.20: two in the B cell panel, two in the lymphocyte panel, two in the CD38br panel, one in the PB/PC AC panel, one in the monocyte panel, and one in the T cell panel. Our findings suggest that GAD pathogenesis may decrease PB/PC levels in B cells ($\beta=-0.289$, 95% CI=0.044~0.194, $P_{FDR}=0.061$, $P=0.002$, Figure 2, Supplementary Tables 1, 2), as well as decreased PB/PC AC levels ($\beta=-0.270$, 95% CI=0.77~0.92, $P_{FDR}=0.111$, $P=0.000$, Figure 2, Supplementary Tables 1, 2). PB/PC of lymphocytes was also decreased in GAD patients ($\beta=-0.315$, 95% CI=0.77~0.93, $P_{FDR}=0.133$, $P=0.001$, Figure 2, and Supplementary Tables 1, 2). the causal effect of GAD on CD20- %B cells on transitional cells was estimated to be 0.85 ($\beta=-0.105$, 95% CI=0.77~0.94, $P_{FDR}=0.191$, $P=0.002$, Figure 2, and Supplementary Tables 1, 2). A similar association was found on CD27 of IgD-CD38br ($\beta=-0.155$, 95% CI=0.78~0.94, $P_{FDR}=0.191$, $P=0.002$, Supplementary Table 2), and a positive association was also observed on IgD-CD38br% lymphocytes ($\beta=-0.305$, 95% CI=0.79~0.95, $P_{FDR}=0.192$, $P=0.002$, Supplementary Table 2). Among the immune cell types positively associated with GAD, our findings suggest that the occurrence of GAD may increase the levels of FSC-A in granulocytes ($\beta=0.200$, 95% CI=0.75~0.91, $P_{FDR}=0.191$, $P=8.35\times10^{-5}$, Figure 2 and Supplementary Tables 1, 2) and CD4RA in TD CD4+ ($\beta=-0.150$, 95% CI=0.82~1.02, $P_{FDR}=0.192$, $P=0.099$, Supplementary Table 2). The

results of the other three methods and sensitivity analyses confirmed the robustness of the observed causal associations (Supplementary Tables 1, 3). Specifically, the MR-Egger intercept and MR-PRESSO global tests ruled out the possibility of horizontal pleiotropy (Supplementary Table 1). In addition, scatterplots and funnel plots showed the stability of the results (Supplementary Figure 2).

Examination of the causal relation of immunophenotypes on GAD

By FDR correction ($P_{FDR}<0.05$), we found two immunophenotypes to be protective against generalized anxiety disorder: CD24+CD27+B cells and CD28+CD4+T cells. In particular, the ratio of CD24+CD27+B cells to GAD risk (OR) was assessed with the IVW method and was 0.94 (95% CI=1.04~1.10, $P_{FDR}=0.009$, $P=1.237\times10^{-5}$, Supplementary Table 4). Three other methods yielded similar results: weighted mode (OR=1.077, 95% CI=1.02~1.14, $P=0.010$); weighted median (OR=1.075, 95% CI=1.02~1.13, $P=0.004$); and MR-Egger (OR=1.070, 95% CI=1.01~1.15, $P=0.031$). By applying the IVW technique, the OR of CD28+CD4+ T cells on GAD risk was calculated to be 0.064 (95% CI=0.89~0.96, $P_{FDR}=0.030$, $P=8.085\times10^{-5}$, Supplementary Table 4). The results were similar for weighted mode (OR=0.944, 95% CI=0.89~1.00,

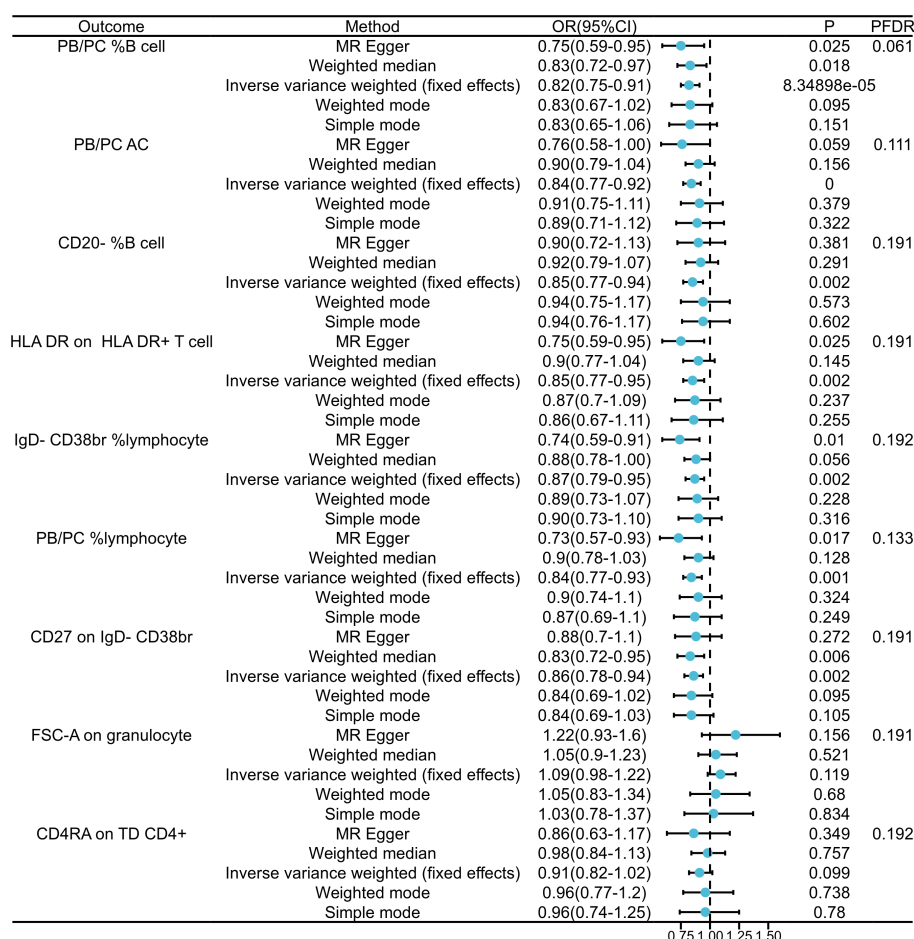


FIGURE 2

Forest plots showed the causal relations between GAD and immune cell traits.

$P=0.057$), weighted median ($OR=0.927$, 95% $CI=0.87\sim0.99$, $P=0.015$), and MR-Egger ($OR=0.965$, 95% $CI=0.91\sim1.03$, $P=0.279$). In addition, for all four associations, the MR-Egger intercept and MR-PRESSO global tests excluded the notion of horizontally collapsed products. Sensitivity analyses provided comprehensive details that validated the strength of the causal relationships found (Figure 3, Supplementary Table 5). The stability of the data was further demonstrated using scatterplots and funnel plots (Supplementary Figure 1).

Discussion

We investigated the causal relationship between 731 immune cell characteristics and GAD using a large amount of publicly available genetic data. To date, this is the only MR investigation that has examined the causal connection between multiple immune phenotypes and GAD. The study included four immune trait categories (MFI, RC, AC, and MP). Among them, GAD showed a causal effect on nine immunophenotypes ($P_{FDR} < 0.20$) and two immunophenotypes showed a strong causal effect on GAD ($P_{FDR} < 0.05$).

Our research indicates that an increased PB/PC%B cell percentage is associated with a reduced risk of GAD. PB/PC refers to two types of cells: plasmablasts (precursors of plasma cells) and plasma cells, and PB/PC%B cells represent the percentage of B cells in peripheral blood and spleen. Under normal circumstances, the ratio of PB/PC to lymphocytes in peripheral

blood remains at a relatively high level, which is crucial for antibody production and immune cell clearance (26). Fluctuations in PB/PC %B cells often impact immune regulation and the intensity of immune responses (27), for instance, clinically predicting an increased risk of developing multiple sclerosis due to excessive immune reactions when the baseline PB/PC percentage falls below 0.1% (28), another study on anxiety comorbidity in adolescents showed a significant increase in lymphocyte ratios, suggesting a potential link between anxiety disorders and inflammatory responses (29).

The link between HLA-DR (human leukocyte antigen-DR) and neuropsychiatric disorders has attracted considerable attention. HLA-DR gene variants have been associated with susceptibility to specific neuropsychiatric disorders, such as schizophrenia and autism spectrum disorders (30). According to earlier research, HLA-DR may be a significant factor in the immunological abnormalities connected to GAD (31). The abnormally elevated expression of peripheral blood CD3~+ HLA-DR cells in patients with anxiety disorders and its correlation with immune disorders in patients suggests that it may have an impact on the development of GAD through its involvement in the modulation of immune responses.

Distinguishing specific cell subgroups is particularly important for immune profiling, such as the use of CD27-expressing IgD-CD38br cells to differentiate various lymphocyte subgroups. Studies have indicated the involvement of HLA-G5 as an immune regulatory molecule in the inflammatory cell response of anxiety disorders (32). The close association between the exposure to HLA-

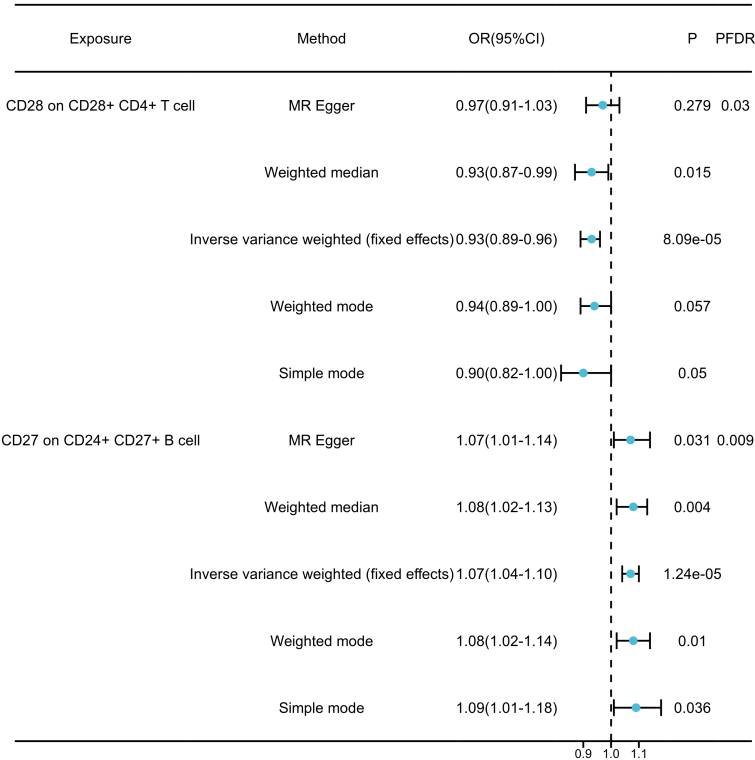


FIGURE 3
Forest plots showed the causal associations between immune cell traits and GAD by using different methods.

G5 and the decreased expression of IgD-CD38^{br} cell subgroups highlights the potential role of immune cell subgroups in mediating anxiety disorders.

Reduced CD28 on CD28⁺ CD4⁺ T cells is linked to the usage of GAD as an exposure factor. The majority of research studies have demonstrated that patients with GAD have higher than normal blood levels of inflammatory cytokines, that the degree of clinical anxiety symptoms is significantly and positively correlated with the patient's age (33), peripheral blood levels of IL-2, IL-4, and TF- α , and that CD28⁺ CD4⁺ T cells inhibit persistent inflammatory processes, thereby reducing the negative symptoms associated with GAD (34). Conversely, the percentage of CD24⁺ CD27⁺ B cells was significantly higher in GAD patients compared to healthy controls. This could be attributed to B cell activating factor (35), which regulates B cell survival and differentiation and plays a regulatory role in both natural and adaptive immunity. It is also involved in the development of a number of chronic inflammatory disorders, so it is likely that CD24⁺ CD27⁺ B cells also influence the inflammatory process of GAD via this pathway.

This study employed two-sample Mendelian randomization analysis using data from a large genomic research cohort of approximately 342,243 individuals, assuring great statistical efficiency. The outcomes of the study were based on genetic instrumental variables, and causal inferences were conducted by various robust Mendelian randomization analysis techniques, which were unaffected by horizontal pleiotropy and other variables. Moreover, to control for false positive results during multiple hypothesis testing, we adopted a false discovery rate (FDR) to control for statistical bias due to multiple comparisons.

However, This study does have several drawbacks, though. First, a thorough evaluation of horizontal pleiotropy is still difficult to achieve, even after several sensitivity studies. Second, stratified population analyses were not feasible due to the lack of individual-level data. Third, the study's reliance on European databases limits the generalizability of the findings to other ethnic groups. Finally, The study's flexible outcome assessment criteria may have led to more false positives, but they also made it easier to evaluate the full extent of the strong relationship between immunological traits and GAD. Overall, a randomized controlled trial of GAD would be the next step in this study in order to reduce the potential impact of confounding factors and thus obtain a higher level of evidence for causality.

Conclusion

In summary, our comprehensive bidirectional MR analysis has revealed causal links between various immunophenotypes and GAD, shedding light on the intricate web of relationships between GAD and the immune system. Moreover, Reverse causality, other variables, and other unavoidable confounding factors have all been successfully reduced in impact by our study, offering a fresh perspective for researchers to delve into the

biological underpinnings of GAD and potentially pave the way for early intervention and treatment strategies. These findings expand the realm of psychoimmunology and offer valuable insights for GAD prevention.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Author contributions

MZ: Data curation, Writing – original draft. ZM: Formal Analysis, Writing – original draft. HZ: Methodology, Writing – original draft. NQ: Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1338083/full#supplementary-material>

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Investigating the shared genetic architecture between hypothyroidism and rheumatoid arthritis

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Background: There is still controversy regarding the relationship between hypothyroidism and rheumatoid arthritis (RA), and there has been a dearth of studies on this association. The purpose of our study was to explore the shared genetic architecture between hypothyroidism and RA.

Methods: Using public genome-wide association studies summary statistics of hypothyroidism and RA, we explored shared genetics between hypothyroidism and RA using linkage disequilibrium score regression, p-HESS, Pleiotropic analysis under a composite null hypothesis (PLACO), colocalization analysis, Multi-Trait Analysis of GWAS (MTAG), and transcriptome-wide association study (TWAS), and investigated causal associations using Mendelian randomization (MR).

Results: We found a positive genetic association between hypothyroidism and RA, particularly in local genomic regions. Mendelian randomization analysis suggested a potential causal association of hypothyroidism with RA. Incorporating gene expression data, we observed that the genetic associations between hypothyroidism and RA were enriched in various tissues, including the spleen, lung, small intestine, adipose visceral, and blood. A comprehensive approach integrating PLACO, Bayesian colocalization analysis, MTAG, and TWAS, we successfully identified *TYK2*, *IL2RA*, and *IRF5* as shared risk genes for both hypothyroidism and RA.

Conclusions: Our investigation unveiled a shared genetic architecture between these two diseases, providing novel insights into the underlying biological mechanisms and establishing a foundation for more effective interventions.

KEYWORDS

hypothyroidism, rheumatoid arthritis, genome-wide association studies, shared genetic architecture, shared risk genes

1 Introduction

The condition of hypothyroidism is characterized by low thyroid hormone levels and high thyroid-stimulating hormone (TSH) levels. Approximately 4% to 10% of the population is affected by hypothyroidism, and subclinical hypothyroidism has been reported in up to 10% of individuals (1), with a higher prevalence among women and the elderly (2). Hypothyroidism is associated with cardiac dysfunction, atherosclerosis, hypertension, and coagulation disorders, potentially reducing patients' lifespans (3).

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune and autoimmune disease that affects 0.5% to 1% of the world's population (4). It leads to inflammation and structure damage of joints, reduced mobility, and increased disability (5). Even though their precise etiology is not fully understood, these diseases are believed to be associated with an interplay between genetic predisposition and environmental influences (6–9). Despite notable advancements in RA treatment, patients experience less favorable outcomes concerning quality of life, morbidity, and mortality than the general population (10).

Previous studies have demonstrated an elevated rate of hypothyroidism in RA patients compared to controls (11). Recently, Gao Y et al. provided compelling evidence supporting a causal association between hypothyroidism and increased risk of RA (12). In a cohort study, there was no significant difference in the prevalence of hypothyroidism between RA patients and healthy adults (13). However, RA and hypothyroidism have not always been associated consistently in the literature, and there has been a dearth of studies on this association.

There is an elusive relationship between hypothyroidism and RA. Our study aims to clarify the shared genetic architecture and molecular pathways of hypothyroidism and RA, providing new information on their underlying biological mechanisms and paving the way for more effective interventions. Figure 1 illustrates the overall study design.

2 Methods

2.1 Data source

We used genome-wide association studies (GWAS) summary statistics for hypothyroidism involving 462,933 individuals (22,687 cases and 440,246 controls) of European ancestry from the GWAS Catalog (<https://www.ebi.ac.uk/gwas/>). For RA, we applied summary statistics for the discovery dataset from GWAS with 253,417 individuals (12,555 cases and 240,862 controls) from the

Abbreviations: COLOC, Bayesian colocalization analysis; FUMA, Functional Mapping and Annotation of GWAS; GO, Gene Ontology; GWAS, Genome-wide association study; IVW, Inverse-variance-weighted; KEGG, Kyoto Encyclopedia of Genes and Genomes; LD, Linkage disequilibrium; LDSC, Linkage disequilibrium score regression; MAGMA, Generalized gene-set analysis of GWAS data; MR, Mendelian Randomization; MTAG, Multi-trait analysis of GWAS.

FinnGen Consortium (<https://r9.finngen.fi/>) (14). To corroborate the findings from the initial discovery dataset, a replication GWAS of RA was performed. This replication involved 58,284 individuals, comprising 14,361 RA cases and 43,923 controls (15). **Supplementary Table S1** presents a comprehensive description of the characteristics of each dataset utilized in our study. To mitigate the potential bias of ethnic diversity, we focused our investigation on individuals of European ancestry. Ethical clearance was not required, as the study relied on publicly available data.

2.2 Linkage disequilibrium score regression

Heritability estimates for hypothyroidism and RA were derived using LDSC in Python 2.7 (16). Additionally, we calculated the genetic correlation (r_g) between the two diseases, quantifying the shared genetic variance relative to the square root of their respective Single Nucleotide Polymorphism (SNP) heritability estimates. The analysis utilized the 1000 Genomes European Reference dataset to convert GWAS summary statistics into precalculated linkage disequilibrium (LD) scores. Sensitivity analysis was conducted with a single-trait heritability intercept constraint.

2.3 p-HESS

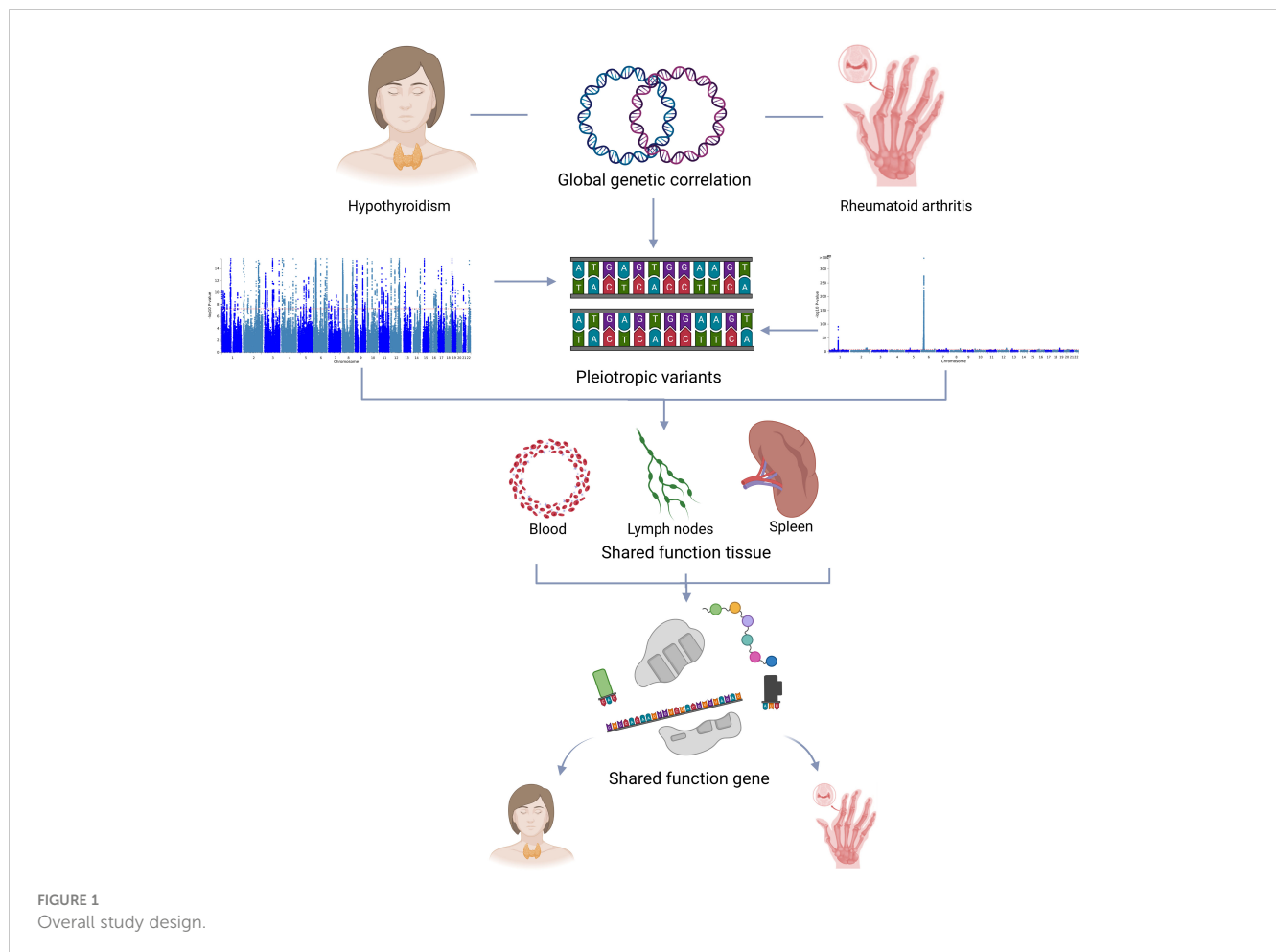
We used the p-HESS approach to investigate the local genetic correlations between hypothyroidism and RA (17), which estimates the local heritability of SNPs and genetic covariance based on genomic references constructed from genomes. We could estimate local genetic correlations by calculating local single-trait SNP heritability and local cross-trait genetic correlation. The algorithm was used to calculate the genome into 1,703 regions and quantifies trait correlations attributable to genetic variation within specific regions.

2.4 Pleiotropic analysis under composite null hypothesis

Identification of potentially pleiotropic single nucleotide variants (SNVs) using genotype-phenotype association statistics at the aggregation level is a novel approach PLACO employs to investigate pleiotropic loci associated with complex traits (18). Significant pleiotropic variants were defined as single-nucleotide variants with P -values less than $P < 5.0 \times 10^{-8}$ for PLACO. Subsequently, to identify common causal variants for each pleiotropic locus, the Functional Mapping and Annotation of Genetic Associations (FUMA) tool was used to identify potential pleiotropic loci, followed by Bayesian colocalization analysis (19, 20).

2.5 Cross-trait meta-analysis

We employed the Multi-Trait Analysis of GWAS (MTAG) (21) in Python 2.7 to identify risk SNPs associated with hypothyroidism



and RA. MTAG allows for joint analysis of GWAS summary statistics for different traits, considering potential sample overlap between GWAS. MTAG presumes that effect sizes across traits have a shared variance-covariance matrix (21). Finally, SNPs associated with joint phenotypes were identified if they exhibited independent associations ($LD\ r^2 < 0.001$) with both diseases ($P\text{-value} < 5.0 \times 10^{-8}$) in MTAG.

2.6 Functional analysis for pleiotropic genes

PLACO was used to identify pleiotropic genes and subsequently subjected to differential expression and gene set enrichment analyses using FUMA (19). Genotype-tissue expression (GTEx) gene expression data from 53 tissues were used in this investigation. Expression normalization was performed as a preliminary step, followed by a two-sided Student's *t*-test to compare each gene in a given tissue with all other genes. It identified differentially expressed genes (DEGs) specific to each tissue type based on genes with a Bonferroni corrected *P*-value of less than 0.05 and an absolute log-fold change of 0.58. These results indicate significant differences in gene expression levels compared to other tissues (22–25).

2.7 Pathway-based functional enrichment analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System version 3.0 online bioinformatics database was used to conduct KEGG pathway enrichment analysis and Gene Ontology (GO) analysis to gain insights into the biological mechanisms associated with hypothyroidism and RA (26).

2.8 Mendelian randomization analysis

We employed MR analysis, a widely used instrumental variable approach for causal inference, to establish causal relationships between hypothyroidism and RA. Exposure-related SNPs served as instruments (27, 28), and data from the GWAS summary were used to identify variants associated with hypothyroidism and RA at $P\text{-value} < 5.0 \times 10^{-8}$. The inverse-variance weighted (IVW) approach was used as a primary method, with LD and physical distance thresholds of 0.001 and 10 MB, respectively, Utilizing the European ancestry reference panel from the 1000 Genomes Project.

2.9 Transcriptome-wide association studies

FUSION software was used to analyze tissue-related TWAS data from the GWAS summary data (29). An analysis of TWAS used pre-computed gene expression weights in conjunction with GWAS summary statistics to determine the associations between genes and diseases. Cortical RNA sequence reference panels from the GTEx Consortium were integrated with GWAS summary statistics of GWAS for TWAS (30).

3 Results

3.1 Global genetic correlation

Based on the baseline LD model and stratification LDSC, we estimated the liability-scale SNP heritability of hypothyroidism and RA (31, 32). Bivariate LDSC methodology was used to estimate the genetic correlations between the two diseases. The analysis revealed a significant positive genetic correlation ($r_g = 0.31$, $P = 6.70 \times 10^{-7}$) between hypothyroidism and RA (discovery) (Supplementary Table S2). The replication aimed to validate the initial observations. Notably, a significant positive genetic correlation was observed between hypothyroidism and RA (replication) ($r_g = 0.35$, $P = 1.13 \times 10^{-10}$), supporting the initial findings. Sensitivity analyses were conducted based on LDSC, with the heritability intercept of a single trait constrained. These results revealed significant genetic associations between hypothyroidism and RA.

3.2 Local genetic correlations

Considering the significant global genetic correlation between hypothyroidism and RA, we looked for specific genomic regions with local genetic correlations. Following multiple tests of correction ($P < 0.05/1703$), strong local correlations were found in 6 different regions, with 6p21.32-21.33 (chr6: 31571218-32682664) showing the most significant correlation between hypothyroidism and RA (discovery) ($P = 3.63 \times 10^{-52}$) (Figure 2A; Supplementary Table S3). Additionally, to further confirm the results of the discovery dataset, twenty-two distinct regions exhibited strong local correlations between hypothyroidism and RA (replication) (Figure 2B; Supplementary Table S4).

3.3 Shared loci between hypothyroidism and RA

PLACO identified 805 single nucleotide variants with potential pleiotropic effects (Supplementary Table S5). MAGMA analysis yielded 483 significant pleiotropic genes by the FUMA platform (Supplementary Table S6). There were 55 independent genomic risk loci identified by FUMA (Supplementary Table S7). Based on colocalization analysis, 9 out of 55 potential pleiotropic loci (16%) had PP.H4 higher than 0.75, candidate-shared causal variants were identified at three top SNVs of corresponding loci (Table 1 and Figure 3B). A similar analysis was conducted on the RA (replication) data to validate these findings. This analysis,

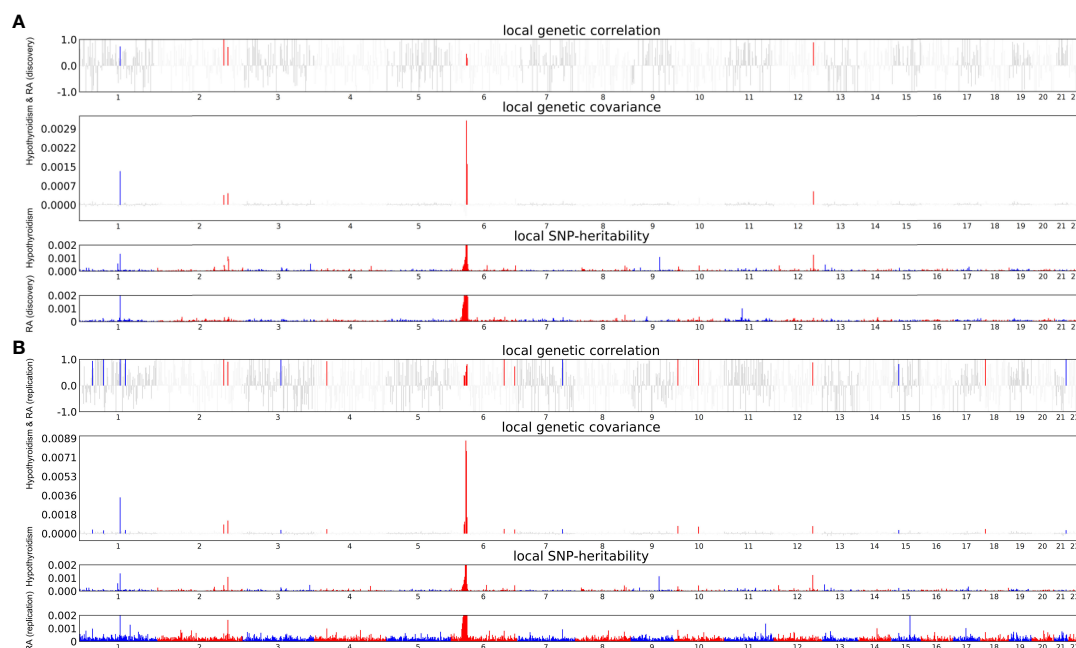


FIGURE 2

Local Genetic Correlation Between Hypothyroidism and RA. (A) Local Genetic Correlation Between Hypothyroidism and RA (discovery). (B) Local Genetic Correlation Between Hypothyroidism and RA (replication). The Manhattan plot illustrates the estimates of local genetic correlation and local genetic covariance between hypothyroidism and RA. It also displays the local SNP heritability of hypothyroidism and RA. The colored bars in the "local genetic correlation" and "local genetic covariance" sections indicate significant regions that share SNP heritability, following multiple adjustments ($P < 5.0 \times 10^{-8}$ in both the local SNP heritability test and $P < 0.05/1703$ in the local genetic covariance test).

facilitated by FUMA, identified 66 independent genomic risk loci (Supplementary Table S8). Notably, loci rs34536443 (located in *TYK2*), rs706778 (located in *IL2RA*), and rs3807307 (located in *IRF5*) were included. Colocalization analysis further indicated that these specific loci (rs34536443 in *TYK2*, rs6454802 in *BACH2*, and rs229544 in *CIQTNF6*) were consistent across both discovery and replication datasets (Supplementary Table S9).

We performed MTAG to identify risk SNPs underlying hypothyroidism and RA(discovery) joint phenotypes. After excluding SNPs in linkage disequilibrium (LD $r^2 \geq 0.001$), a total of 39 shared independent SNPs with genome-wide significance were found (Figure 3B and Supplementary Table S10). Furthermore, the MTAG of hypothyroidism and RA (replication) dataset revealed 53 shared independent SNPs reaching genome-wide significance (Supplementary Table S11). Of these, 12 loci were concurrently identified in both the RA (discovery) and RA (replication) datasets, including SNPs rs11085727 (mapped on *TYK2*), rs3118470 (mapped on *IL2RA*), and rs3807307 (mapped on *IRF5*).

3.4 Mendelian randomization

A bidirectional MR study was conducted to investigate the potential causal relationship between hypothyroidism and RA (discovery). All SNPs were strong instruments in the Mendelian randomization analysis ($F > 10$). Various bi-directional MR methods were employed to ensure result stability. Our findings further validated the causal effect of hypothyroidism on RA (discovery) (Supplementary Table S12), with estimates remaining directionally consistent across the weighted median, weighted mode, and MR Egger approaches. However, no significant effect

of RA (discovery) on hypothyroidism was observed (Supplementary Table S8). Moreover, our findings provided validation of the causal effect of hypothyroidism on RA (replication) (Supplementary Table S13).

3.5 Enrichment analysis for identified pleiotropic genes

Enrichment analyses of pleiotropic genes identified by PLACO were conducted using FUMA. The pleiotropic genes showed differential expression in various tissues, including the spleen, lung, small intestine, adipose visceral, and blood (Figure 4A). GO enrichment analysis revealed significant enrichment of pleiotropic genes in the type I interferon signaling pathway ($P = 1.58 \times 10^{-12}$), T cell activation ($P = 9.25 \times 10^{-11}$), and ubiquitin-protein ligase ($P = 2.25 \times 10^{-3}$). Additionally, KEGG enrichment analysis demonstrated significant enrichment of genes involved in Th cell differentiation ($P = 1.50 \times 10^{-5}$) and the checkpoint pathway in cancer ($P = 7.10 \times 10^{-5}$). The top 10 GO and KEGG pathways in Figure 4B and Supplementary Table S14 provided additional evidence that the identified pleiotropic genes were appropriate.

3.6 Transcriptome-wide association study

Subsequently, we conducted Transcriptome-wide association studies (TWAS) to explore the relationship between genetically predicted mRNA levels and disease risk, utilizing whole blood collection from the GTEx consortium (29). We identified 621 genes whose expression in blood was associated with

TABLE 1 Nine colocalized loci identified through colocalization analysis on 55 pleiotropic loci.

Top SNV	Locus boundary ^a	Region	Nearest gene	Best causal	SNP.PP.H4
rs1559810	3:188072513-188135783	3q28	<i>LPP</i>	rs13319297	0.819
rs2393923;rs57252182;rs72854533;rs9468356;rs112886535;rs3117343;rs116466121;rs116802478;rs1003582;rs362520;rs29242;rs29232	6:27262294-29611431	6p22.1	<i>ZKSCAN3</i> , <i>ZSCAN31</i>	rs72854535	0.925
rs383711;rs2854027;rs1704995;rs9277946;rs34859217;rs9296092;rs78075721;rs2229637;rs13219530;rs10947433;rs12194518	6:33173842-33811790	6p21.32-p21.31	/	rs206763	1.0
rs6908626	6:90880393-91005743	6q15	<i>BACH2</i>	rs6454802	0.922
rs706778;rs41260244	10:6071453-6182251	10p15.1	<i>IL2RA</i>	rs3118470	0.999
rs62623446	12:55358844-55368291	12q13.2	<i>TESPA1</i>	rs62623446	0.999
rs34536443	19:10427721-10586018	19p13.2	<i>TYK2</i>	rs34536443	1.0
rs73068668	19:55739048-55763262	19q13.42	<i>PPP6R1</i>	rs73068668	0.982
rs229527	22:37573712-37609342	22q13.1	<i>CIQTNF6</i>	rs229544	0.874

^aLocus boundary of each pleiotropic genomic risk locus was denoted as “chromosome: start-end” defined by FUMA.

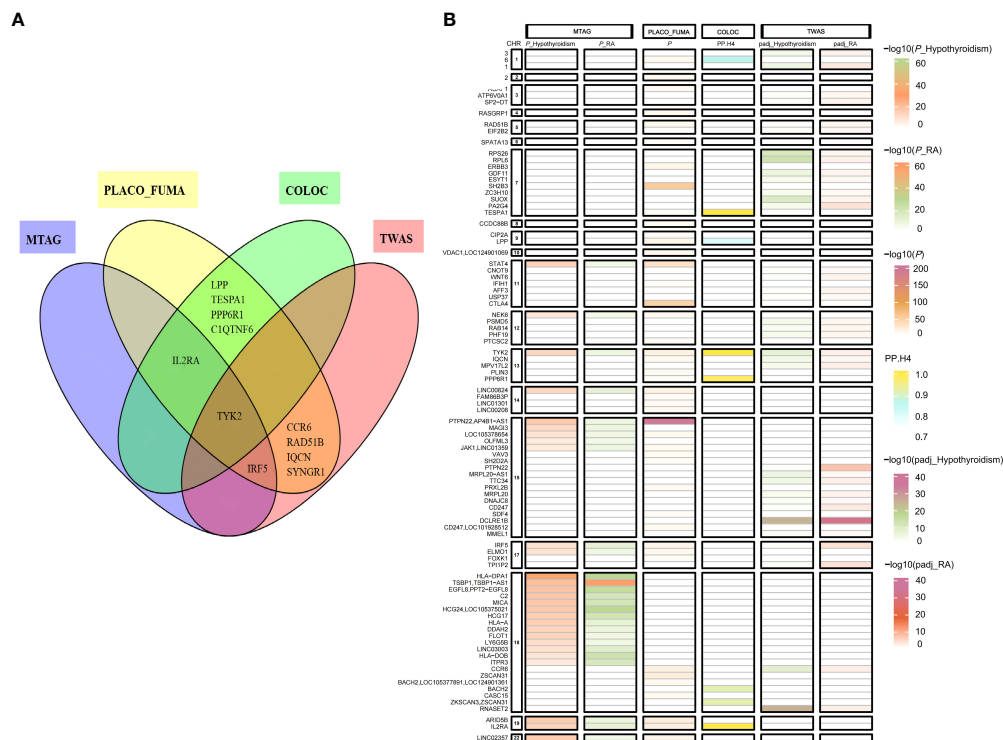


FIGURE 3

Venn diagrams and heat maps showing shared risk genes in hypothyroidism and RA (discovery) using four methods. **(A)** Venn Diagrams Showing Shared Risk Genes in Hypothyroidism and RA (discovery) Using Four Methods. **(B)** Heat Maps Showing Shared Risk Genes in Hypothyroidism and RA (discovery) Using Four Methods, with the y-axis presenting the gene, the x-axis presenting the method, and the change in color being based on the $-\log(P\text{-value})$ or PP.H4 affinities. PLACO_FUMA, Pleiotropic analysis under composite null hypothesis_Funcational Mapping and Annotation of Genetic Associations; MTAG, Multi-Trait Analysis of GWAS; COLOC, Bayesian colocolization analysis; TWAS, transcriptome-wide association study.

hypothyroidism (Supplementary Table S15), 139 genes whose expression in blood was associated with RA (discovery) (Supplementary Table S16) and 68 genes whose expression in blood was associated with RA (replication) (Supplementary Table S17). In total, 37 genes associated with hypothyroidism and RA (discovery) were identified through TWAS (Figure 3B and Supplementary Table S18). In addition, by using TWAS, 27 genes associated with hypothyroidism and RA (replication) were identified (Supplementary Table S19). Among these, 12 genes, including *TYK2* and *IRF5*, were implicated in RA (discovery), RA (replication), and hypothyroidism, according to TWAS.

3.7 Summary findings

We performed an overlap analysis of genes in PLACO_FUMA, MTAG, Bayesian colocolization analyses, and TWAS to identify the most representative genes associated with hypothyroidism and RA. This comprehensive approach identified *TYK2*, *IL2RA*, and *IRF5* in hypothyroidism and RA (discovery) utilizing four or three methods. Similarly, an independent replication dataset was analyzed, confirming the discovery dataset results. *TYK2* was consistently identified by all four methods used, while *IL2RA* and *IRF5* were identified in the hypothyroidism and RA (replication) studies using two or three of these methods. These findings suggest that *TYK2*,

IRF5, and *IL2RA* may be the most representative genes associated with hypothyroidism and RA (Figures 3A, B).

4 Discussion

This study represents the first genome-wide cross-trait analysis investigating the shared genetic basis that underlies hypothyroidism and RA. Our findings provide novel evidence supporting a genetic interrelation between these two diseases, as evidenced by the following key discoveries: Firstly, we observed an association between hypothyroidism and RA in specific genomic regions. Secondly, our Mendelian randomization analysis revealed a causal effect of hypothyroidism on RA. Finally, we focused on the genetic commonality between hypothyroidism and RA across various tissues and identified potentially functional genes associated with both diseases.

There have been varying results reported in different studies about the relationship between hypothyroidism and RA. For instance, RA is an independent predictor of thyroid dysfunction by Mahagna H et al. (11). Previous studies have indicated that RA coexisting with hypothyroidism may increase disease activity and joint tenderness (33). Three large-scale GWAS summary datasets were chosen to ensure robust heritability interpretation for hypothyroidism and RA. A significant global genetic correlation was found between hypothyroidism and RA in our study. In

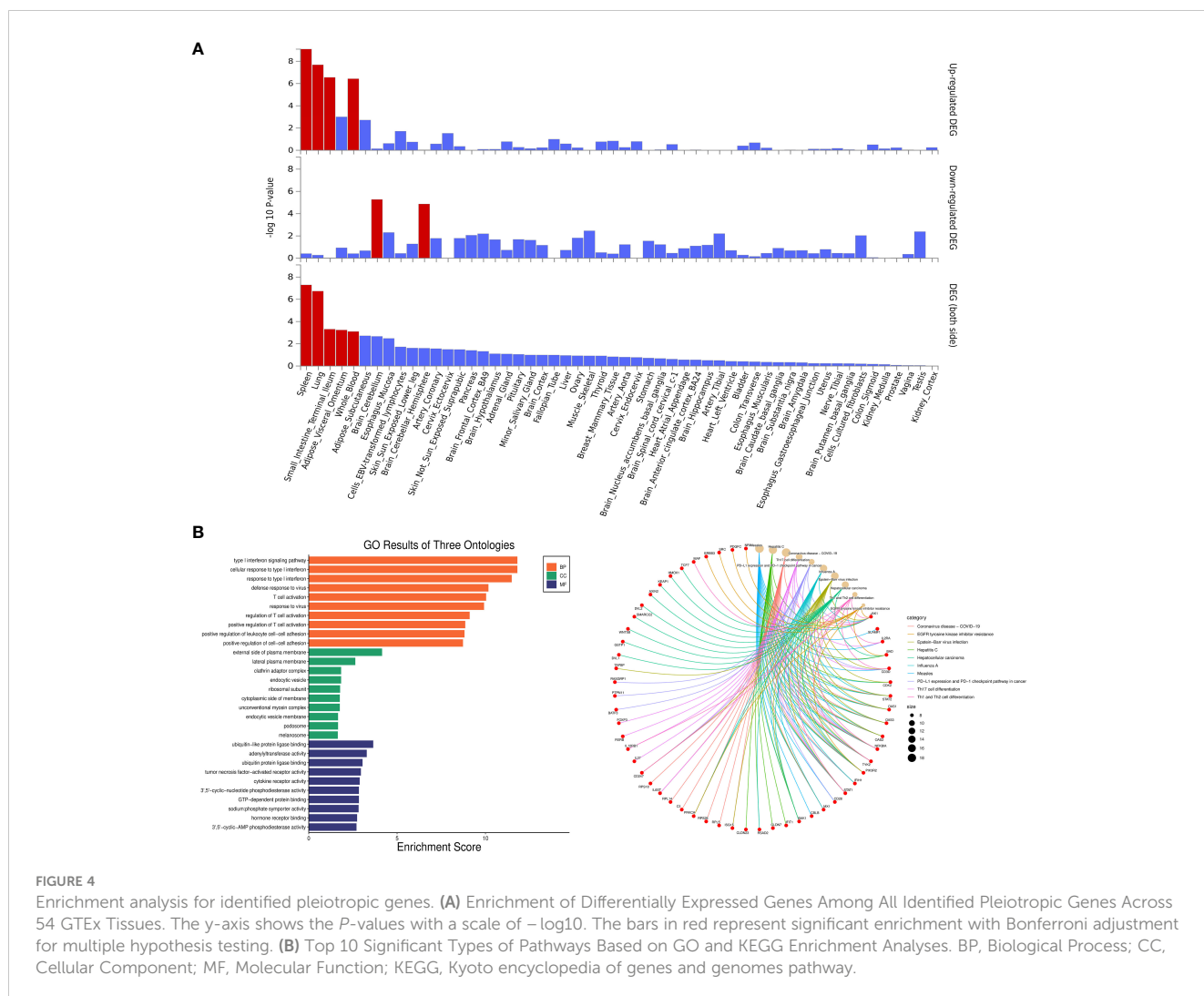


FIGURE 4

Enrichment analysis for identified pleiotropic genes. **(A)** Enrichment of Differentially Expressed Genes Among All Identified Pleiotropic Genes Across 54 GTEx Tissues. The y-axis shows the P -values with a scale of $-\log_{10}$. The bars in red represent significant enrichment with Bonferroni adjustment for multiple hypothesis testing. **(B)** Top 10 Significant Types of Pathways Based on GO and KEGG Enrichment Analyses. BP, Biological Process; CC, Cellular Component; MF, Molecular Function; KEGG, Kyoto encyclopedia of genes and genomes pathway.

our exploratory MR analyses, we found evidence of a causal effect of hypothyroidism on RA, aligning with recent study findings (12). The findings of our study suggest that hypothyroid patients can be monitored for RA risk, which will aid in early detection. The association between hypothyroidism and RA may be attributed to the natural course of autoimmune diseases and their propensity to overlap (34). Our enrichment analysis observed a significant enrichment of genes involved in T cell activation, Th cell differentiation, and the interferon signaling pathway. The peripheral Th1/Th2 cell ratio is linked to the severity of Hashimoto's disease, while the proportion of Th17 cells is associated with the intractability of Graves' disease (35). Koumine, an alkaloid, demonstrates promising therapeutic effects against RA. Yang J et al. provided detailed elucidation of koumine's mechanism of action, revealing its ability to effectively restore the balance of Th subsets and cytokine network systems by inhibiting T cell activation. This process leads to the modulation of Th subset polarization and downstream pro/anti-inflammatory cytokine imbalance, which proves beneficial in RA (36). Additionally, in thyroid tissue, the recruitment of T helper 1 (Th1) lymphocytes appears to be associated with heightened production of IFN- γ and tumor necrosis factor-alpha (TNF- α) (37). Studies have demonstrated that aberrant expression of IFN-Is and type I IFN-

inducible gene signatures in the serum or tissues of patients with autoimmune disorders is closely associated with the pathogenesis, clinical manifestations, and disease activity (38–41). The shared genetic determinants observed in our study reflect common biological pathways that play a crucial role in regulating hypothyroidism and RA.

Among the shared risk genes we identified, Yuan S et al. previously revealed a Mendelian randomized association of *TYK2* loss-of-function variants with hypothyroidism, inflammatory bowel disease, primary biliary cirrhosis, and type 1 diabetes (42). Additionally, in-silico tools were used by Akhtar M et al. to demonstrate the critical role of *TYK2* (rs34536443) in RA pathogenesis (43). The SNP in exon 21 changes proline to alanine at position 1104 of *TYK2*'s kinase domain (44, 45). *TYK2*, a non-receptor tyrosine kinase-linked Janus kinase (JAK), belongs to the Janus kinase/signal transduction and transcription factor 4 (JAK-STAT) pathway (46), mediating cytokine signaling (IL-6, IL-10, IL-12, and IL-23 receptors) and regulating group 1 and 2 cytokine pathways (47). Autoimmune diseases are associated with abnormal expression of IFN-I and other cytokines or members of JAK kinase (48, 49). *TYK2* plays an important role in many immune processes, including natural killer cell activity, maturation of B and Treg cells, and differentiation of Th1 and Th17 cells. Dysregulated *TYK2*

expression has been linked to autoimmune diseases (49). Regarding *IL2RA*, according to Knevel et al., altered genetic constitutions at *IL2RA* may lead to a less destructive course of RA (50). In the Chinese Han population, Yang Y et al. found an association between *IL2RA* and a decreased risk of RA (51). Furthermore, *IL2RA* has been identified as a susceptibility gene for autoimmune thyroid disease (52). *IL2RA* (CD25) is highly expressed in regulatory T cells of CD4CD25 (Tregs) as an important regulator of immune homeostasis and suppressing autoimmune responses (53). In our study, for *TYK2* and *IL2RA*, the most likely risk genes were identified through four methods (PLACO_FUMA, MTAG, Bayesian colocalization analysis, and TWAS) and three methods (PLACO_FUMA, MTAG, and Bayesian colocalization analysis), respectively. This consistency with previous studies adds to the reliability of our integrative analysis. Our study emphasized their significance in hypothyroidism and RA, suggesting their potential role as important drug targets for these diseases. Furthermore, it can be postulated that different SNPs in *TYK2* and *IL2RA* may play different roles in various types of autoimmune diseases.

IRF5 has been confirmed as a RA disease-associated loci (54, 55). However, its relationship with hypothyroidism remains undefined. *IRF5* is a transcription factor involved in inflammation and autoimmune response, mediating the induction of pro-inflammatory cytokines such as IL-6, IL-12, IL-23, and TNF- α (56, 57). As a consequence of *IRF5* overexpression, IL6 and IFN are produced at higher levels, playing a crucial role in the pathogenesis of RA (58). Our study identified *IRF5* as a potential risk gene for hypothyroidism and RA, but further validation with larger sample sizes and functional experiments is needed.

There are several limitations to our study, which we acknowledge. Firstly, due to the use of predominantly European ancestry data sources, our findings may not be generalizable to other ethnic groups. Secondly, due to limited data availability, we could not perform phenotype-specific analyses for hypothyroidism's primary and secondary forms, and did not separate the datasets of people with both diseases for analysis. Third, while we identified genes relevant to hypothyroidism and RA, future longitudinal studies and experimental investigations are necessary to understand the underlying biological mechanisms fully.

5 Conclusion

In conclusion, we provide evidence of genetic correlation, identify shared loci, and infer causal relationships between hypothyroidism and RA in our study. In the future, large prospective longitudinal clinical studies should be conducted to determine if hypothyroidism patients with specific genotypes are more likely to develop RA. These findings may have potential implications for future therapeutic strategies and risk prediction in patients with these diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

ZP: Conceptualization, Methodology, Visualization, Writing – original draft, Writing – review & editing. WH: Conceptualization, Writing – review & editing. MT: Data curation, Writing – review & editing. BC: Data curation, Visualization, Writing – review & editing. RY: Formal analysis, Writing – review & editing. QL: Formal analysis, Writing – review & editing. CL: Visualization, Writing – review & editing. PL: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1286491/full#supplementary-material>

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Complete Freund's adjuvant as a confounding factor in multiple sclerosis research

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Complete Freund's adjuvant (CFA) is used as a standard adjuvant for the induction of experimental autoimmune encephalomyelitis (EAE), the most commonly used animal model in multiple sclerosis studies. Still, CFA induces glial activation and neuroinflammation on its own and provokes pain. In addition, as CFA contains Mycobacteria, an immune response against bacterial antigens is induced in parallel to the response against central nervous system antigens. Thus, CFA can be considered as a confounding factor in multiple sclerosis-related studies performed on EAE. Here, we discuss the effects of CFA in EAE in detail and present EAE variants induced in experimental animals without the use of CFA. We put forward CFA-free EAE variants as valuable tools for studying multiple sclerosis pathogenesis and therapeutic approaches.

KEYWORDS

multiple sclerosis, experimental autoimmune encephalomyelitis, complete Freund's adjuvant, antigen, pain

1 Introduction

Experimental autoimmune encephalomyelitis (EAE) is an animal model of the central nervous system (CNS) autoimmune diseases. It is mostly used to study the pathogenesis of multiple sclerosis and to explore therapeutic approaches for the disease (1). EAE was invented by Rivers and colleagues in 1933 as a consequence of studying the neurological side effects of vaccination against rabies (2). For more details on the timeline of the origin and development of EAE, readers are referred to a comprehensive review by Alan G. Baxter (3). Complete Freund's adjuvant (CFA) was introduced as an essential component for EAE induction as early as 1947 (4, 5), just 5 years after its invention (6). CFA is used in most variants of EAE, including the most prominent ones, such as myelin oligodendrocyte glycoprotein (MOG)_{35–55}-induced chronic EAE in C57BL/6 mice, proteolipid protein (PLP)_{139–151}-induced relapsing-remitting EAE in Swiss Jim Lambert (SJL) mice, and myelin basic protein (MBP)-induced monophasic acute EAE in Lewis rats. Moreover,

T-cell lines produced for adoptive transfer in passive EAE are originating from animals immunized with CNS antigens emulsified in CFA (7).

CFA is a suspension of desiccated heat-inactivated mycobacterium (most commonly used is *M. tuberculosis*, followed by *M. butyricum*) in paraffin oil and mannide monooleate. It has been regularly used as an adjuvant of choice for induction of autoimmune disorders in experimental animals (8). CFA extends injected autoantigen lifetime, galvanizes the effective delivery of the antigens to the immune system, and stimulates the innate compartment of the immune system (8), which, in turn, results in the effective induction of autoimmune disorders in experimental animals, including EAE. Still, in studies where EAE is used as a multiple sclerosis model, CFA introduces numerous confounding effects, including immune reactivity against antigens unrelated to the CNS, activation of glia independent of the autoimmune process, and induction of pain (Figure 1). These and other confounding effects of CFA are discussed in detail in the following sections.

2 Confounding effects of CFA in EAE

2.1 Mycobacteria

One of the confounding effects of CFA arising from the presence of mycobacteria is the stimulation of lymphocytes specific for non-CNS antigens that contribute to the inflammatory response in EAE (8). As an example, it has been reported that lymphocytes obtained from the lymph nodes draining the site of immunization or from the CNS of EAE rats immunized with MBP + CFA exhibited reactivity against mycobacterial purified protein derivative (PPD) (9). Moreover, the addition of a PPD-specific T-cell line to MBP-specific T cells in passive EAE induction resulted in increased blood-brain barrier disruption, suggesting that PPD-specific T cells contribute to the CNS autoimmunity in EAE

(10). On the contrary, reactivity to mycobacterial antigens homologous to mammalian proteins, such as heat-shock proteins (HSPs), may interfere with EAE induction. Indeed, prophylactic application of mycobacterial HSP was shown to inhibit the development of EAE (11, 12).

Mycobacteria are intracellular bacteria, and the dominant immune response against them is performed by activated T helper 1 (Th1) cells. Therefore, there is a concern that CFA skews immune response toward Th1 and away from Th17 arm. Both Th populations are involved in multiple sclerosis pathogenesis, and each population has been associated with different clinical expressions of the disease and/or different mechanisms of effector actions within the CNS (13, 14). Still, CFA does not contain live mycobacteria, and it is more likely that it shapes immune response through mycobacterial pathogen-associated molecular patterns, such as muramyl dipeptide (MDP), trehalose dimycolate (TDM), and lipoarabinomannan (LAM), which are recognized by nucleotide oligomerization domain (NOD), C-type lectin-type receptors, or Toll-like receptor 2 (TLR2), respectively (8, 15–17). Whereas TLR2 stimulation enhances production of cytokines interleukin (IL)-12, interferon (IFN)- γ , and IL-18, promoting Th1 differentiation, TDM induces production of TNF, IL-6, and CXCL2, promoting development of both Th1 and Th17 cells (8, 17). Similar to TDM-induced stimulation, acute NOD2 activation by MDP results in induction of TNF, IL-12, IL-6, IL-8, and IL-10 (18). Th1-dominated cytokine pattern is also induced through TLR9 stimulation by unmethylated 5'-C-phosphate-G-3' (CpG) oligodeoxynucleotides and TLR4 stimulation by mycobacterial HSP (19–21). Thus, it seems that CFA is able to produce both Th1 and Th17 responses. As an example, both Th1 and Th17 T cells are present in the CNS in EAE induced in Dark Agouti (DA) rats with spinal cord homogenate (SCH) + CFA (22). Still, predominance of Th1 response cannot be excluded as a confounding factor of CFA.

The other important question is if mycobacteria are involved in the aetiology and pathogenesis of multiple sclerosis. There were

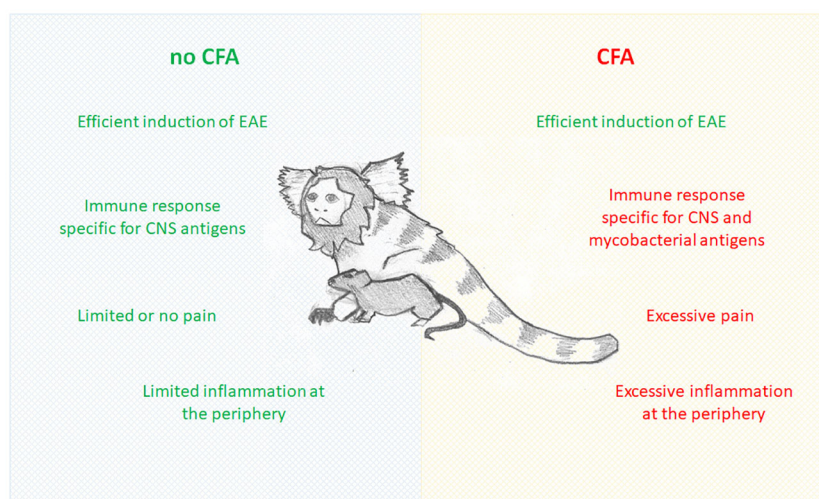


FIGURE 1
Comparison of EAE induced without and with CFA.

studies on the role of mycobacterial HSP70 in multiple sclerosis (23, 24), not only on the human zoonotic infection with *M. avium* subsp. *paratuberculosis* as a cause of multiple sclerosis (25, 26) but also on the protective role of vaccination with attenuated strain of *M. bovis* (BCG vaccine) on disease activity in patients with multiple sclerosis (27, 28). In short, it seems that *M. avium* subsp. *paratuberculosis* might be a causal agent in genetically predisposed individuals that appear to be restricted to certain human populations, such as those of Sardinia and Japan. As for BCG, epidemiological data support its protective role in multiple sclerosis, but the mechanisms behind its effects are still elusive. For more details on the possible role of mycobacteria in multiple sclerosis etiopathogenesis, readers are referred to a review by Cossu and colleagues (29). Thus, it seems that there is insufficient evidence on the role of mycobacteria in multiple sclerosis pathogenesis to support the use of CFA in EAE.

In addition, one should be careful when studying gut/lung microbiota role in the pathogenesis of the CNS autoimmunity using EAE induced with CFA, as there are studies showing that CFA is affecting microbiota in experimental animals (30, 31). Overall, as different findings demonstrated diverse relationship between mycobacteria and multiple sclerosis, the presence of mycobacterial components in CFA during EAE induction could potentially serve as a disease-modifying factor. This could pose challenges in conclusively identifying the precise pathological mechanisms driving the CNS autoimmunity and in evaluating potential mechanisms for multiple sclerosis therapy.

2.2 Pain, glial activation, and neuroinflammation

CFA is known to induce pain in experimental animals (8). Continual release of antigens from the oily deposit induces a delayed hypersensitivity reaction characterized by intense inflammation and hyperalgesia at the injection site (8). Noted responses to CFA include local acute and chronic inflammation, granulomatous reactions, skin ulceration, local abscess, and sloughing. Systemic reactions include diffuse systemic granulomas resulting from the migration of the oil emulsion, adjuvant-related arthritis, and chronic wasting disease (32). Inflammatory pain is influenced by various modulators, including neurotransmitters, receptors, ion channels, and signaling pathways (33). In a study exploring effects of CFA on various metabolites, a decrease of arginine levels and an increase in histidine, phenylalanine, and tyrosine levels were found in response to CFA injection (34). These changes in amino acid levels have been associated with alterations in neurotransmitter levels and, subsequently, with potentiation of chronic inflammatory pain (34). Additional mechanisms involved in CFA-induced pain include the production and release of prostaglandin E₂, NO, leukotriene B₂, TNF, IL-2, and IL-17 (35). These mediators contribute to synovitis, polyarticular inflammation, bone resorption, periosteal bone proliferation, and consequently to joint degeneration (36).

CFA is typically used to induce peripheral inflammation that can subsequently affect the CNS. Peripheral inflammation induced by

CFA leads to the release of inflammatory mediators, including cytokines that can affect the blood-brain barrier and influence communication between the immune system and the CNS. Indeed, it was previously demonstrated that the permeability of the blood-brain barrier increased after CFA administration (37). This can affect both glial cells and neurons and ultimately induce pain. Accordingly, CFA-induced pain in experimental animals is paralleled with the activation of glia and the production of inflammatory mediators in the spinal cord (38). Microglia and astrocytes are recognized as active participants in the initiation and maintenance of pain facilitation triggered by inflammation and damage to peripheral tissues, peripheral nerves, spinal nerves, and spinal cord (39–42). Upon activation, glial cells release a variety of mediators, including proinflammatory cytokines that can enhance pain transmission by activating and sensitizing neurons (41–44). In turn, activated neurons can have reciprocal effects on glial cells, thus maintaining persistent inflammation and prolonged pain sensitization (45). Specifically, increased expression of IL-1 β and IL-1RI was found in glial cells and sensory neurons in an articular arthritis model induced with CFA (46). Furthermore, intraplantar administration of CFA led to elevated expression of microglial markers (Mac-1, CD11b/c, TLR4, and CD14) in the spinal cord and brain during all stages of inflammation (47). In contrast to microglia, increased expression of astrocytic markers, glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein B (S100B), was detected only at the later stages, indicating delayed astrocytic activation. Having in mind all before mentioned, immunization with CFA represents a standard model for studying pain (48).

Considering the wellbeing of experimental animals, the ability of CFA to cause inflammatory pain in experimental animals is problematic *per se* and should be avoided to prevent unnecessary suffering and additional distress in animals. Furthermore, the enduring inflammatory pain induced by CFA interferes with various classical tests assessing exploratory behavior, stress coping, and naturalistic behavior. A systematic review and meta-analysis, encompassing numerous experiments with hundreds of mice and rats, distinctly revealed that CFA markedly reduces exploratory behavior and heightens immobility in the tail suspension test (49). The most pronounced negative impact was observed in naturalistic behaviors like burrowing and wheel running. Finally, pain induced by CFA interferes with the studies of pain caused by CNS autoimmunity and, consequently, with translation to multiple sclerosis. Neuropathic pain is a common symptom in patients with multiple sclerosis, affecting between 28 and 87% of individuals (50, 51). It stems from damage of the central or peripheral somatosensory systems, including the hyperexcitability of neurons within pain pathways (52). As CFA induces pain on its own, EAE induced with CFA is not a reliable animal model for analyzing the mechanism underlying chronic neuropathic pain frequently registered in patients with multiple sclerosis.

3 EAE models without CFA

EAE can be induced in monkeys and rats with IFA (incomplete Freund's adjuvant) as adjuvant or even without adjuvant at all.

Although EAE cannot be induced in mice without adjuvant, transgenic mice develop EAE without induction (*vide infra*). The list of actively induced EAE variants in experimental animals without adjuvant or with IFA is provided in [Table 1](#). Importantly, the idea of using CFA-free animal models in the study of CNS autoimmunity is not novel, having been proposed by pioneers in the field, Levine and Wenk, some 60 years ago ([65](#)). These authors were able to show that EAE can be induced by intracutaneous injection of neural tissue homogenates without adjuvant in various strains of rats, with Lewis rats being the most susceptible. In addition, they tested SCH of different origins, including various rodents, dogs, bovines, guinea pigs, and even humans. Their results showed that guinea pig and rat SCH were by far the most efficient.

DA rats were not investigated in their study, but we were able to convincingly demonstrate that these rats are also highly susceptible to CFA-free EAE ([66–68](#)). Classically, EAE in DA rats is induced with SCH (either guinea pig or rat) emulsified in CFA ([69–71](#)). Moreover, if immunization of DA rats is performed with SCH mixed with carbonyl iron, then strong and highly lethal EAE is induced ([72](#)). Still, EAE is easily induced in DA rats by rat SCH immunization even without adjuvant. The incidence higher than 90% and the clinical course that is prolonged in comparison to SCH + CFA immunization was determined in DA rats immunized with SCH ([67](#)). Infiltrates were observed in both the white and the gray matter of the spinal cord and also in the brain ([68](#)). This model has proven useful for evaluating the therapeutic efficacy of novel agents ([73, 74](#)). We are currently investigating cellular and molecular mechanisms behind the pathogenesis of CNS autoimmunity in this model in detail. We are particularly interested in the effects of immune cells on various brain structures, such as the cortex, the

cerebellum, and the hippocampus. Moreover, antigen specificity of T and B cells activated by immunization with SCH in DA rats is underway.

Furthermore, DA rats immunized with rat SCH and IFA develop severe protracted and relapsing EAE characterized by extensive demyelinating inflammatory lesions and autoreactivity to the rat-specific neuro antigens MOG, MBP_{69–87}, and MBP_{87–101} ([58](#)). Axonal loss in DA rats immunized with MOG in IFA correlates with clinical severity and number of relapses ([61](#)). A similar mechanism of inflammatory demyelination involving different components of endoplasmic reticulum stress has been proposed for demyelination in both the MOG + IFA rat EAE model and in multiple sclerosis ([62](#)). Recently, a novel low-dose MOG + IFA-induced EAE rat model was introduced in DA and SD rats to investigate pain with minimal motor impairment/disability and to find a potential treatment for multiple sclerosis-related pain ([63, 64](#)). The high susceptibility of DA rats to EAE was also confirmed by the induction of severe disease after injection of an encephalitogen in Titermax, an adjuvant consisting of the block copolymer CRL-8300, squalene, and a sorbitan monooleate ([75](#)).

EAE in marmosets immunized with rhMOG peptides in IFA is characterized by demyelinating cortical gray matter and prevalent white matter lesions ([53–55](#)). Interestingly, the cross-reactivity of MOG-specific T cells with the effector memory cells that control latent CMV infection was determined in rhesus monkeys ([76](#)). Although, this cross-reactivity was not investigated in marmosets, the possibility exists that CMV infection, which is common in these monkeys, contributes to the rhMOG peptide + IFA-induced EAE model. The variable clinical presentation of EAE in monkeys is related to inflammation and demyelination in the CNS ([56](#)),

TABLE 1 Active EAE models with IFA or no adjuvant.

	Model	Antigen	Adjuvant	Clinical Course	Reference
Monkeys	Marmosets	rhMOG ₂₀₋₅₀ rhMOG ₁₄₋₃₆ rhMOG ₃₄₋₅₆ rhMOG ₇₄₋₉₆	IFA	Chronic/progressive	(53–55)
	Marmosets Rhesus Cynomolgus	rhMOG ₁₋₁₂₅	IFA	Monophasic/relapsing/ progressive	(56, 57)
Rats	DA	rSCH	IFA	Protracted/relapsing	(58)
	DA Lewis	rSCH	IFA	Relapsing	(59)
	Lewis	rrMOG ₁₋₁₂₅	IFA	Chronic/progressive	(60)
	DA	rmMOG ₁₋₁₁₆	IFA	Chronic/relapsing	(61, 62)
	DA SD	rhMOG ₁₋₁₂₅	IFA	Benign to progressive	(63, 64)
	Lewis CD F	gpSCH	No IFA	NA	(65)
	DA	rSCH gpSCH	No	Chronic/relapsing	(66–68)

CD F, Fischer; DA, Dark Agouti; EAE, experimental autoimmune encephalomyelitis; gp, guinea pig; IFA, incomplete Freund's adjuvant; MOG, myelin oligodendrocyte glycoprotein; NA – not addressed; r, rat; rh, recombinant human; rm, recombinant mouse; rr, recombinant rat; SCH, spinal cord homogenate; SD, Sprague Dawley.

whereas the occurrence of an early anti-rhMOG IgM response is associated with a more severe disease course (76). However, the purity of the antigen is critical for the development of a mild form of EAE using IFA, as low traces of LPS in rhMOG can increase the severity of clinical and histologic features of the disease in cynomolgus monkeys (57).

Finally, resistance of mice to EAE induction with MOG_{35–55} or PLP_{139–151} in IFA can be abolished by addition of the bacterial component peptidoglycan from *S. aureus* (77) or by co-injection of pertussis toxin (78, 79), respectively. EAE can also be induced in mice with MOG_{35–55} and quillaja bark saponin as an adjuvant followed by pertussis toxin injections (80, 81). Microbial products seem to be essential for efficient antigen-presenting cell activity and, consequently, for the induction of CNS autoimmunity in mice (77–79). Thus, it can be concluded that active EAE induction in mice is not possible without adjuvant or with IFA as the only adjuvant. However, there are transgenic mouse models that develop EAE without any immunization, such as double-transgenic TCR^{MOG35–55} × IgH^{MOG} C57BL/6 or non-obese diabetic (NOD) mice (82–84) and TCR^{MOG92–106} transgenic SJL mice (85, 86). These models are very useful for studying the pathogenesis of CNS autoimmunity yet with limitations introduced by intrinsic restriction in the specificity of their antigen recognition receptors.

Importantly, different models presented in Table 1 cover the full spectrum of multiple sclerosis expression, from benign/mild, through the most common relapsing-remitting, and to the chronic and progressive forms. For a comprehensive understanding of the complexity of clinical manifestations of multiple sclerosis and their significance for the pathogenesis and treatment of the disease, please refer to a review by Confavreux and Vukusic (87). This versatility of the CFA-free animal models makes them useful for studying and addressing specific questions related to different clinical forms of multiple sclerosis.

4 Discussion

Numerous variants of EAE are available for the investigation of multiple sclerosis. Given the diversity in the pathogenesis of multiple sclerosis as well as the elusive etiology of the disease, each of the EAE variants is a valuable tool for the studies. We strongly support the use of CFA-free EAE variants as complementary models in multiple sclerosis studies. This approach can help to eliminate the potential effects of CFA, which were described in detail above. Interestingly, there were important early reports showing that EAE can be induced in rats by immunization with an emulsion made of CFA and lung homogenate (88). In addition, a demyelinating disease was induced in Syrian hamsters and guinea pigs by liver homogenate emulsified in CFA (89). It seems unlikely that CNS and lung or liver share some common antigens that could serve as autoantigens in CNS autoimmunity. It is more likely that CFA leads to neuroinflammation independent of the presence of CNS antigens

in the reported studies. This is one more reason to consider CFA as a confounding factor in multiple sclerosis studies based on EAE. Thus, we propose that CFA-free animal models should be used more frequently in the exploration of multiple sclerosis pathogenesis and therapeutic opportunities. Contemporary studies of multiple sclerosis should provide a means to prevent demyelination and neurodegeneration and to promote remyelination and neuroregeneration. We strongly suggest CFA-free EAE variants for such studies as they are superior to CFA-based EAE variants, which are discredited by the weaknesses introduced into the model by the use of CFA, as discussed above.

Author contributions

ML: Writing – original draft, Writing – review & editing. SS: Writing – original draft, Writing – review & editing. NN: Writing – original draft, Writing – review & editing. MD: Writing – original draft, Writing – review & editing. DM: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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When inflammatory stressors dramatically change, disease phenotypes may transform between autoimmune hematopoietic failure and myeloid neoplasms

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Aplastic anemia (AA) and hypoplastic myelodysplastic syndrome are paradigms of autoimmune hematopoietic failure (AHF). Myelodysplastic syndrome and acute myeloid leukemia are unequivocal myeloid neoplasms (MNs). Currently, AA is also known to be a clonal hematological disease. Genetic aberrations typically observed in MNs are detected in approximately one-third of AA patients. In AA patients harboring MN-related genetic aberrations, a poor response to immunosuppressive therapy (IST) and an increased risk of transformation to MNs occurring either naturally or after IST are predicted. Approximately 10%–15% of patients with severe AA transform the disease phenotype to MNs following IST, and in some patients, leukemic transformation emerges during or shortly after IST. Phenotypic transformations between AHF and MNs can occur reciprocally. A fraction of advanced MN patients experience an aplastic crisis during which leukemic blasts are repressed. The switch that shapes the disease phenotype is a change in the strength of extramedullary inflammation. Both AHF and MNs have an immune-active bone marrow (BM) environment (BME). In AHF patients, an inflamed BME can be evoked by infiltrated immune cells targeting neoplastic molecules, which contributes to the BM-specific autoimmune impairment. Autoimmune responses in AHF may represent an antileukemic mechanism, and inflammatory stressors strengthen antileukemic immunity, at least in a significant proportion of patients who have MN-related genetic aberrations. During active inflammatory episodes, normal and leukemic hematopoieses are suppressed, which leads to the occurrence of aplastic cytopenia and leukemic cell regression. The successful treatment of underlying infections mitigates inflammatory stress-related antileukemic activities and promotes the penetration of leukemic hematopoiesis. The effect of IST is similar

to that of treating underlying infections. Investigating inflammatory stress-powered antileukemic immunity is highly important in theoretical studies and clinical practice, especially given the wide application of immune-activating agents and immune checkpoint inhibitors in the treatment of hematological neoplasms.

KEYWORDS

aplastic anemia, myelodysplastic syndrome, acute myeloid leukemia, inflammatory stressors, leukemic transformation, antileukemic immunity

General overview of aplastic anemia and myeloid neoplasms

Aplastic anemia (AA) is an acquired form of autoimmune hematopoietic failure (AHF). Traditionally, AA has been considered a benign hematological disease. AA pathogenesis results from the destruction of hematopoietic progenitor cells (HPCs) by deranged autoimmune responses that occur mainly in the bone marrow (BM) environment (BME). After an initial insult by pathogenic environmental challenges, such as genotoxic exposure or microbial infection, the activated immune responses perpetuate and result in immune-mediated impairment of blood cell production and subsequent clinical presentations of BM hypoplasia/aplasia and peripheral cytopenia in one or more cell lineages (1, 2). Currently, the diagnosis of AA is largely based on markedly decreased BM cellularity and hematopoietic volume with fatty replacement and the absence of overt dysplastic hematopoiesis in HPCs (3, 4). AA is a highly heterogeneous disease entity that converges on a pathophysiological process of persistently deranged autoimmune responses and heavily exhausted HPCs. Patients with AA exhibit varying clinical presentations regarding disease phenotype, disease severity, disease course, treatment response to immune suppressants, and the likelihood of transformation to myeloid neoplasms (MNs). This high heterogeneity is driven by the diversity of the constitutional genetic background, the competence of innate immune cells, the appropriateness of adaptive immune responses to environmental challenges, basic functions of other organ systems, events that guide immune responses, and perhaps most importantly, conditions that sustain deranged autoimmune reactions (1–4).

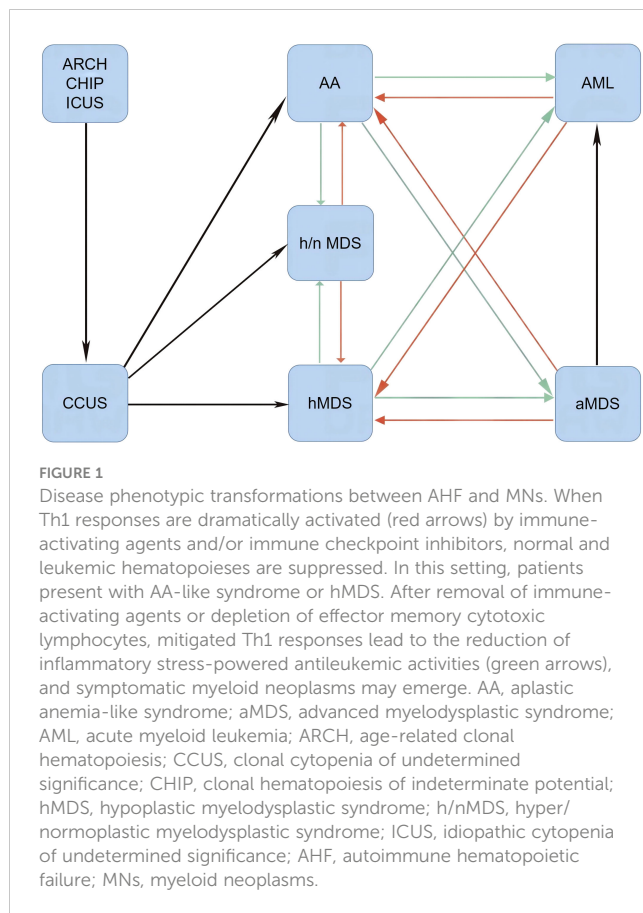
Myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), and acute myeloid leukemia (AML) are unequivocal MNs. MNs result from the acquisition and accumulation of somatic mutations in HPCs. Genetic damage is generated from the exposure of HPCs to various oncogenic agents, and the emergence of leukemic clones arises from the selective advantage of transformed HPCs in the context of chronic inflammatory BME. Oncogenic mutations in transformed HPCs lead to altered proliferation, self-renewal, and antiapoptotic

capacity. The dysregulated growth of leukemic cells results in the overgrowth of their normal counterparts and the suppression of normal blood cell production, clonal hematopoiesis, and corresponding hematological presentations (5–8). The presence of dysplastic features indicates a diagnosis of MNs, and an increased percentage of myeloblasts is the most significant biomarker representing leukemic hematopoiesis and entry into advanced stages (9, 10). Immunological, cytogenetic, and biological molecular analyses can provide valuable information for diagnosis, classification, and risk stratification. MDS, MPN, and AML are also highly heterogeneous with somatic mutations involving hundreds of genes in association with their pathogenesis. Each somatic mutation plays a distinctive role in malignant transformation or affects clinical complications, hematological presentations, cellular components, immunological signatures, disease progression, and treatment response (5–8).

Accumulating evidence supports that there is an intrinsic relationship between AHF and MNs regarding their clonal hematopoiesis, immunological profile, and disease phenotypic transformations.

Reciprocal transformations between AA and MNs

By definition, AA is distinct from MDS, MPN, and AML with differences in fundamental pathogenesis, biological behaviors, laboratory features, treatment strategies, and prognostic significance. However, disease phenotypes can transform between AHF and MNs. The natural transformation of AA to MNs has been widely investigated (11–14) and is generally ascribed to the acquisition and accumulation of oncogenic mutations due to selective pressure and immune exhaustion in the setting of a chronic inflammatory milieu (15–18). It is well known that antithymocyte globulin (ATG) plus cyclosporine-based immunosuppressive therapy (IST) promotes leukemic transformation. Approximately 10%–15% of patients with severe aplastic anemia (SAA) experience leukemic transformation following IST, and in some patients, this transformation emerges



during or shortly after IST (19–23). Such a short interval that spans the transformation process indicates that leukemic clones preexist in the SAA stage (24–27) and rapidly expand due to depletion of cytotoxic T lymphocytes (CTLs) or the induction of immune tolerance (28–32) rather than due to the acquisition of novel molecular events. In patients with genetic aberrations typically observed in MNs, there is a greater frequency of transformation to MNs. Moreover, 20%–30% of SAA patients fail to respond to IST, and these patients have a significantly greater frequency of MN-related genetic aberrations compared to patients who achieve a good treatment response. Even in patients who achieve a hematological response, the presence of MN-related genetic aberrations is associated with a reduced sensitivity to IST, a low rate of deep remission, a high frequency of disease relapse, and a significantly increased risk of leukemic transformation (19–23).

Leukemic transformation in SAA patients can also occur after successful treatment of underlying infections and is frequently separated by a short duration of hematological remission. A rapid increase in leukemic blasts is the representative presentation of leukemic hematopoiesis (33–35). Moreover, patients with definitively diagnosed MNs can develop an aplastic crisis during infectious episodes. During aplastic crisis, the cytological and immunological features resemble those of SAA with a regression of leukemic blasts (35, 36). It remains unclear why patients with a similar genetic landscape or even an identical genetic background exhibit variable disease phenotypes. Furthermore, the significance

of disease phenotypic transformations has not been fully elucidated through basic research and clinical practice. It is possible that environmental factors, most likely changes in inflammation strength or depletion of effector immune cells, affect the disease phenotype and contribute to phenotypic transformations (as schematized in Figure 1).

Clonal hematopoiesis in AA

With the wide application of next-generation sequencing (NGS) in the diagnosis, mutational analysis, and risk stratification of hematological diseases (37, 38), it has been shown that more than one-third of patients definitively diagnosed with AA carry genetic aberrations that are well-known driver mutations in MNs (19–27). Although the number and clone size of mutant genes in AA are much smaller than those in MDS and AML, the number and clone size of these mutant genes can increase in patients who respond to IST (20–22). Currently, AA is also known to be a clonal hematological disease. Clonal HPCs gain a selective proliferative and survival advantage over their normal counterparts in a chronic inflammatory setting and become the dominant hematopoietic components (24–27). Moreover, some patients with MDS or AML can present heavily reduced BM cellularity at initial diagnosis or during disease progression, and patients with low-risk MDS have an immunological landscape similar to that of SAA (39–43).

Immune dysregulation and an inflamed BME in AHF

AA and hypoplastic MDS (hMDS) are typical forms of immune-mediated AHF, and they share similar cytological and immunological landscapes (39, 40). In AHF, autoimmune CTLs (43–45) and natural killer (NK)/NKT cells (46–48) become activated and expanded. Trained CTLs [effector memory CTLs (emCTLs)] and NK/NKT cells (effector memory NK/NKT cells) exhibit elevated killing activities (43–48), increased secretory capacities (49–51), and enhanced sensitivities to antigen stimulation (52, 53). The oligoclonal feature of expanded emCTL infiltrating the BM suggests the presence of chronic stimulation that is driven by an antigen from the BME (43, 50). The excessive production of hematopoietic inhibitory mediators (54–57) due to T helper type 1 (Th1) immune responses (58–60) plays a central role in AHF pathogenesis. Additionally, upregulated expression of proapoptotic molecules (61–63) and decreased telomerase activity (64, 65) accelerate the death of HPCs. Upregulated expression of Toll-like receptors (TLRs) and costimulatory molecules in response to chronic inflammation enhances sensitivity to antigen stimulation, propagates inflammatory responses, induces the production of reactive oxygen species, augments apoptotic signals, amplifies pyroptotic cell death, and favors a selective survival advantage, which forms a positive feedback loop to cause ineffective hematopoiesis and sustain clonal proliferation (66–69). Moreover,

the number and function of regulatory T cells (Tregs) and regulatory B cells (Bregs) are dramatically decreased (70–73), as is the downregulation of anti-inflammatory mediators (74–76). Defects in HPCs and dysregulation of immune responses are also present in complete remission following IST (77, 78). Autoimmunity targeting HPCs by autoantigen-specific lymphocytes (50, 59) attracts a variety of immune cells (79–81). Immune cell infiltration and activation create an immune-active BME (79–83), which forms an inflammatory background and may determine a BM-predominant autoimmune impairment (39–42). The BM-predominant autoimmunity is amplified by an additional antigenic stimulation (84–87). Th1 immune responses suppress the production of erythrocytes, granulocytes, platelets, and B lymphocytes (49, 55–57, 87), which results in clinical and laboratory features of AA and hMDS (3, 4, 9, 10).

The development of an immune-active BME can be achieved in several ways. 1) Pattern recognition receptors (PRRs) sense pathogen-associated molecular patterns (PAMPs), or antigen-specific CTLs target pathogenic antigens of microbes that can survive and smolder in BM cells (84–87). 2) Cellular components that cross-react with microbial epitopes are targeted by autoimmune lymphocytes trained in active infectious diseases (88), particularly inflammatory conditions involving the gastrointestinal tract (89, 90), which can continuously supply intestine-derived antigens from pathogenic or commensal microbes in the context of increased epithelial permeability (90–92). 3) Somatic mutations in epigenetic modulators or transcription factors lead to the excessive production of inflammatory cytokines (93, 94). 4) PRRs sense damage-associated molecular patterns (DAMPs) (95–98), or autoimmune CTLs target neoplastic antigens of genetic or epigenetic products (40, 59, 99–101) in transformed HPCs. If autoimmune responses target DAMPs or neoplastic antigens on leukemic HPCs, the role of autoimmune responses in AHF may be antileukemic (95, 97, 102).

Contribution of inflammatory stressors to autoimmune diseases

Chronic intracellular infections in AID pathogenesis

Chronic infectious diseases, especially intracellular infections, are closely associated with the development, persistence, and acceleration of autoimmune-related impairments in various organ systems (103–110). Autoimmune diseases (AIDs), which usually manifest as organ-specific impairments, are frequently localized at sites away from infectious niduses. The severity of AIDs commonly parallels the severity of chronic infectious conditions (111, 112), which provides persuasive evidence that chronic infections contribute to the perpetuation and acceleration of autoimmune responses. Low-affinity autoimmune lymphocytes can escape the process of clonal selection and clonal deletion (113–116), and the

development of AIDs critically requires the engagement of environmental factors to disturb immune homeostasis and generate high-affinity autoimmune lymphocytes by somatic mutations in the antigen-binding region (affinity mutation) (117–119).

Mechanisms by which infectious diseases induce AID pathogenesis

Several hypotheses have been proposed to explain the relationship between the occurrence of infectious diseases and the generation of autoimmune responses. 1) When infected by microbes or inoculated with vaccines, hosts generate high-affinity antibodies or emCTLs through class switching and affinity maturation in the fight against invading pathogens. High-affinity antibodies or emCTLs cross-react with epitopes of autoantigens in certain tissues (molecular mimicry) (120–123). After being trained in response to microbial infections, these autoreactive lymphocytes migrate to remote organs, exert an immunological attack, and induce tissue damage when recognizing cognate autoantigens (117–119). 2) When tissue is infected by pathogenic microbes, autoreactive lymphocytes in the inflamed niche can be activated and trained by antigen-presenting cells and autoantigens, which form high-affinity autoimmune B and T cells and exert an immunological attack on corresponding tissues (bystander activation) (124–126). 3) In the inflamed niche, cryptic epitopes can be exposed, recognized, and presented to immune cells as exotic antigens to elicit immunological reactions. This immunological attack on cryptic epitopes sustains inflammatory impairment in affected tissues (epitope spreading) (127, 128). 4) During chronic infections, antigens or antibodies can be modified, frequently by glycosylation, which results in altered antigenicity of tissue components or an altered affinity of autoantibodies (posttranslational modifications) (129–132). 5) Certain components of infecting microbes can activate polyclonal lymphocytes and result in the generation of self-reactive antibodies and autoimmune CTLs that can bind to tissue components. These microbial components are called superantigens. Superantigen-induced self-reactive antibodies or autoimmune CTLs initiate an immunological attack (superantigen stimulation) (133–135). The same microbes can trigger AIDs in different organ systems through different mechanisms, and AIDs in certain organs can be induced by different microbial infections through different mechanisms.

After pathogenic antigens have been cleared, the host immune system rapidly returns to a homeostatic state by complex immune regulatory mechanisms to limit inflammatory damage (136–138). However, effector memory lymphocytes have the ability to respond promptly and play a major role when the host encounters the same or cognate epitopes (139–141). Chronic infectious diseases can continuously supply pathogenic antigens or create an inflammatory milieu in which host immune cells persistently produce inflammatory cytokines (84–92) and thereby synergistically sustain the activated form of host autoimmune cells in cognate antigen-dependent or antigen-independent pathways (139–141) to attack antigen-specific cells and innocent bystander tissues (142, 143).

Gut inflammatory conditions in AID pathogenesis

In addition to chronic intracellular infections, the gastrointestinal tract is another common chronic inflammatory nidus. As an important organoid in the human body (144, 145), the gut microbiota plays an essential role in the stimulation, education, and maturation of the host immune system and is the most important element that dynamically affects host immune homeostasis throughout life (146–148). Indeed, host peripheral lymphatic tissues develop poorly in the absence of the gut microbiome (149–151). The associations of AIDs with gut dysbiosis and gut inflammatory conditions have been investigated extensively (90, 92, 147, 152, 153). Patients with inflammatory bowel disease (IBD) have a high frequency of extraintestinal autoimmune manifestations including systemic connective tissue diseases, seronegative spondyloarthritis, systemic vasculitis, and organ-specific autoimmunity in different organ systems (154, 155).

The critical requirement of the gut microbiome for inducing and sustaining autoimmunity in genetically susceptible animal models has been verified in multiple AIDs and provides affirmative evidence for the contribution of gut dysbiosis and gut inflammatory disorders to the development of autoimmune pathogenesis. These animal models spontaneously develop AIDs under conventionally reared conditions, but autoimmune impairment does not occur in germ-free or specific pathogen-free conditions (155–161). In animal models of AIDs, the clearance of the gut microbiome can effectively alleviate disease severity (157). Even in conventionally reared animals, administration of probiotic antibiotic agents can significantly delay disease penetration (162). This is not surprising because the gastrointestinal tract harbors the most abundant lymphoid tissues and microbial community (163, 164). The microbial community and gut homeostasis are constantly subject to various environmental challenges (165, 166), including pathogen intruders (167–170), chemical damage (171, 172), drug exposure (173–175), and nutrient delivery (176–178). Harmful challenges can compromise the integrity of the intestinal epithelium, induce dysfunction, cause dysbiosis, and trigger gut inflammatory disorders; these can occur not only by injuring diseased segments but also by affecting other segments through disruption of microbial ecology (179–181). Gut dysbiosis can initiate immune responses and cause inflammatory lesions in the gastrointestinal tract (181–184). In the setting of a compromised intestinal structure, impaired barrier functions allow host immune cells to come into intimate contact with intestine-derived antigens (89–92). This occurs not only from pathogenic agents but also from commensal microbes and undigested food (185–187) and thereby activates host immune cells, disturbs immune homeostasis, overrides immune tolerance, and triggers deranged immune responses (188–192).

The gastrointestinal tract can continuously supply a large amount of intestine-derived antigens and persistently activate a substantial number of immune cells. The structural and functional features of the gastrointestinal tract render the organoid vulnerable to be an active inflammatory nidus in which host immune

homeostasis is disturbed and inflammatory stresses are sustained with sufficient inflammation strength to perpetuate autoimmune responses in remote organ systems (163, 164). In this process, probiotic commensal microbe-derived metabolites play important roles in maintaining epithelial integrity, regulating host immune responses, and modulating autoimmunity (177, 178, 193–196). An inadequate dietary supply can induce gut dysbiosis and immune dysregulation and affect epithelial repair function (197–200). Chronic gut inflammatory disorders likely work as amplifiers linking systemic immune dysregulation with environmental factors to augment the intensity of extraintestinal autoimmune responses and thereby power autoimmune impairment (201–203). Gut microbiota also plays an indispensable role in combating intracellular infections (204–206) and in eliciting anti-infection responses through vaccine inoculation (207, 208), which reinforces the role of gut microbiota in amplifying systemic immune activities.

Non-specific antigen stimulation in AID pathogenesis

Autoimmune impairments in genetically predisposed animals can also be initiated by adjuvant agents, PAMPs, recombinant inflammatory cytokines, and immune checkpoint inhibitors (ICIs). The *Bacillus Calmette–Guerin* (BCG) vaccine has long been recognized in the induction of AIDs (209, 210), and Freund's adjuvant has been widely used in experimental models to trigger autoimmunity (211, 212). Autoimmune responses are common complications that occur following not only BCG but also other inoculations. This leads to the creation of the specific term “autoimmune syndrome induced by adjuvant (ASIA)” (213–216). Polyribonucleic acid:polyribocytidylic acid (polyI:C) (217, 218), endotoxins (219–221), and fungal peptidoglycans (222–224) activate different PRRs. These PAMPs can trigger and sustain immune derangement by disrupting immune homeostasis (225, 226). ICIs (227, 228) and even hematopoietic growth factors (229, 230) can promote the penetration of AIDs. The initiation of AIDs by non-specific antigen stimulation suggests that systemic inflammatory stressors, rather than specific antigen stimulation, can synergize with a primary immune-active background to trigger deranged organ-specific autoimmune processes (125, 126, 139).

Potential antileukemic mechanism in AHF

In 2009, Nissen (231) proposed that autoimmune responses occurring in acquired AA may function as an antileukemic mechanism against malignant proliferation. In the SAA stage, the decreased number and function of Tregs and Bregs, the decreased production of inhibitory cytokines, the reduced expression of coinhibitory molecules, the increased Th1 responses, the increased number and function of CTLs and NK/NKT cells, the

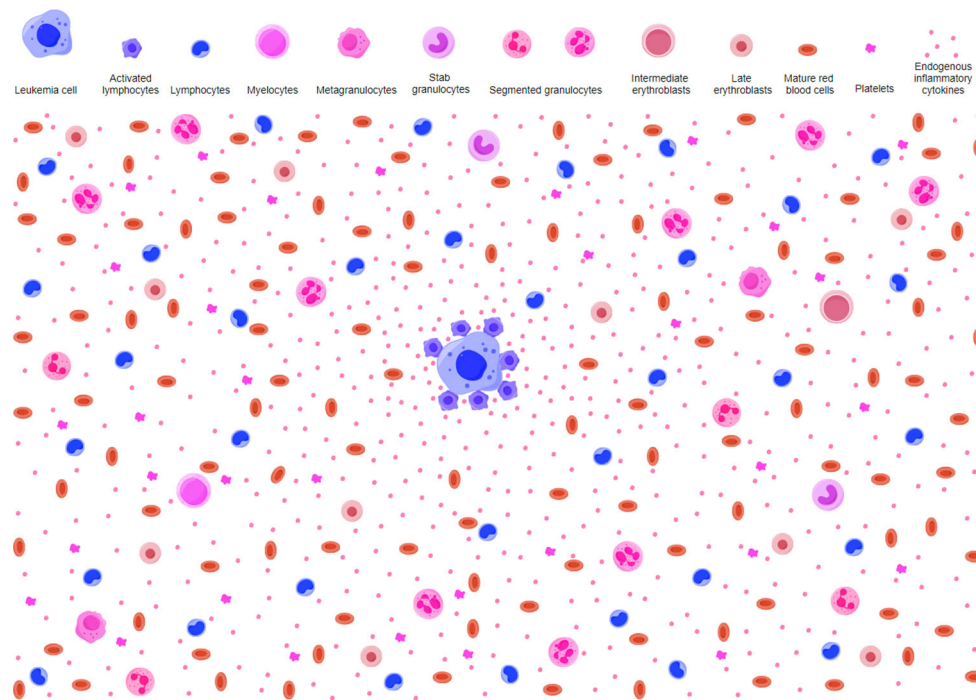


FIGURE 2

Potential antileukemic mechanism in AA-like syndrome. AA-like syndrome is characteristic of reduced HPCs and relatively increased lymphocytes without overt dysplasia. Early hematopoietic progenitors are rare. AA-like syndrome is caused by host immune surveillance against transformed HPCs. Immune responses to neoplastic molecules on leukemic HPCs activate innate and adaptive immune cells. Activated lymphocytes secrete proinflammatory cytokines and create an inflammatory background in the BME. Normal HPCs are suppressed by hematopoietic inhibitory mediators as innocent bystander victims in the process of host immune cells attacking leukemic HPCs. Because of the preferential suppressive effects on transformed HPCs, myeloblasts are hardly observed. The small number of nucleated hematopoietic cells and more intensive responses to transformed HPCs make it difficult to identify overt aplasia, which results in a diagnosis of AA in most circumstances. AA, aplastic anemia; HPCs, hematopoietic progenitor cells; BME, bone marrow environment.

increased antigen-presenting activities, and the excessive expression of proapoptotic and costimulatory molecules on HPCs may represent an adaptive process in response to ongoing antigen stimulation. Along with disease progression, the immunological signature in the BME is transformed from a Th1-predominant response to an immune exhaustion state in which the function of infiltrated autoimmune lymphocytes is inhibited and immune surveillance is gradually dampened in adaptation to the chronic inflammatory milieu. The immune exhaustion state favors leukemic cell survival and facilitates the penetration of symptomatic MNs (15–18). The extrapolation was mainly drawn from tumor regression and concomitant autoimmune phenomena in diseased organs during the treatment of non-hematological malignancies with immunological therapy. The author also described examples of hematological neoplasms in which leukemic cell regression was observed along with the appearance of prolonged aplastic cytopenia and autoimmune reactions (AA-like syndrome) after intensive chemotherapy or other treatment modalities. The author coined the phrase “pancytopenia of unknown significance” to describe this clinical phenomenon. Severe BM hypoplasia, systemic inflammatory symptoms, increased autoreactive CTLs, and decreased Tregs were observed in this AA-like syndrome. These cytological and immunological features indicate that antileukemic immunity has been reinvigorated.

Indeed, AHF can occur in response to neoplastic antigens or DAMPs (95, 96, 102, 232). The acquisition of autoantibodies against the neoplastic antigen carbonic anhydrase I induces AA-like syndrome and leukemic cell regression (102). Constitutive activation of the RAS-MAPK pathway can induce HPC senescence (96), and the absence of PRRs that sense neoplastic molecules results in accelerated leukemia cell proliferation (97). Similar to solid tumors (233–235), immune responses to neoplastic antigens (99–102) and stress in response to HPC damage (95–97) establish a chronic inflamed BME (93, 94, 236, 237). The inflamed BME enhances sensitivity to exogenous antigen stimulation (52, 66, 67) and accelerates apoptotic death in leukemic (95, 96, 238, 239) and normal (95, 96, 142, 143) HPCs, which are the common clinical and laboratory features of AA and low-risk MDS (39, 40, 49, 240) (as schematized in Figure 2).

Antileukemic immunity-induced Th1 responses preferentially repress leukemic cells and their normal counterparts, which can lead to pronounced neutropenia (42, 102, 232). Autoimmune neutropenia can persist for many years prior to the emergence of symptomatic MNs. In this stage, the BM is hypocellular, and cytological and immunological features exhibit immune-mediated hematopoietic impairments in the absence of morphologically identifiable leukemic blasts (241, 242). A significant proportion of MDS patients with overt dysplasia present with hypocellular BM

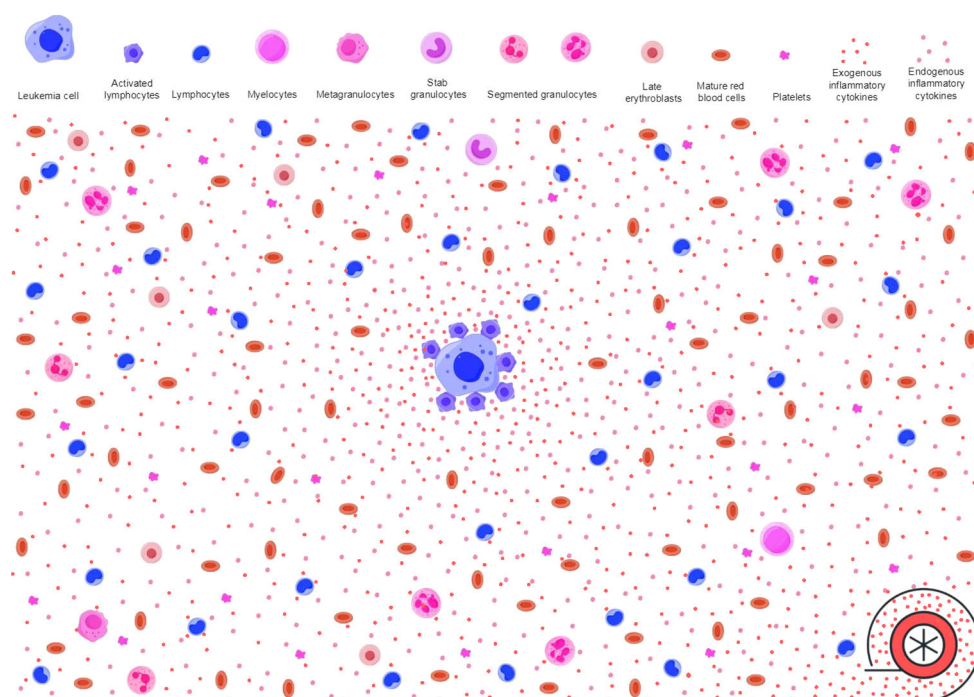


FIGURE 3

Inflammatory stressors power antileukemic activities. As described in Figure 2, immune surveillance in response to neoplastic molecules releases proinflammatory cytokines (endogenous inflammatory cytokines as shown in pink dots) and creates an inflammatory background. However, the baseline antileukemic immunity may not be sufficient to effectively suppress leukemic HPCs. Immune responses to invading microbes produce additional activated immune cells and proinflammatory cytokines (exogenous inflammatory cytokines as shown in red dots). These pathogen-activated immune cells and the proinflammatory cytokines migrate to the inflamed BME through blood circulation, power the primary inflammatory milieu, and enable the antileukemic immunity to achieve an intensity that is sufficient to effectively suppress leukemic HPCs. The primary inflamed BME and extramedullary inflammatory stressors synergistically trigger and sustain AA-like syndrome. HPCs, hematopoietic progenitor cells; BME, bone marrow environment; AA, aplastic anemia.

and peripheral pancytopenia at initial diagnosis or during disease progression and these patients frequently have longer survival (21, 23, 42, 240). Prolonged neutropenia after intensive chemotherapy has been associated with deep remission, longer remission duration, reduced frequency of relapse, and restoration of Th1 immune responses (243, 244). Substantial evidence supports an antileukemic role of autoimmune responses in AHF (42, 95, 102, 232), at least in a large number of AHF patients who have MN-related genetic aberrations (19–26). However, factors underlying the exaggerated autoimmune responses that influence antileukemic activities and disease phenotypes were not discussed in Nissen's manuscript (231).

Inflammatory stressors power antileukemic immunity

The transformation between AHF and clinically overt MNs (MDS with excessive blasts or AML in which an increased percentage of myeloblasts is the most representative parameter in determining leukemic hematopoiesis) can reciprocally occur. The switch that shapes the disease phenotype is a change in the strength of extramedullary inflammatory conditions (33–36). Extramedullary infectious diseases have a conspicuous impact on

phenotypic presentations. During active inflammatory episodes, normal and leukemic hematopoieses are simultaneously suppressed with regression of leukemic blasts on cytological examination and Th1-predominant responses on immunological analysis. Aplastic cytopenia, the disappearance of leukemic blasts, Th1-predominant responses, and a small number of nucleated blood cells for identification of dysplasia result in cytological and immunological features that meet the criteria for the diagnosis of AA (33–36). Following successful control of the inflammatory episodes and a short duration of hematological remission, quickly expanded leukemic clones lead to the appearance of substantial leukemic blasts and symptomatic MNs (33–35). Patients with clinically overt MNs can develop an aplastic crisis during active inflammatory episodes in which systemic inflammatory stress is dramatically aggravated (35, 36). Within this period, the BM becomes aplastic/hypoplastic, and leukemic blasts disappear with the infiltration of autoimmune lymphocytes. These phenomena indicate that autoimmune responses in AHF are an inflammatory stress-powered antileukemic mechanism. Immune surveillance against leukemic blasts contributes to BM-specific autoimmune impairments, while inflammatory stressors strengthen autoimmune responses (as schematized in Figure 3).

This process simulates spontaneous remission in AML patients after an infectious episode and aplastic crisis (245–252). The

mechanism is presumed to be due to reversion of the immune exhaustion state and restoration of antileukemic immunity through activation of immune cells and their secretion of proinflammatory cytokines in response to invading pathogens (250–253). Another frequent situation that induces spontaneous remission is that patients with hypoplastic AML (hAML) are treated with hematopoietic growth factors. To date, spontaneous remission has been reported in more than 200 AML patients. The remission duration usually lasts for 2–6 months, which indicates that leukemic clones cannot be eradicated even under inflammatory stress-powered antileukemic immunity (245, 254). This may be due to the low expression of neoplastic antigens on dormant leukemic cells (255–258). However, repeated spontaneous remissions occur in a paucity of patients (245, 247), which suggests that patients do not completely lose the hematopoietic regulatory mechanisms and the inflammatory stress-powered antileukemic immunity. Another interesting phenomenon also suggests the existence of inflammatory stress-powered antileukemic activities (243, 244). Prolonged neutropenia due to hematopoietic suppression in AML patients after intensive chemotherapy is usually complicated by durable or repeated infectious episodes, which frequently result in death. If patients survive prolonged neutropenia, they may achieve deep remission, longer remission duration, and a reduced frequency of relapse. Recombinant type I inflammatory cytokines (259–262), interferon inducers (263–265), and BCG inoculation (266–268) have been used to treat hematological and non-hematological neoplasms and have shown efficacy in the control of tumor growth or even the clearance of malignant clones. Other vaccine inoculations that can stimulate Th1 responses have also been shown

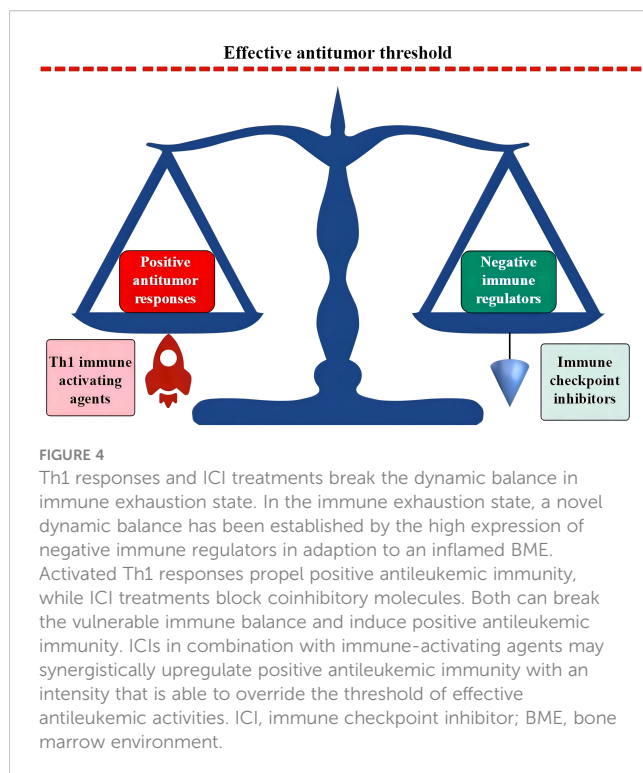
to have antitumor effects (269–271). These non-specific immunological therapies and their treatment effects indicate that inflammatory stressors, rather than antigen-specific responses to invading pathogens, augment antitumor activities (139).

Reversion of the immune-exhausted state by Th1 responses and ICI treatment

Immune exhaustion in adaptation to persistent inflammatory stimulation is a common mechanism underlying the escape of leukemic clones from immune surveillance (17, 18). Hyporesponsiveness is characterized by a high infiltration of negative immune cells, excessive secretion of anti-inflammatory cytokines by immune regulatory cells, and high expression of immune checkpoint molecules on tumor cells in the tumor microenvironment, which work to limit the magnitude of inflammatory responses, avoid excessive tissue damage, and maintain a dynamic immune balance in the context of chronic inflammatory stressors (272, 273). In an immune exhaustion state, dampened antileukemic immunity favors leukemic cell escape from immunological attack and facilitates the penetration of symptomatic MNs. ICIs block coinhibitory molecules and reinvigorate antitumor immunity. ICIs have been successfully used to treat MNs (273–275), and a high-grade inflammatory profile in the BME is a predictor of a good therapeutic response to ICI treatment (276, 277) and suggests that antileukemic immunity is closely related to an inflamed BME. The relationship between an inflamed microenvironment and ICI treatment response has been more thoroughly studied in solid tumors (234, 278–280).

It is widely accepted that dysbiotic gut microbiota plays an important role in promoting tumor cell escape from immune attack in solid tumors (281–283), and the response to ICI treatment is strongly influenced by the abundance and diversity of the gut microbiome (284, 285). This also indicates that antitumor immunity necessitates the engagement of additional antigenic stimulation for immune surveillance against neoplastic antigens. A reduced gut microbial abundance and diversity due to antibiotic treatment can dramatically dampen ICI treatment responses (286). Modulation of the gut microbiota can reverse the dampened responses (287–290). Some pathogenic (291–295) and commensal (296, 297) microbes can stimulate Th1 immune responses and thereby power antitumor immunity (298–300) and enhance ICI treatment responses (301–303). One of the major adverse effects of ICI treatment is hematological toxicity (304–306), especially when treating hematological malignancies and when combined with other antileukemic agents (306). This reinforces the role of Th1 responses in AHF pathogenesis and inflammatory stress-related antileukemic immunity.

Blood cells are immune cells, and their production is regulated largely in response to microbial invasion. Therefore, hematopoietic cells are more sensitive to inflammatory mediators. When



confronting an acute and limited infection, HPCs sense microbial antigens and inflammatory cytokines. With the pathogenic stimulation, host hematopoiesis proliferates and polarizes the differentiation toward the production of effector immune cells to fight invading microbes and replenish the consumed innate immune cells (307–310). However, chronic inflammatory stress in response to persistent intracellular infections can induce Th1-predominant immune responses and exhaust host hematopoietic functions. These responses predominantly exhaust granulopoiesis, erythropoiesis, megakaryocytopoiesis, and B lymphopoiesis (311–316) and result in the morphological and immunological signatures shown in AHF (3, 4, 10, 240). The upregulated expression of HLA-DR, TLRs, and the Nlrp3 inflammasome due to the primary immune-active BME increases sensitivity to antigen stimulation (50, 52, 66, 67) and apoptotic death of HPCs (50, 62, 63). In combination with extramedullary inflammatory stressors, the immune-active BME leads to BM-specific and inflammatory stress-powered autoimmune responses. In the absence of an inflamed BME, some infectious diseases, although able to induce Th1 immune responses, may not seriously injure hematopoietic function. Evidence for this is that patients with systemic rheumatic diseases are seldom complicated by AHF. Along with the persistence and exacerbation of inflammatory stressors, the host immune system can be impaired, which changes the immunological landscape from the proinflammatory phenotype of AHF to the immune exhaustion phenotype of advanced MN (317–320). Exhausted immune responses and DNA damage occurring under chronic inflammatory conditions are considered major contributors to the immune escape of leukemic cells (15, 16, 321, 322). The role of immune exhaustion in advanced MN may cause an increased threshold for effective antileukemic immunity (323). Enforcing an additional antigen stimulation or administering ICIs can reverse the immune exhaustion state and thereby overcome the raised threshold and restore missed antileukemic immunity (35, 95, 250, 253, 323). Combination of immune-stimulating agents and ICI treatments may further potentiate antitumor activities (as schematized in Figure 4).

From this point of view, AHF and MNs have complex intrinsic relationships, and some patients with definitively diagnosed AA may be hMDS (39, 40, 324, 325) or hAML (102, 322). The absence of overt leukemic hematopoiesis in AHF likely results from the small number of nucleated cells available for identification of aplasia and leukemic blasts due to severe BM hypoplasia and a preferential attack on transformed HPCs by autoimmune CTLs and their secretion of hematopoietic inhibitory mediators (238, 239, 259–262). Indeed, neutropenia is a common preleukemic presentation in MNs, and in a significant proportion of MDS patients, neutropenia can be a solitary hematological abnormality that persists for many years (241, 242). Furthermore, a poor response to hematopoietic growth factors predicts a high frequency of disease progression (242). A decreased white blood cell count and concealed leukemic clones make it difficult to distinguish this AA-like syndrome from genuine AA (39, 40, 324, 325), which leads to a diagnosis of AA in most circumstances (3, 4), especially before the wide application of NGS in the diagnosis and stratification of hematological diseases.

Decreased inflammatory strength facilitates leukemic transformation

AHF is an organ-specific AID. Autoimmune responses occur predominantly in the BM and result in the exhaustion of HPCs and impairment of mature blood cell production (1, 2, 39, 240). AA and hMDS are clonal hematological diseases (5, 25, 27, 240). Genetic aberrations in clonal hematopoiesis affect cell proliferation, self-renewal, and apoptosis (5–8). While hMDS is an unequivocal MN, AA has a high frequency of transformation to MNs. The natural evolution from AA to MNs is usually a long-term process (11–14) and is frequently attributed to genetic damage or immune exhaustion due to an inflamed BME (15–18). However, leukemic transformation can occur during or shortly after IST (19–23). Effective treatment of underlying infections can also induce leukemic transformation (33–35). Prior to the emergence of symptomatic MNs, patients usually experience a short duration of hematological remission. Leukemic transformation shortly after IST or treatment of underlying infections may better reflect the intrinsic relationship between AHF and MNs with regard to the influence of the host immunological state on the disease phenotype. This is because IST and treatment of underlying infections can rapidly change the host immune state without inducing novel genetic damage. An aplastic crisis with leukemic cell regression in definitively diagnosed MNs during inflammatory episodes provides a more persuasive argument for the influence of the host immune state on the disease phenotype (35, 36).

How should we interpret BM-predominant autoimmune impairment in AHF when considering the very high prevalence of chronic inflammatory disorders in the gastrointestinal tract and other organ systems?

A reasonable explanation is that, similar to the microenvironment in solid tumors (233, 234, 272, 279), primary autoimmune pathogenesis targets specific antigens or DAMPs on HPCs or other blood cells in the BM, regardless of whether the targeted antigens are infectious (84–87) or neoplastic (95–102). Interactions between autoimmune CTLs and targeted antigens (50, 59, 100, 232) or between innate immune cells and DAMPs (95–98) evoke an inflammatory background in the BME (27, 93, 94, 236), which determines the BM-predominant autoimmunity. Defects in HPCs (77) and derangement of immune responses (78) are present not only in active SAA but also in hematological remission following IST, which suggests that intrinsic abnormalities in HPCs are responsible for the primary autoimmune response. Like patients with AA (326–331), patients with MDS and AML have also been shown to have a high frequency of autoantibodies that can bind erythrocytes, erythroblasts, and platelets (42, 99–102, 258), and autoantibodies against hematopoietic progenitors can directly induce Th1 immune responses (100, 102, 332, 333). Patients with these autoantibodies frequently present with hypoplasia and

absence of dysplasia, which resembles a clinical phenotype observed in patients with AA (21, 42, 102).

How should we interpret leukemic cell regression in the SAA stage and their dysregulated proliferation after the successful control of underlying inflammatory stressors?

A reasonable explanation is that targeted molecules are present in leukemic blasts (50, 59, 88, 100, 102). Neoplastic epitopes from genetic or epigenetic products of transformed HPCs (42, 93, 95, 97, 100, 232) are likely the targeting molecules responsible for the primary inflammatory BME. However, effective repression of leukemic blasts also requires the engagement of additional inflammatory stressors that are strong Th1 responses for the sustenance of antileukemic immunity (95, 161, 245, 246, 250). This is perhaps due to the low immunogenicity of neoplastic antigens on leukemic cells (255–258). Additional inflammatory stressors may play a role in sustaining a systemic inflammatory state (201–203), which together with the primary inflammatory BME creates an inflammatory niche with an intensity sufficient to repress normal and leukemic hematopoieses and synergistically forms the characteristic immunological signature in AHF. Th1 responses repress granulopoiesis, erythropoiesis, megakaryocytopoiesis, and B lymphopoiesis (311–317) in accordance with the cytological signature in AHF (3, 4, 10, 240). Th1 responses from extramedullary infectious diseases may preferentially strengthen antileukemic immunity in these lineages. ICIs for the treatment of hematological and non-hematological malignancies may also benefit from inflammatory stress-powered antitumor immunity. The administration of ICIs reverses the immune exhaustion state, promotes the infiltration of proinflammatory immune cells into the tumor microenvironment, and reduces the threshold for inducing effective antileukemic immunity (272–275). Inflammatory strength in the tumor microenvironment predicts the treatment response to ICIs (276–280). However, effective antitumor immunity requires additional antigen stimulation especially from the gastrointestinal tract (281–285), and the dynamic microbial ecosystem is the most important environmental factor for maintaining host immune homeostasis (146–148). Some pathogenic (291–295) and commensal (296, 297) microbes can stimulate Th1 responses, enhance ICI treatment-induced antitumor immunity (301–303), and even directly overcome the immune exhaustion state (298–300). This mechanism may play a more important role in MNs because blood cells are immune cells and their production is regulated largely in response to pathogen invasion (307–310). During active inflammatory episodes, Th1 immunity in response to invading pathogens powers antileukemic effects, which results in the regression of leukemic clones (33–36). After successful control of inflammatory diseases, suppressive effects on leukemic clones are mitigated and result in the penetration of morphologically identifiable leukemic hematopoiesis (35).

How should we interpret the very short interval spanning the leukemic transformation process when considering that the development of symptomatic MNs through the acquisition and accumulation of novel oncogenic mutations is unlikely within such a short interval?

A reasonable explanation is that leukemic clones preexist but are repressed in AHF stages (19–27) and rapidly expand after the resolution of inflammatory stressors (95, 245, 250, 254). The persistence of autoantibodies (42, 99, 100, 258, 330) and autoimmune responses (59, 78, 93, 273, 321) in AA, MDS, and AML suggests that autoantigens are persistent and their immunogenicity is less intensive. Leukemic clones cannot be eradicated even in inflammatory stress-powered antileukemic immunity, which is perhaps due to the low expression of neoplastic antigens and immune molecules in dormant leukemic cells (35, 245, 250, 254). During an inflammatory episode against invading pathogens that can stimulate strong Th1 responses, a patient's immune system is mobilized, and the vulnerable immune balance in adaptation to chronic inflammation is disrupted, which results in the recall of antileukemic immunity and concealment of leukemic blasts. This process is similar to that observed in spontaneous remission in AML patients after an infectious episode (35, 245, 250). During infectious episodes, patients demonstrate positive immune responses and frequently experience an aplastic crisis. After successful treatment of underlying infections, dampened inflammatory stress-powered antileukemic immunity leads to the expansion of previously suppressed leukemic clones and facilitates the appearance of symptomatic MNs, which is distinct from the naturally occurring clonal evolution in AA (11–14) due to the acquisition of novel somatic mutations and immune exhaustion in the chronic inflammatory milieu (16, 17, 321, 323).

How should we interpret the short duration of hematological remission before the emergence of symptomatic MNs?

Prior to the emergence of symptomatic MNs, whether following IST (19–23) or effective treatment of underlying infectious diseases (33–35), patients experience a short duration of hematopoietic remission. A reasonable explanation for this short remission duration is that inflammatory stress-powered antileukemic activities can simultaneously repress normal (54–57) and malignant (245–254) hematopoiesis, which results in BM hypoplasia/aplasia with the infiltration of autoimmune lymphocytes and an increase in inflammatory intensity (34, 35). The absence of leukemic blasts may be due to the preferential repression of malignant proliferation (238, 239, 259, 260) and the small number of nucleated blood cells available for the identification of dysplastic features (232, 240, 324, 325). Following

the effective treatment of underlying infections, inflammatory stress-mediated antileukemic activities are mitigated, which leads to the simultaneous proliferation of normal and leukemic hematopoieses and transient clinical remission (33–35) due to the prolonged proliferative cycle of damaged HPCs (334–336). Along with an increase in leukemic cell burden due to dysregulated proliferative capacity (5–8), leukemic cells compromise the hematopoietic pool and outcompete their normal counterparts. Normal hematopoiesis is subsequently suppressed (337–340), which leads to the emergence of symptomatic MNs. The panorama of inflammatory stress-powered antileukemic immunity is better displayed in our reported patient with definitively diagnosed MDS with excessive blasts-1 who developed an aplastic crisis during an inflammatory episode (35). During the aplastic crisis, leukemic blasts regressed with atypical lymphocyte infiltration in the BM. Tentative antituberculosis treatment resulted in not only the disappearance of the atypical lymphocytes but also an increase in leukemic blasts.

How should we interpret leukemic transformation during or shortly after ATG-based IST?

Approximately 70%–80% of SAA patients respond to ATG-based IST (19–23). However, dysregulation of the immune system and defects in HPCs persist even in remission patients (77, 78), and relapse eventually occurs in 30%–40% of responding patients (19–23), which indicates that the primary pathogenic factor is not removed by IST. In approximately 10%–15% of SAA patients, the disease phenotype transforms to MNs. In some cases, leukemic transformation occurs during or shortly after IST (19–23). Some MN-related somatic mutations in the SAA stage predict a lower frequency of deep hematologic response and a higher rate of disease relapse and leukemic transformation. Non-responding patients carry a higher frequency of these unfavorable mutations compared to responding patients (21–24). The presence of MN-related genetic aberrations influences treatment responses, which indicates that genetic damage in HPCs plays an important role in eliciting autoimmune pathogenesis. The pharmacologic role of ATG-based IST in AHF treatment is to deplete effector CTLs and induce immune tolerance; thus, IST functions as an immune suppressant to relieve autoimmune impairment in HPCs (28–32). At the same time, inflammatory stress-mediated antileukemic immunity can be compromised. After IST, the number and size of somatic mutations are massively increased (20, 42) due to the mitigation of inflammatory stress-powered antileukemic immunity. From this point of view, IST promotes the penetration of symptomatic MNs by interfering with the process of immunological attack, which is distinct from the removal of antigen stimulation by treating underlying infections. However, their effects are similar. Both IST and treatment of underlying infections can mitigate the intensity of the inflamed BME and promote the penetration of symptomatic MNs. Intriguingly, approximately one-third of patients who respond to IST do not relapse or progress to MNs, which supports the heterogeneous nature of the etiology and pathology that initiates

and sustains autoimmune-mediated hematopoietic injuries. More interestingly, transient genetic aberrations occasionally occur in a paucity of patients after IST (19, 20). It is possible that some genetic aberrations are not correlated with or not sufficient for leukemic transformation (24, 25, 27).

Particular attention should be given to gut inflammatory disorders and disseminated tuberculosis in AHF patients

Although patients with MNs may benefit from inflammatory stress-powered antileukemic immunity, an excessive inflammatory cascade and severe cytopenia can be harmful or even fatal. Like in other AIDs (156–161), AHF pathogenesis necessitates an active chronic inflammatory condition in addition to the inflamed BME to sustain BM-predominant autoimmune impairment (90, 95, 161, 313, 315). When AHF patients present with active inflammatory symptoms and their cytopenia is dramatically exacerbated, identifying and disposing of inflammatory niduses are urgent tasks that are needed to save patients' lives. In this situation, particular attention should be given to inflammatory diseases in the gastrointestinal tract and disseminated tuberculosis. Tuberculosis reactivation may be the result of an immune exhausted state and/or the administration of immune suppressants. Disseminated tuberculosis can worsen hematopoietic function and become a major health problem in AHF patients.

Association of gut inflammation with AHF pathogenesis

An association between AHF and gut inflammatory conditions has long been proposed (89, 90, 341). Patients with AA have a dysbiotic gut microbiota (342), and the immunological signature of AA is the same as that of IBD (343), which suggests that AA and IBD may share similar initiating factors and common pathogenic agents (1, 2, 344, 345). A gluten-free diet for coeliac disease-associated aplastic cytopenia (346) or resection of diseased colonic segments in patients with neutropenic enterocolitis (347) can effectively relieve hematopoietic suppression. In our previous report (348), two cycles of intermittent hematopoietic recovery and disease relapse occurred in a patient with refractory SAA following intermittent treatment with a gut-cleansing preparation, which reinforces the definitive role of gut inflammatory conditions in AHF pathogenesis. The contribution of the gut microbiome to sustaining the pathogenesis of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation, a pathogenic mechanism similar to that of AA and frequently used to study AHF pathogenesis in animal models, was reported several decades ago (161). In our retrospective study, all patients with SAA had imaging abnormalities that reflected the existence of chronic inflammatory lesions and acutely aggravated inflammatory damage in the gastrointestinal tract during inflammatory episodes and exacerbated cytopenia (349). Similar to

non-hematological AIDs (156–160), growing evidence demonstrates that the gastrointestinal tract is the most common inflammatory nidus that sustains AHF pathogenesis in patients with a background of an inflamed BME (89, 90). Aggravated gut inflammatory disorders can strengthen the immune-active BME and suppress normal and leukemic hematopoieses, which leads to AHF and regression of leukemic clones (33–36). The effective treatment of gut inflammatory conditions can ameliorate AHF (348) and promote leukemic transformation (33).

Hematopoietic suppression by Th1 responses to tuberculosis

The Th1 immune response is the major mechanism involved in defense against *Mycobacterium tuberculosis*, with IFN- γ production playing a pivotal role in combating *M. tuberculosis* infection (291, 350, 351). IFN- γ is the most important mediator of AHF development (55, 56, 240) and links AHF pathogenesis with chronic infectious diseases that provoke Th1-predominant immune responses. Tuberculosis is one of the most common chronic intracellular infections (106, 209, 315). Over 90% of the world's population has been infected by *M. tuberculosis*. Despite great therapeutic advances, nearly a quarter of individuals remain latently infected after the initial infection (352–354). Latent tuberculosis can be reactivated under certain circumstances such as with aging, undernourishment, prolonged psychological stress, long-term administration of immune suppressants, or coinfections with HIV or other pathogenic microbes (355–357). In this situation, antituberculosis immunity by trained Th1 cells (291, 358), CTLs (359, 360), NK/NKT cells (361, 362), unconventional lymphocytes (363, 364), and even CD5+ B cells (365) can be recalled. These memory immune cells rapidly expand and produce a substantial amount of IFN- γ , TNF- α , and other proinflammatory cytokines in response to increased *M. tuberculosis* antigen load. Excessive proinflammatory cytokines strengthen the primary immune-active BME and repress normal and leukemic hematopoieses (34, 35, 106, 315). Antigens of *M. tuberculosis* may also cross-react with autoantigens on HPCs. With the cross-reaction, reactivated effector immune cells induce an immunological attack on HPCs and strengthen the inflamed BME (88). Even in latent tuberculosis, chronic antigen stimulation may persist due to the high heterogeneity of this chronic infection (356, 365–367) and fluctuations in host performance status, which leads to fluctuating chronic inflammation.

The more important role of gut involvement in tuberculosis infection

Disseminated tuberculosis (368–371) or even disseminated BCG vaccination (372) can induce BM hypoplasia and peripheral cytopenia. The fact that tuberculosis-induced AHF occurs only in disseminated tuberculosis instead of in isolated pulmonary tuberculosis suggests that tuberculosis can infect hematopoietic tissues (373) or the gastrointestinal tract (34, 35, 181, 349). Gut involvement and subsequent dysbiosis (181) likely play more important roles in inducing systemic inflammatory disorders

(374, 375). In our retrospective study investigating infectious niduses during flared inflammatory episodes and accelerated cytopenia, five of 17 recruited SAA patients had imaging abnormalities suggestive of tuberculosis reactivation, all of which involved inflammatory diseases in the gastrointestinal tract (349). The incidence of tuberculosis reactivation may be even greater in advanced MN patients (376–378). It is not surprising that there is a high incidence of chronic *M. tuberculosis* infection in AHF patients because patients with HLA-DR15 have weaker responses to *M. tuberculosis* (379, 380), and AHF patients have a high frequency of HLA-DR15 phenotype (52, 53). Gastrointestinal and even isolated pulmonary tuberculosis can induce pathological processes in the gastrointestinal tract, not only alone but also in combination with gut dysbiosis, which results in diffuse gut inflammatory lesions (181, 381, 382). In this setting, both pathogenic and commensal (185, 186) microbes, in addition to *M. tuberculosis* infection (315, 316), become exogenous antigens to sustain deranged immune responses.

Summary and perspective

Autoimmune responses in AHF may represent an inflammatory stress-powered antileukemic mechanism, at least in a significant proportion of patients whose pathogenesis is associated with MN-related genetic aberrations. With age, the incidence of clonal hematopoiesis increases, which is known as mutation-specific adaptation to environmental stressors and is consistent with a high incidence of AHF and inflammatory disorders in old age. However, age-related clonal hematopoiesis also increases the risk of malignant evolution (383–385). A dynamic balance between antileukemic activities and negative immune regulators may play a more important role in the suppression of transformed HPCs and the maintenance of effective hematopoiesis in old age. In this process, immune surveillance against neoplastic antigens or DAMPs initiates a primary inflammatory background in the BME and may contribute to the BM-specific autoimmune impairment. Extramedullary inflammatory stressors generated from exogenous antigen stimulation, especially from the gastrointestinal tract and chronic intracellular infections, play a critical role in the sustenance of autoimmune responses and the apoptosis of HPCs and promote the development of AA-like syndrome.

Although AA and MNs are generally considered distinctive disease entities, the phenotype of aplastic cytopenia can transform into MNs and vice versa. The switch that shapes the disease phenotype is a change in inflammation strength. During active infectious episodes, leukemic blasts can regress due to the reversion of the immune exhaustion state and the restoration of antileukemic activities. At this time, patients manifest aplastic cytopenia. After successful treatment of underlying infections, leukemic clones rapidly expand, and patients manifest symptomatic MNs, which commonly follow a short duration of hematological remission. Miscellaneous microbes or even endotoxins can induce antitumor immunity. This indicates that not antigen-specific stimulation but rather inflammatory stressors in response to microbial invasion are responsible for sustaining antitumor activities. Although they occur through different mechanisms, the effect of ATG-based IST is

similar to that of treating underlying infections. Spontaneous remission in AML patients and non-specific immunological therapies in treating hematological malignancies may share an inflammatory stress-powered antileukemic mechanism.

An in-depth investigation of inflammatory stress-powered antileukemic immunity is highly important for theoretical research and clinical practice. Understanding the mechanisms of immune surveillance against malignant proliferation and the rational administration of immune-activating agents and ICIs in the treatment of hematological malignancies will be helpful, especially when aplastic cytopenia appears to be the major adverse effect. Some pathogenic microbes can stimulate Th1 responses. Inoculation of vaccines from these microbes has direct antileukemic effects and enhances ICI treatment efficacy. The inflammatory stress-powered antileukemic mechanism raises many questions.

- 1) Despite powering antileukemic immunity, chronic antigen stimulation may further exhaust the host immune system and upregulate the responsive threshold. These effects may lead to a requirement for more intensive antigen stimulation to achieve effective antileukemic strength, which forms a vicious cycle and attenuates antileukemic activities. This raises the question of how to take advantage of non-specific inoculation for the treatment of MNs without inducing immune exhaustion; intermittent inoculation in combination with ICI treatment is likely a superior option (348, 386, 387).
- 2) ICI treatments lower the response threshold, while inoculations enforce the response strength. ICI treatments in combination with inoculation may synergistically reinvigorate missed antileukemic activities and further enhance treatment efficacy. This combination therapy may produce an overwhelming inflammatory cascade and heavy hematopoietic suppression. This fatal side effect raises the question of how to tip the balance between benefits and severe toxicity and how to combine immunological therapies.
- 3) While some commensal microbes can stimulate Th1 responses and exhibit antileukemic activities, some microbes can suppress Th1 responses and favor the maintenance of an immune exhaustion state. However, the abundance and diversity of the gut microbiota are critical for the integrity and function of the gastrointestinal tract. Gut dysbiosis and excessive Th1 responses disrupt the intestinal integrity and barrier function and induce gut inflammatory conditions (147, 183, 197, 345). Furthermore, chronic gut inflammatory conditions can induce systemic immune dysregulation and immune exhaustion. This raises the question of how to exploit immune responses to “beneficial microbes” and avoid severe gut inflammatory damage.
- 4) Both AHF and MNs are fatal diseases. The resolution of inflammatory stressors by either IST or treatment of underlying diseases may lead to phenotypic

transformation from AHF to clinically overt MNs, which complicates the patients’ disease course. This raises the question of how to assess the benefits and risks of leukemic transformation prior to IST. Additionally, underlying inflammatory conditions should also be properly managed.

- 5) Different lineages of immune cells are regulated by different cytokines in response to different antigen stimulation (307–310). Blood cells are immune cells, and MNs can be derived from different cell lineages (10). Some cytokines inhibit certain lineages of leukemic clones but may promote the proliferation of others (388). This raises the question of what leukemic cells of origin can benefit from and which ones may be promoted by the treatment with immune-activating agents.
- 6) It is unclear what antigens are neoplastic, what neoplastic antigens are immunogenic, and what intensity of immunogenic antigens can provoke antileukemic immunity. Although antileukemic immunity is powered by non-specific antigenic stimulation and immunogenic antigens responsible for antileukemic immunity may be miscellaneous in different neoplasms, is there any advantage in selecting an inoculation microbe with epitopes that cross-react with neoplastic antigens if the neoplastic antigens are identified?

Collectively, inflammatory stress-powered antileukemic immunity warrants extensive investigation. This may explore a new way of MN treatment. With in-depth investigations, many questions will emerge, and better strategies for improving treatment responses and managing adverse complications will be found.

Author contributions

X-CZ: Conceptualization, Writing – original draft. BJ: Data curation, Writing – original draft. N-NX: Data curation, Writing – original draft. X-YS: Data curation, Writing – original draft. F-JM: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prognostic significance of lymphocytic foci composition in minor salivary gland biopsies for severe disease flare and severity in Sjögren's syndrome: a 3-year follow-up cohort study

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Introduction: This was an ambispective cohort study evaluating the prognostic significance of lymphocytic foci and its lymphoid composition in minor salivary gland biopsy (MSGB) for short-term disease flare and severity in Sjögren's syndrome (SS).

Methods: The inclusion criteria comprised individuals meeting the ACR/EULAR 2016 criteria who underwent MSGB with an infiltration of more than 50 lymphocytes and received clinical diagnosis between September 2017 and December 2018. Patients with inadequate biopsy samples were excluded. The number of lymphocytic foci and their lymphoid composition in MSGB were assessed using immunofluorescence staining. Major organ damage and improvements in the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) were measured. Statistical analyses, including Cox and linear regressions, were conducted.

Results: A total of 78 patients with at least one lymphocytic focus were included in the study. The presence of higher T-cell counts in lymphocytic foci in MSGB was associated with severe disease flare, and a logarithmic transformation of T-cell count indicated increased risk (HR 1.96, 95% CI 0.91-4.21). Improvements in the ESSDAI were associated with higher total lymphocyte count and T- and B-cell numbers in the lymphoid composition of the lymphocytic foci. Seropositive patients exhibited higher T CD4+ cell numbers. Correlation analysis showed negative associations between age and lymphocytic foci and the T-cell count.

Positive correlations were observed between antinuclear antibody (ANA) titers and total lymphocyte numbers.

Discussion: Patients with a higher number of T cells in the lymphocytic infiltrates of lymphocytic foci may have a two-fold risk of severe disease flare. The number of B cells and T CD4+ cells in the lymphocytic infiltrates of lymphocytic foci showed a weak but positive relation with the ESSDAI improvement during follow-up. Age and seropositivity appeared to influence the lymphoid composition of the lymphocytic foci.

KEYWORDS

Sjögren's syndrome, immunofluorescence staining, fluorescent antibody technique, lip biopsy, minor salivary gland biopsy, histopathology, lymphocyte infiltration, lymphoid organization

1 Introduction

Sjögren's syndrome presents a diagnostic challenge due to the lack of a definitive gold standard test and the diverse range of clinical manifestations. The 2016 classification criteria for Sjögren's syndrome aims to facilitate earlier detection in young patients with systemic symptoms. This is crucial for identifying candidates for clinical trials and introducing new therapeutic options. The immunological profile now focuses solely on anti-Ro antibodies, excluding antinuclear antibodies, rheumatoid factor, and isolated anti-La due to concerns about specificity, deviating from previous criteria (1). The diagnosis relies on the minor salivary gland biopsy (MSGB) since 20-40% of the patients are Ro/La antibody negative (2-5). It has proved to be especially useful when seronegative patients present with high clinical suspicion (6, 7). The results of the MSGB in these patients show a distinct feature consisting of a focal lymphocytic sialadenitis (FLS) characterized by the presence of one or more inflammatory infiltrates of at least 50 cells present in 4mm² of gland surface (8, 9). Macrophages, dendritic cells, and natural killer cells are also present in a smaller proportion.

Traditionally, the focus score (FS), based on the quantification of lymphocytic foci in the MSGB (10), has been widely employed for diagnostic purposes, but it is frequently failed to be applied correctly in clinical settings due to miscalculation and difficulties in interpretation if other histopathologic alterations are present, and it fails to include important considerations such as the size of infiltrates in the foci (11, 12). It was also found that interobserver reliability was good, but there were disparities between assessment methods used by local pathologists (13). According to a retrospective study, the percentage of MSGBs with a focus score higher than one increased from 63% to 83% when using immunohistochemical staining, allowing the highlighting of small clusters of lymphocytes hardly visible when laying among the salivary ducts or acini (14).

At our institution, MSGB evaluations occur in the immunology department, involving a meticulous process overseen by two independent evaluators and an expert immunologist. Sequential biopsy sections are completely devastated for lymphocytic foci using immunofluorescence staining, with a comprehensive description of lymphoid composition. To ensure precision, only lymphocyte aggregates containing over 50 cells are considered foci, validating Sjögren's syndrome diagnoses and minimizing false positives. This criterion was met by all patients, each presenting at least one lymphocytic focus. Immunofluorescence staining, by enabling the visualization and quantification of specific cellular components and lymphoid composition within salivary gland tissue, may improve diagnostic accuracy and discrepancy between observers. It permits the detection of well-circumscribed mononuclear inflammatory cell infiltrates (>50 cells), exhibiting a tightly packed dark zone and a more loosely packed light zone within salivary gland epithelium. Lymphoid composition and lymphocytic foci are believed to hold prognostic value for disease evolution and severity, yet evidence remains inconclusive.

The objective of this study is to assess the risk between significant disease flare and the lymphoid composition of the lymphocytic foci in the MSGB among Sjögren's Syndrome patients over a 3-year follow-up period. The findings of this study will shed light on the potential value of immunofluorescence staining as a diagnostic technique with practical implications for clinical practice.

2 Materials and methods

2.1 Participants

This ambispective cohort study took place at the Systemic Autoimmune Disease Outpatient Clinic in the Rheumatology

Department of the Hospital Universitari de la Santa Creu i Sant Pau, Barcelona, a regional referral unit for rare diseases and a healthcare provider for 450,000 AIS Dreta district residents under universal health coverage.

Patients aged 18 or older were screened for Sjögren's syndrome diagnosis confirmation, primarily due to glandular or extraglandular organ symptoms with or without serological biomarkers. They underwent clinical assessments, including serological tests, by expert rheumatologists in the autoimmune disease clinic. Those with high clinical suspicion proceeded to routine clinical practice minor salivary gland biopsies (MSGBs) at the plastic surgery department.

Inclusion criteria required MSGBs with over 50 lymphocytes and compliance with the 2016 EULAR/ACR classification criteria, alongside clinical diagnosis by treating rheumatologists. Patients with inadequate biopsy samples were excluded. Recruitment spanned from September 1, 2017, to December 31, 2018, with data collection concluding in May 2022 after a 3-year follow-up period.

Patients were regularly monitored every 3–12 months based on disease severity, with data recorded until outcome onset, death, loss, or the observation period's end.

2.2 Variables

We collected demographic data and data on the presence of other systemic autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, idiopathic inflammatory myopathies, antiphospholipid syndrome, or undifferentiated connective tissue disease). At each visit, we recorded the ESSDAI, concurrent use of corticosteroids, conventional or biologic disease-modifying anti-rheumatic drugs (DMARDs), and serological variables, including complement levels, immunoglobulins (IgA, IgM, and IgG), antinuclear antibodies, anti-Ro/SSA, anti-La/SSB, and rheumatoid factor (RF). Electronic medical records from the hospital were used for data collection.

Variables related to the lymphoid composition of the MSGB were the number of lymphocytic foci, the number of total lymphocytes, T cells, B cells, CD4+ cells, CD8+ cells, and the presence of inflammatory infiltrate that does not form lymphocytic foci.

2.3 Outcome

The primary outcome of this study was to evaluate if the number of lymphocytic foci and the lymphoid composition of the MSGB are prognostic factors for severe disease flare requiring treatment or new organ involvement and ESSDAI changes until flare or end of follow-up.

The secondary outcome of this study was to evaluate if the number of lymphocytic foci and the lymphoid composition of the MSGB show an association with the clinical characteristics and severity of Sjögren's syndrome at diagnosis.

Severe disease flare was defined as new major organ damage, such as lymphoma, arthritis, nervous system, and renal or pulmonary affection, or a flare requiring moderate to high doses of steroids or

add-on DMARD. The ESSDAI score was evaluated for every visit until the end of follow-up or until severe disease flare. Moreover, meaningful improvement of the ESSDAI was collected if there was a decrease of 3 points from the baseline compared to the baseline visit (15, 16). We defined the study's outcomes to encompass different clinical scenarios: severe disease flare and a clinically meaningful ESSDAI improvement, which may include fluctuation without treatment.

2.4 MSGB and immunofluorescence staining

In our clinical practice, a plastic surgeon obtained four fragments of minor salivary glands, each with a diameter of 2 mm. The immunology department was responsible for the immunofluorescence staining of the glands. The entire minor salivary gland piece was sequentially sliced into 7-micron sections, and the biopsy was completely devastated. For every 10 sections, 1 was stained with methylene blue and observed under the microscope. In the presence of infiltrates, the slide was prepared for further staining with antibodies. If there was no infiltrate, the cutting process continued until an infiltrate was identified. For each sample, two slides from two different points of the infiltrate were processed. The numbers reported in the manuscript correspond to the largest infiltrate observed in the total of the three fragments. The cell numbers were manually counted in the largest infiltrate within one of the glands. If there were any variations or differences in the cell composition among the infiltrates, these were described independently.

Both cellular infiltrate and its lymphoid composition were assessed by immunofluorescence staining of sequential sections with monoclonal antibodies recognizing CD45, CD3, CD4, CD8, CD20, HLA-DR, kappa, and lambda immunoglobulin chains.

Firstly, MSGB was embedded in O.C.T. Compounds (Sakura Finetek USA), frozen in liquid nitrogen, and cross-sections of 7 µm thickness were obtained (Figure 1). MSGB sections were fixed with acetone (Panreac Applichem, Barcelona, Spain) and incubated with the following primary antibodies: anti-CD45 for leukocyte identification (clone 2B11 and PD7/26), anti-CD3 for T lymphocytes (clone F7.2.38), anti-CD4 for T-helper lymphocytes (clone 4B12), anti-CD8 for cytotoxic T lymphocytes (clone C8/144B), anti-CD20 for B lymphocytes (clone L2), anti-HLA-DR for detecting MHC class II expression (clone TAL.1B5), and polyclonal anti-kappa and anti-lambda light chains for detecting immunoglobulins (DAKO, Glostrup, Denmark). After washing with PBS (Immuno concepts, Sacramento, California), sections were incubated with polyclonal rabbit anti-mouse immunoglobulins/FITC (DAKO, Glostrup, Denmark). All sections were randomly analyzed by two expert observers and blinded to clinical and molecular data. Each sample was independently evaluated, and any discrepancies were resolved by agreement.

A biopsy was considered lymphocytic foci positive when displaying at least one focus of lymphocyte infiltration with more than 50 cells within normal minor salivary gland tissue. Cellular infiltration that did not meet this criterion was classified as negative.

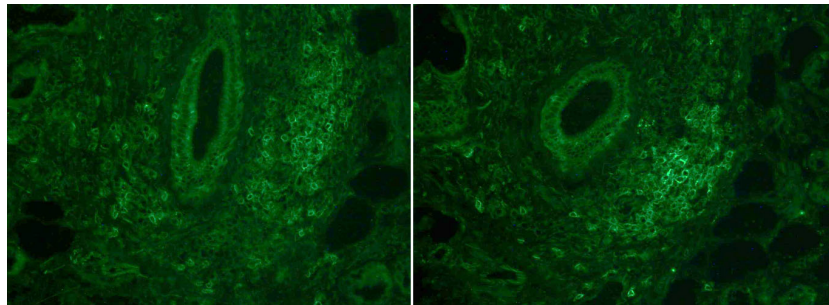


FIGURE 1
Immunofluorescence staining with anti-CD45 (left) and anti-CD20 (right).

A complete description of the characteristics of the lymphoid composition was performed.

2.5 Statistical analysis

We carried out a crude analysis using Student's *t*-test, the Fisher–Pitman test was used to compare quantitative variables, and the χ^2 test was employed to compare dichotomous variables according to outcomes of interest. Analysis was not carried out for variables with a small number of cases where assumptions were not met. The Shapiro–Wilk test and a box plot were used to check the distribution of the variables. For variables related to the MSGB findings, if log-normal distribution was observed, logarithmic transformation was carried out previously to apply regression or correlation tests.

For the primary outcome of the study, Cox regression was carried out to evaluate the risk of severe disease flare according to the number of lymphocytic foci and their lymphoid composition. Linear regression was used to evaluate the association between the ESSDAI changes and MSGB findings. Pearson and Kendall's Tau correlation were calculated to evaluate the strength of association.

For the secondary outcome of the study, linear regression was used to evaluate the association between the ESSDAI at diagnosis, clinical variables, and MSGB findings.

In the previous statistical plan established, the presence of other systemic autoimmune diseases, years since first symptoms, steroid treatment, DMARDs, sex, and age were selected as clinically relevant variables for adjustment if meaningful association was found from the crude analysis. A two-sided 95% confidence interval was calculated, and a *p*-value <0.05 was considered significant. Data analysis was performed using Stata 16.0.

3 Results

3.1 Baseline population characteristics

A total of 139 individuals met the eligibility criteria, of whom 81 fulfilled the inclusion criteria. Ultimately, 78 patients were included after excluding 3 individuals due to insufficient salivary gland tissue

samples. There was no loss during the 3-year follow-up period. The baseline characteristics and comparison of groups according to the main outcomes are shown in [Table 1](#). In all, 13 patients tested positive for Ro/La antibodies, with 3 showing positivity for all three antibodies (Ro52/Ro62/La). Additionally, 6 patients were positive for both Ro52 and Ro60, while 4 patients tested positive for either Ro52 or Ro60 alone. [Supplementary Figure s 1–9](#) summarizes the distribution of the cellular infiltration of the lymphocytic foci.

When comparing patients who experienced severe disease flare with those who maintained stable disease, a higher prevalence of male sex, history of lymphoma, and treatment with DMARDs and steroids was observed. When comparing patients who demonstrated significant ESSDAI improvement at the end of the 3-year follow-up period, a higher prevalence of steroid use at diagnosis and a higher baseline ESSDAI were observed, as one would expect.

Within our cohort, it is noteworthy that nearly 44.59% of patients demonstrated ANA titers lower than 1/160, and 87.38% of patients were negative for Ro and La antibodies. The lymphoid infiltration in the biopsy was similar between patients with low and high ANA titers and between Ro/La antibody-positive and -negative groups. No significant differences were observed when comparing the lymphoid composition of the biopsy between groups.

3.2 Association with disease severity at diagnosis and severe disease flare

Among patients who exhibited severe disease flare during the 3-year follow-up period, higher total lymphocyte counts, along with elevated T-cell, T CD4+, and T CD8+ cell counts, were observed in the lymphocytic foci when compared to patients with stable disease. No significant differences were noted in the lymphoid composition of the lymphocytic foci between patients who demonstrated ESSDAI improvement and those who did not. [Table 2](#) provides a summary of the key findings concerning the lymphoid composition of the lymphocytic foci of the MSGB, along with a comparison based on the main outcomes.

Cox regression was employed to examine the prognostic value of lymphoid composition of the lymphocytic foci and severe disease flare. Among the studied risk factors, only the number of T cells

TABLE 1 Baseline characteristics and comparison of main outcomes.

Variable	Progression (n=16)	Stable disease (n=62)	p	ESSDAI improvement (n=8)	No ESSDAI improvement (n=70)	p	Total (n=78)
Male sex, (n, %)	4 (25)	4 (6.45)	0.03	1 (12.5)	7 (10)	0.83	8 (10.26)
Age, mean (SD)	56.35 (14.60)	61.73 (12.05)	0.13	64.67 (5.89)	60.17 (13.21)	0.35	60.63 (12.70)
Years since disease symptoms, p50 (IQR)	3.5 (2)	4.5 (10)	0.30	3 (2.5)	4 (9)	0.12	4 (9)
Baseline ESSDAI, mean (SD)	3.81 (3.97)	1.06 (1.85)	0.01	6.75 (1.98)	1.04 (2.01)	0.00	1.63 (2.65)
Inactive to low disease, (n, %)	8 (50)	56 (90.32)		0	64 (91.43)		64 (82.05)
Moderate or higher activity, (n, %)	8 (50)	6 (9.68)		8 (100)	6 (8.57)		14 (17.95)
Follow-up ESSDAI, mean (SD)	3.5 (4.49)	0.53 (1.16)	0.00	0.88 (0.83)	1.17(2.66)	0.29	1.14 (2.54)
Other autoimmune disease, (n, %)	4 (25)	9 (14.52)	—	2 (50)	11 (15.71)	—	13 (16.67)
Lymphoma, (n, %)	3 (18.75)	2 (3.23)	—	0	5 (7.14)	—	5 (6.41)
ANA titers, (n, %)	5 (31.25)	14 (22.58)	0.52	3 (37.5)	16 (22.86)	0.87	19 (24.36)
<1/80	3 (18.75)	15 (24.19)		1 (12.5)	17 (24.29)		18 (23.08)
1/80	1 (6.25)	14 (22.58)		1 (12.5)	14 (20)		15 (19.23)
1/160	0	7 (11.29)		0	7 (10)		7 (8.97)
1/320	7 (43)	12 (19.36)		3 (37.5)	16 (22.86)		19 (24.36)
≥1/640							
Ro/La antibody, (n, %)	7 (43.75)	6 (9.68)	0.07	3 (37.5)	9 (12.86)	0.23	13 (16.67)
Rheumatoid Factor, (n, %)	4 (25)	10 (16.12)	0.50	2 (25)	12 (17.14)	0.66	14 (17.95)
Normal gammaglobulinemia, (n, %)	12 (75)	47 (75.81)	0.08	6 (75)	61 (87.14)	0.17	67 (85.90)
Hypergammaglobulinemia, (n, %)	0	4 (6.45)		0	4 (5.71)		4 (5.13)
Hypogammaglobulinemia, (n, %)	4 (25)	3 (4.84)		2 (25)	5 (7.14)		7 (8.97)
Hypocomplementemia, (n, %)	6 (37.5)	13 (20.97)	0.34	4 (50)	15 (21.43)	–	19 (23.36)
History of steroid treatment, (n, %)	2 (12.5)	9 (14.52)	–	1 (12.5)	10 (14.29)	–	11 (14.10)
History of DMARD use, (n, %)	2 (12.5)	2 (3.23)	–	0	4 (5.71)	–	4 (5.13)
Steroid treatment at diagnosis, (n, %)	6 (37.5)	0	–	2 (25)	4 (5.71)	–	6 (7.69)
DMARD use at diagnosis, (n, %)	6 (37.5)	2 (3.23)	–	1 (12.5)	7 (10)	–	8 (10.26)

Comparison was not carried out in a small number of cases.

exhibited statistical significance for severe disease flare, displaying a hazard ratio (HR) of 1.01 (95% CI 1.00-1.01); this effect, however, was clinically insignificant. Given the lognormal distribution of variables related to total cell counts (Figure 1), a logarithmic transformation was applied to these variables. The HR for the logarithmically transformed T-cell count was 1.96 (95% CI 0.91 – 4.21), indicating a moderate risk effect that approached statistical significance. No discernible prognostic correlation was identified for the count of T CD4+ cells, T CD8+ cells, or the total number of lymphocytes, consistent with the results of the initial analysis. Furthermore, no associations were evident in relation to the proportion of each cell subtype.

Linear regression was employed to analyze differences in ESSDAI scores between follow-up and baseline visits. A higher difference signifies an enhancement, while a lower difference indicates deterioration in the ESSDAI. Variations in ESSDAI scores showed positive associations with total lymphocyte count, T- and B-cell numbers, and the logarithm of T CD4+ cells (suggesting a potential exponential relationship for the latter) within the lymphocytic foci. Table 3 summarizes the results of the regression analysis for the main outcomes. Although the strength of

association was assessed through correlation analysis, no statistically or clinically significant relationships were observed.

3.3 Association with clinical and serological manifestations

The relationship between the clinical characteristics and lymphoid composition of the lymphocytic foci was explored using group comparisons, correlation analysis, and regression analysis. Seropositive patients displayed a higher number of T CD4+ cells, with a mean of 100.83 (SD 122.71), in contrast to seronegative patients with a mean of 43.49 (SD 36.81); this difference was statistically significant (p=0.04). Correlation analysis revealed that age exhibited a negative correlation with the number of lymphocytic foci with a tau-b score of -0.17 (p=0.05) and a negative correlation with the number of T cells with a tau-b score of -0.15 (p=0.05). Antinuclear antibody (ANA) titers demonstrated positive correlations with the total number of lymphocytes with a tau-b score of 0.1768 (p=0.04), a positive correlation with the number of T cells with a tau-b score of 0.22 (p=0.01), and a positive correlation

TABLE 2 Lymphoid composition of the lymphocytic foci of the MSGB and comparison of main outcomes.

Variable	Progression (n=16)	Stable disease (n=62)	p	ESSDAI improvement (n=8)	No ESSDAI improvement (n=70)	p	Total (n=78)
Number of lymphocytic foci, (n, %)	1.5	1	0.10	1	1	0.94	1.53 (0.95)
0	1 (6.25)	6 (9.68)		1 (12.5)	6 (8.57)		7 (8.97)
1	7 (43.75)	33 (53.23)		4 (50)	36 (51.43)		40 (51.28)
2	2 (12.5)	13 (20.97)		1 (12.5)	14 (20)		15 (19.23)
≥3	6 (37.5)	10 (16.13)		2 (25)	14 (20)		16 (20.51)
Total number of lymphocytes, mean (SD)	191.56 (256.17)	121.04 (112.52)	0.12	100.63 (101.26)	139.5 (158.68)	0.58	85 (120)
Number of T cells, mean (SD)	115 (157.05)	58.95 (38.77)	0.03	45.63 (40.57)	73.29 (83.80)	0.29	55 (55)
Number of T CD4 cells, mean (SD)	82.11 (103.20)	41.4 (25.14)	0.03	32.92 (25.16)	51.26 (54.78)	0.33	35 (36.67)
Number of T CD8 cells, mean (SD)	49.32 (67.12)	24.69 (17.67)	0.04	27.92 (21.79)	29.86 (35.88)	0.85	18.33 (16.25)
Number of B cells, mean (SD)	76.56 (132.71)	62.10 (84.06)	0.57	55 (76.25)	66.21 (72.25)	0.86	30 (90)
% T cells/Total lymphocytes, mean (SD)	69.48 (29.91)	63.40 (27.98)	0.46	0.53 (0.37)	0.66 (0.27)	0.26	0.65 (0.28)
% B cells/Total lymphocytes, mean (SD)	30.52 (29.91)	36.60 (27.98)	0.46	0.47 (0.37)	0.34 (0.27)	0.26	0.35 (0.28)
% T CD4 cells/Total T cells, mean (SD)	64.29 (11.98)	62.64 (15.29)	0.73	0.56 (0.18)	0.64 (0.14)	0.19	0.63 (0.15)
% T CD8 cells/Total T cells, mean (SD)	35.71 (12.0)	37.36 (15.29)	0.77	0.44 (0.18)	0.36 (0.14)	0.25	0.37 (0.15)
Presence of isolated infiltrate without lymphocytic foci, (n, %)*	3 (18.75)	11 (17.74)	—	1 (12.5)	13 (18.57)	—	14 (17.95)

Comparison was not carried out in a small number of cases (<5).

with the number of T CD8+ cells with a tau-b score of 0.19 (p=0.03). However, the linear regression analysis did not reveal any significant association between the clinical variables and lymphoid composition of the lymphocytic foci including serological variables, DMARD, or steroid treatment. No further multiple analysis was performed for this reason, but confounder analysis revealed that these variables were not found to be confounders or modifiers of effect.

4 Discussion

Our study found significant associations between clinical factors and severe disease flare, with male sex, lymphoma history, and DMARDs/steroid use being more common in progressing patients. Increased lymphocyte and T-cell counts within lymphocytic foci suggest immune cell involvement in exacerbations. The total T-cell count within lymphocytic foci was significantly associated with severe disease flare (HR 1.01) after logarithmic transformation. The subsequent Cox regression showed a substantial risk between T-cell count within lymphocytic foci and severe disease flare (HR 1.96, approaching significance).

Regarding ESSDAI score changes and lymphoid composition of lymphocytic foci, there was a positive link between ESSDAI improvement and T CD4+ and B-cell counts post-logarithmic transformation, with weak associations with T cells and overall lymphocytes within lymphocytic foci. No significant relationships

emerged with cell subtype proportion or cellular infiltrates lacking germinal centers.

Concerning the correlation between clinical and serological variables and the MSGB's lymphoid composition of lymphocytic foci, seropositive patients exhibited elevated T CD4+ cell counts. Age was inversely correlated with the number of lymphocytic foci and its T-cell counts. ANA titers positively correlated with total lymphocyte, T-cell, and T CD8+ cell counts. It is important to note that these correlations are not sufficient for making predictions or definitive conclusions. No significant relationships were observed with the duration of disease symptoms, disease biological activity, or steroid/DMARD treatment.

Our study population exhibited characteristics consistent with prior research, confirming a higher prevalence of male sex, lymphoma history, and the use of steroids or DMARDs in patients with poorer prognosis. Our study included incident cases of Sjögren's syndrome, accompanied by a low prevalence of treatment history that could potentially impact the results of the MSGB. Despite a relatively short median time from disease symptom onset, our study population encompassed patients ranging from less than 1 year to 30 years after initial manifestation. Severe disease flare was considered only if new major organ damage was observed or if moderate to high doses of steroid or DMARD treatment were required.

The focus score, based on the histopathologic criteria established by Greenspan et al. in 1974 (10), has been known to potentially overestimate cell infiltrate extent (11, 12) with poorer

TABLE 3 Evaluation of the lymphoid composition of the lymphocytic foci using Cox regression for severe disease flare and linear regression for ESSDAI differences.

Variable	HR (95% CI) for severe disease flare	p	β (95% CI) for ESSDAI difference from baseline	p
Number of lymphocytic foci, (n, %)	1.57 (0.92 - 2.69)	0.10	0.59 (-0.00 - 1.18)	0.05
0	1.22 (0.15 - 10.12)	0.89	-0.24 (-2.28 - 1.80)	0.82
1	1.02 (0.09 - 11.24)	0.99	0.31 (-1.97 - 2.59)	0.78
2	2.59 (0.29 - 23.32)	0.40	1.45 (-0.83 - 3.73)	0.21
≥3	14.20 (0.84 - 240.23)	0.07	0.71 (-4.6 - 6.04)	0.79
Total number of lymphocytes, mean (SD)	1.00 (1.00 - 1.01)	0.17	0.01 (0.00 - 0.01)	0.00
Log total lymphocytes, mean (SD)	1.13 (0.61 - 2.08)	0.70	0.52 (-0.14 - 1.18)	0.12
Number of T cells, mean (SD)	1.01 (1.00 - 1.01)	0.01	0.01 (0.00 - 0.15)	0.03
Log T cells, mean (SD)	1.96 (0.91 - 4.21)	0.09	0.70 (-0.11 - 1.51)	0.09
Number of T CD4 cells, mean (SD)	0.87 (0.53 - 1.43)	0.58	0.26 (-0.30 - 0.82)	0.35
Log T CD4+ cells, mean (SD)	1.79 (0.82 - 3.89)	0.14	0.91 (0.09 - 1.73)	0.03
Number of T CD8 cells, mean (SD)	0.74 (0.30 - 1.84)	0.50	-0.06 (-0.96 - 0.83)	0.89
Log T CD8+ cells, mean (SD)	1.68 (0.82 - 3.42)	0.16	0.61 (-0.20 - 1.42)	0.14
Number of B cells, mean (SD)	1.00 (1.0 - 1.01)	0.80	0.01 (0.00 - 0.015)	0.00
Log B cells, mean (SD)	0.71 (0.39 - 1.31)	0.27	0.71 (0.07 - 1.34)	0.03
% T cells/Total lymphocytes, mean (SD)	2.51 (0.33 - 18.91)	0.37	0.04 (-2.09 - 2.16)	0.97
% B cells/Total lymphocytes, mean (SD)	0.40 (0.53 - 2.99)	0.37	-0.04 (-2.16 - 2.09)	0.97
% T CD4 cells/Total T cells, mean (SD)	1.01 (0.20 - 49.98)	1.00	1.31 (-2.65 - 5.27)	0.51
% T CD8 cells/Total T cells, mean (SD)	0.99 (0.02 - 49.38)	1.00	-1.31 (-5.27 - 2.65)	0.51
Presence of isolated infiltrate without lymphocytic foci, (n, %) ⁺	1.42 (0.39 - 5.11)	0.61	0.07 (-1.42 - 1.56)	0.92

interobserver reliability than GC-like structure assessment (13). In routine hematoxylin and eosin (H&E) staining, most leukocytes generally appear quite similar in terms of their staining characteristics. While this staining method is excellent for visualizing tissue architecture, it may be challenging to distinguish lymphocytes from other leukocyte subtypes. Immunofluorescence staining, allowing for the visualization and quantification of specific cellular components and lymphoid composition within salivary gland tissue, has the potential to significantly improve diagnostic accuracy while reducing interobserver discrepancies. As demonstrated in a systematic review, the percentage of MSGBs with a focus score exceeding 1 increased from 63% to 83% when immunohistochemical staining was used to highlight small clusters of lymphocytes that might otherwise go unnoticed when intermingled with salivary ducts or acini (14).

Thus, the use of immunofluorescence to evaluate cell subtypes aimed to enhance diagnostic accuracy. This approach elucidates why our study population included patients with infiltrates exhibiting a high lymphocyte count, accompanied by the presence of lymphocytic foci in all enrolled patients.

At our institution, MSGB evaluation is conducted within the immunology department as part of standard clinical practice. This evaluation process is meticulous and involves two independent evaluators supervised by an expert immunologist. Sequential sections of the biopsy sample are completely devastated, encompassing the

identification of lymphocytic foci and a detailed description of lymphoid composition. To ensure the highest accuracy, only lymphocyte aggregates containing over 50 cells are considered as foci indicative of Sjögren’s syndrome to minimize the risk of false positive diagnoses. In our study, a unique characteristic of the enrolled patient population was the presence of at least one lymphocytic focus in all patients meeting this criterion. Biopsies with cellular infiltrates lacking lymphocytic foci composed of fewer than 50 lymphocytes were considered inconclusive for Sjögren’s syndrome diagnosis. The lymphocytic foci described in our study resemble the germinal center (GC)-like structures defined by Costa et al. (17). However, further investigations by Carubbi et al. (18), Haacke et al. (19), and Kroese et al. (11) emphasize the importance of additional staining techniques, including CD21, CD3, CD20, and BCL-6, for a more precise characterization of germinal centers. Immunofluorescence serves to distinguish these lymphocytic foci that resemble GC-like structures from the diffuse lymphocytic infiltration of the gland. Interestingly, according to a systematic review, previous studies evaluating the MSGB had reported a low prevalence of germinal center-like structures, ranging from 18% to 59% (20).

Our study did not reveal any prognostic or clinical associations pertaining to the number of lymphocytic foci over the 3-year follow-up period. The infrequent instances of lymphoma cases in our study can be attributed to the relatively brief follow-up period, which was deliberately chosen to address the primary aim of evaluating the

practical utility of the MSGB findings for short-term decision-making. Nonetheless, the prognostic value of the MSGB concerning its response to lymphoma treatment remains inconclusive (17). Our results show that the higher number of T cells in the lymphocytic infiltrates of lymphocytic foci may have an exponential relationship with clinically relevant severe disease flare in 3 years. Possibly, patients with a higher number of T cells in the lymphocytic infiltrates of lymphocytic foci may have two-fold risk of severe disease flare and are correlated with younger age. Moreover, in our population, the number of B cells and T CD4+ cells in the lymphocytic infiltrates of lymphocytic foci showed a weak but positive relation with ESSDAI improvement during follow-up. This result is different from some previous research where B-cell predominance was observed for longer disease evolution, extraglandular manifestations, and lymphoma (17, 21). The results also differ from studies that conclude that the number of B cells and T cells was not related to any clinical variables of severity, as in Carubbi et al. and Christoudoulou et al. (18, 22).

The natural history of minor salivary gland alterations in SS patients remains unclear. Until now, the FS and fibrosis were believed to increase over time and with severe disease flare (23, 24). However, in a recent study of serial MSGBs, neither the infiltration grade nor the FS values changed significantly, although significant severe disease flare occurred (24). Neither did significant changes develop for lymphocytic foci, fibrosis, or adiposis. This would indicate that SS lesions are slowly progressing alterations.

This study's notable strength lies in its pioneering approach, being the first to examine the prognostic significance of the lymphoid composition within the lymphocytic foci of the MSGB in a population where all individuals exhibit lymphocytic foci. Additionally, the study's robustness stems from its deliberate selection of severe disease flare as the primary outcome, driven by its clinical utility and relevance. The relatively brief follow-up duration was strategically chosen to capture the biopsy's potential for guiding short-term decision-making, further enhancing the study's practical applicability.

The primary limitation of this study was the sample size. It was conducted as a pilot study with consecutive patient inclusion over a 1-year recruitment period. As a result, it was not feasible to incorporate some statistical analysis due to the limited number of cases as indicated in the methods. Moreover, only univariate regression was possible. These limitations were addressed by employing non-parametric tests, which offer a robust method for comparing distributions without necessitating specific distributional assumptions. We acknowledge another limitation stemming from its monocentric nature. Our institution, a prominent referral tertiary hospital catering to a population of 450,000 residents, serves as an expertise unit and regional referral center in Catalonia. Despite this specialized role, the single-center design of our study might raise questions about the broader applicability of our findings.

However, it is important to note that this limitation is mitigated by several factors. Firstly, our hospital's role as a comprehensive referral center ensures that a diverse range of patients seeking evaluation for Sjögren's syndrome, and subsequently undergoing MSGB, were included in our study. This, in turn, contributes to the representation of a wider spectrum of cases. Moreover, the standardized protocol followed for MSGB examination across all

patients ensures consistency in the procedure, minimizing variability in this critical aspect of our study.

In light of these considerations, while the monocentric nature of our study could potentially influence the external generalizability of our findings, the comprehensive scope of our institution's services and the standardized procedures employed help mitigate this limitation to some extent.

In conclusion, our study has revealed increased counts of lymphocytes, T cells, T CD4+, and T CD8+ cells in the lymphocytic foci of the MSGB in patients with progressing disease, suggesting an active role for immune cells in exacerbations. Particularly, the total T-cell count exhibited a significant association with severe disease flare, indicating its potential as a prognostic marker. Our subsequent Cox regression analysis unveiled a substantial risk between T-cell count and severe disease flare with a HR of 1.96, though statistical power limitations might have hindered reaching full significance.

Analyzing the relationship between the ESSDAI score changes and lymphoid composition of the lymphocytic foci, our linear regression uncovered a positive link between ESSDAI improvement and post-logarithmic transformation T CD4+ and B-cell counts. While similar associations were found with T cells and overall lymphocytes, their coefficients were indicative of weak connections. Importantly, no significant relationships emerged regarding cell subtype proportions or the presence of isolated lymphocytic infiltrate without foci.

Future research endeavors involving multiple centers and bigger sample sizes could offer a broader perspective and further validate the implications of our study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by IIB Research Institute Sant Pau. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from a by-product of routine care or industry. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

H-SP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. LM-M: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. BM:

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Deep analysis of skin molecular heterogeneities and their significance on the precise treatment of patients with psoriasis

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Background: Psoriasis is a highly heterogeneous autoimmune inflammatory disease. At present, heterogeneity in disease has not been adequately translated into concrete treatment options. Our aim was to develop and verify a new stratification scheme that identifies the heterogeneity of psoriasis by the integration of large-scale transcriptomic profiles, thereby identifying patient subtypes and providing personalized treatment options whenever possible.

Methods: We performed functional enrichment and network analysis of upregulated differentially expressed genes using microarray datasets of lesional and non-lesional skin samples from 250 psoriatic patients. Unsupervised clustering methods were used to identify the skin subtypes. Finally, an Xgboost classifier was utilized to predict the effects of methotrexate and commonly prescribed biologics on skin subtypes.

Results: Based on the 163 upregulated differentially expressed genes, psoriasis patients were categorized into three subtypes (subtypes A–C). Immune cells and proinflammatory-related pathways were markedly activated in subtype A, named immune activation. Contrastingly, subtype C, named stroma proliferation, was enriched in integrated stroma cells and tissue proliferation-related signaling pathways. Subtype B was modestly activated in all the signaling pathways. Notably, subtypes A and B presented good responses to methotrexate and interleukin-12/23 inhibitors (ustekinumab) but inadequate responses to tumor necrosis factor- α inhibitors and interleukin-17A receptor inhibitors. Contrastly, subtype C exhibited excellent responses to tumor necrosis factor- α inhibitors (etanercept) and interleukin-17A receptor inhibitors (brodalumab) but not methotrexate and interleukin-12/23 inhibitors.

Conclusions: Psoriasis patients can be assorted into three subtypes with different molecular and cellular characteristics based on the heterogeneity of the skin's immune cells and the stroma, determining the clinical responses of conventional therapies.

KEYWORDS

gene expression profiling, machine learning, psoriasis, stratification, unsupervised clustering

1 Introduction

Psoriasis is a common chronic auto-inflammatory skin disease characterized by obviously erythematous and scaly skin lesions accompanied by prominent skin and joint manifestations (1, 2). Chronic plaque or psoriasis vulgaris is the most common form of psoriasis, where lesions arise from hyperproliferation and disrupted differentiation of epidermal keratinocytes provoked by innate and adaptive immune system dysfunction (3, 4). The American Academy of Dermatology proposes biologics as a first-line treatment option for moderate to severe plaque psoriasis owing to their superior efficacy and acceptable safety profile compared to other treatment options. Specifically, the most commonly prescribed biologics are inhibitors to tumor necrosis factor- α (TNF- α ; including etanercept and adalimumab) and cytokine-targeting treatments such as the p40 subunit of interleukin (IL)-12/23 (ustekinumab) and IL-17 (brodalumab) (5). The systemic anti-inflammatory response caused by most of these biologics in treating psoriasis decreases the severity of comorbidities. Nevertheless, a considerable number of patients with psoriasis still present an inadequate response to these therapies (6–8), which may result from the heterogeneous pathophysiological background of psoriasis.

Gene expression profiling of psoriasis skin tissues has been used to provide pathophysiological insights for explaining the variations in treatment outcomes. Ainali et al. have revealed two distinct molecular subgroups from a single chronic plaque psoriasis skin transcriptomic profile and noted that one of these subgroups enriched in the transforming growth factor- β and ErbB signaling pathways might be more amenable to biologics therapies (9). Wang et al. have delineated two distinct immune phenotypes and constructed a psoriatic microenvironment score from a meta-analysis of psoriasis skin transcriptomic profiles (10). There is a correlation between improved clinical outcomes and overexpression of genes responsible for keratinocyte differentiation in the nonlesional

phenotype. Some characteristics are common among the various subtypes, but their conclusions are inconsistent. Consequently, gaining more profound and comprehensive mechanistic insights into divergent and shared features of the psoriasis skin subtypes is necessary for determining the pathobiological approaches for treatment-resistant patients with psoriasis.

In this study, we aimed to elucidate the cellular, molecular, and clinical features of three newly identified psoriasis skin subtypes using an unsupervised machine learning method performing a comprehensive meta-analysis of publicly available microarray datasets published thus far. Finally, we sought to apply a machine-learning model to identify the psoriasis skin subtypes for evaluating the therapeutic efficacy of biologics.

2 Methods

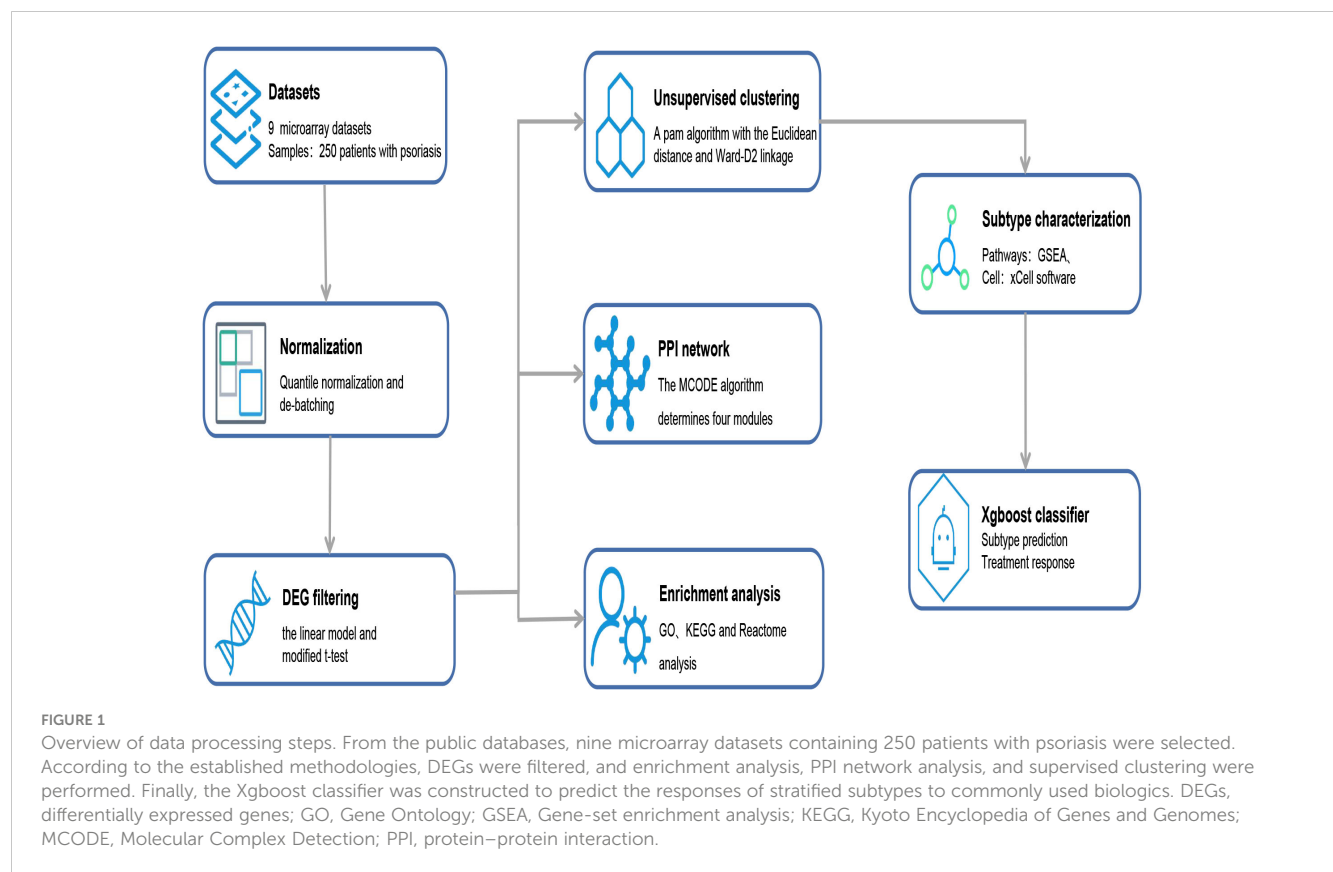
2.1 Systematic search, data selection, and preprocessing

The study design is outlined in Figure 1. We retrieved the microarray gene expression datasets of psoriasis skin datasets from the Gene Expression Omnibus database. Nine microarray datasets (GSE30999 (11), GSE13355 (12), GSE14905 (13), GSE41662 (14), GSE53552 (15), GSE34248 (14), GSE67853 (16), GSE47751 (17), and GSE50790 (18)) of eligible psoriasis skin tissues were collected for this study (Supplementary Table 1). The independent cohorts for treatment include 66, 85, 73 and 15 psoriasis lesions that were treated with etanercept (14, 19), ustekinumab (19, 20), brodalumab (20), and methotrexate (21), respectively, to which therapeutic responses were measured using the Psoriasis Area and Severity Index (PASI) score endpoint after the initiation of therapy. Here, patients were considered responders when there was an improvement of at least 75% on the PASI score from baseline and were considered non-responders otherwise.

Using the affy R package, microarray raw data from Affymetrix was processed by employing the robust multi-array average algorithm for background correction, quantile normalization, and probe-set summarization. Normalized matrix files are downloaded directly from Illumina into raw microarray data.

Residual technical batch effects from different data sets were corrected using the 'ComBat' function in the sva R package to

Abbreviations: DEGs, differentially expressed genes; MTX, methotrexate; PASI, Psoriasis Area and Severity Index; PCA, Principal components analysis; PPI, protein–protein interaction; MCODE, Molecular Complex Detection; GSEA, Gene-set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; CDF, cumulative distribution function; IFN, type I interferon; IL, interleukin.



reduce the systematic, dataset-specific bias (22). Quality assurance and distribution bias were assessed using the principal component analysis (PCA) of the same datasets before and after normalization and batch correction (Supplementary Image 1).

2.2 Filtering of differentially expressed genes and functional comments

The limma R package performed gene identification by differential expression analysis of microarray data from lesional and non-lesional psoriasis skin tissues using a linear model and modified t-test (23). To control for the proportion of false positive findings, we adjusted P-values using the Benjamini-Hohberg correction (24), and adjusted P-values <0.05 and absolute fold change values ≥ 1.5 were considered statistically significant. Then, the functional enrichment analysis of the upregulated DEGs lists in psoriatic lesions skin was performed via the Metascape online website. Adjusted $p < 0.05$ was deemed to be significant for enriched functional pathways (25).

2.3 Construction of protein-protein interaction network and identification of the important modules

To visualize the interconnectedness of DEGs in the psoriasis skin samples, we constructed a protein-protein interaction (PPI)

network based on the STRING and BioGrid databases and Cytoscape software (26, 27). The network is described by proteins (i.e., nodes) and their relationships (physical or functional interactions) (i.e., edges). Four significant modules were identified during the analysis using the Molecular Complex Detection (MCODE) algorithm (28). The biological functions of important module genes were identified by enrichment analysis. Based on the P value, the three highest-scoring terms were retained to describe the function of the corresponding module.

2.4 Gene-set enrichment analysis

Gene-set enrichment analysis (GSEA) ranked the list of upregulated DEGs in psoriasis-lesioned skin using a predefined gene set database to identify potential biologically critical pathways related to psoriasis (29, 30). Information on gene sets for signaling pathways or biological processes was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome databases. Terms with a false discovery rate below 0.25 were considered significant.

2.5 Unsupervised clustering for psoriasis skin gene expression profiles

To classify patients with psoriasis into subtypes based on the skin transcriptomic signatures, we performed hierarchical

agglomerative clustering using the ConsensusClusterPlus R package (31). The procedure was repeated for 1000 iterations to ensure the stability of the subtype stratification. The clustering approach adopted a Partitioning Around Medoids algorithm with the Euclidean distance and Ward-D2 linkage, and the optimal and stable number of clusters was selected by the cumulative distribution function (CDF). We then used the PCA to confirm the results of unsupervised clustering. DEGs were differentially expressed in the stroma- and immune-enriched subtypes compared to those differentially expressed in the other subtypes. An adjusted P-value <0.05 and absolute fold-change ≥ 1.5 were used to identify enriched pathways in the Gene Ontology Biological Process (GO-BP) and Reactome databases.

2.6 Inference of cell types and pathways activated in psoriasis skin subtypes

To explore the specific biological pathways associated with the subtypes, we used single-sample GSEA to condense information from gene expression profiles into pathway or marker gene sets (32). Here, the enrichment score is defined as the absolute enrichment of the gene set in each sample of a given dataset. Psoriasis skin-related pathways were collated from the published literature and GSEA results, with gene sets from KEGG and response group databases. We used the “xCell” algorithm to calculate the enrichment scores for immune and stroma cell-type signatures for elucidating the cellular composition of the three subtypes (33). Enrichment scores representing given cell types and pathway activities were compared among the three subgroups using the Wilcoxon and Kruskal-Wallis test.

2.7 Construction of multi-classification model for psoriasis skin subtype prediction

The Xgboost [extreme gradient boosting] (version 1.5.0.2) is a machine learning method that assembles weak prediction models to build more robust prediction models. We made a prediction model based on 163 gene features using the Xgboost-tree method with softmax objective function. The predictive power of the system was then measured using the average area under the receiver operating characteristic curve (AUC). For training the classifier, 250 psoriasis skin samples were divided into training ($n=176$) and testing ($n=74$) sets in 70% and 30% proportions, respectively, which used the caret R package. We controlled overfitting in the model using 10-fold cross-validation and used the fitted model to select subtypes for the new samples. Finally, we applied the 163-gene classifier for categorizing the samples into subtypes.

2.8 Statistical analysis

All statistical analyses were performed using the R software (version 4.0.3). We used the Kruskal-Wallis test to compare differences among two or more samples and the Wilcoxon test to

compare two samples. The chi-square or Fisher's test was applied to analyze the relationship between the psoriasis skin subtypes and clinical measures. Statistical significance was set at $P<0.05$ (two-tailed test).

3 Results

3.1 DEGs, PPI network, and enriched signaling pathways

On comparing the gene expression profiles of lesional and non-lesional skin samples from 250 patients with psoriasis, 163 upregulated DEGs were obtained (Figures 2A, B). Gene ontology [GO] annotation demonstrated that these upregulated DEGs in lesional skin were appreciably enriched in biological processes (BP) such as keratinization; inflammatory response; response to viruses, bacteria, and fungi; and type I interferon (IFN) production (Figure 2C). The KEGG and Reactome analysis indicated that the upregulated DEGs primarily enriched the IL-17 signaling, p53 signaling, neutrophil degranulation, and IFN- α/β signaling pathways (Figures 2D, E).

In total, 188 interactions among 82 DEGs were identified in the PPI networks constructed using these DEGs, and 81 genes were isolated without any direct relation to each other. The MCODE analysis identified 29 hub genes and clustered them into four highly correlated protein clusters (Figure 2F). Enrichment analysis was performed separately for each module, and a functional descriptor was chosen for each module based on the three terms that scored best based on the P-value. In line with our expectations, combining the result of GSEA, IFN-related (IFN- $\alpha/\beta/\gamma$ signaling and response to viruses) genes, neutrophil-related (neutrophil degranulation and response to bacteria and fungi) genes, toll-like receptor (TLR), NOD-like receptor (NLR), RIG-I-like receptor (RLR) signaling pathways, and keratinization were significantly enriched in lesional skin, adequately explaining the molecular mechanism of psoriasis skin.

3.2 Identification of skin gene expression-driven subtypes

Using the “ConsensusClusterPlus” R package of 1,000 iterations, we evaluated the number of clusters from $k = 2$ to $k = 6$. The CDF value and the delta area were used to measure the robustness of clustering results. The results show that when $K=3$, the consensus matrix (Figure 3A), consensus CDF plot (Figure 3B), and delta area plot (Figure 3C) all showed stable results, and the clustering consistency scores of each subgroup exceeded 0.8 (Figure 3D). Psoriasis skin subtype segregation patterns were revealed using the PCA (Figure 3E). The heatmap plot showed upregulated DEG in the three subtypes, showing the gene-level variability of the three subtypes (Figure 3F).

We compared each cluster with the others (such as subtype A vs subtype B and subtype C) to determine the specific upregulation of DEG signatures in each psoriasis skin subtype and to evaluate the

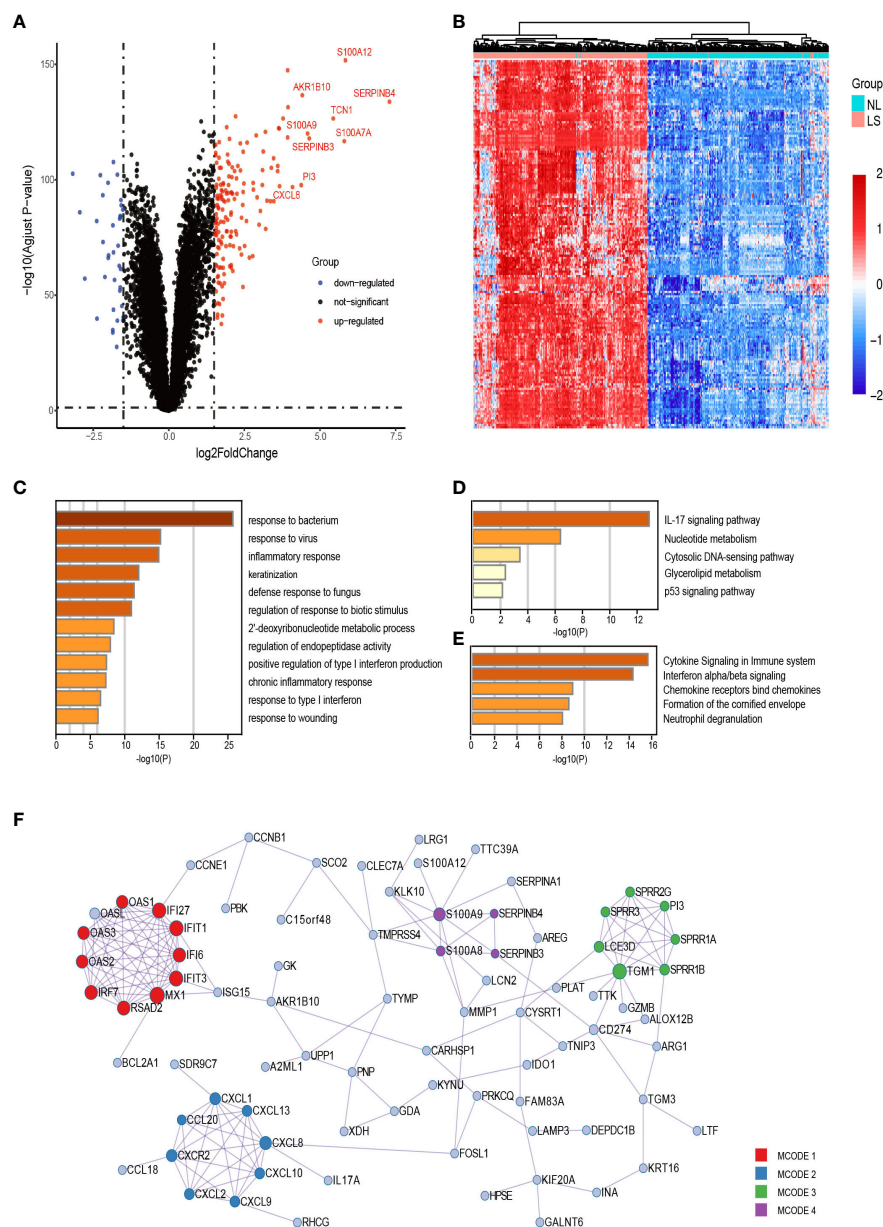


FIGURE 2

Upregulated DEGs analysis between lesional and nonlesional skin samples of patients with psoriasis. (A, B) Volcano plot and heatmap of all the DEGs. (C) Functional enrichment analysis of upregulated DEGs. The top 20 most significant biological processes in the GO-BP database. (D, E) Pathway enrichment analysis of upregulated DEGs. Top 5 most considerable signaling pathways in the KEGG and Reactome databases. (F) Protein-protein network of upregulated DEGs. The nodes and edges of the network represent genes and the functional or physical relationships between them, respectively. Four modules were found to be significant using the MCODE algorithm. GO-BP, Gene Ontology Biological Process.

molecular signatures and biological processes of the corresponding subtype. Here, 144 DEGs were significantly upregulated in subtype A, only 1 in subtype B, and 305 in subtype C using the same filtering threshold (Figure 4A). Thereafter, the Metascape was used to enrich the most obviously dysregulated biological processes and signaling pathways of each subtype from the Gene Ontology Biological Process (GO-BP), KEGG, and Reactome databases. To be specific, there was a significant activation of canonical immune pathways in subtype A, such as neutrophil-related pathways (including neutrophil degranulation and response to bacteria or fungi) and

IFN-related pathways (including response to IFN- $\alpha/\beta/\gamma$ and response to viruses) (Figures 4B–D). There was an enrichment of stroma proliferating pathways in subtype C, including cornified envelope formation, peroxisome proliferator-activated receptor (PPAR) signaling pathway, and Wnt signaling pathway (Figures 4E–G). Additionally, the mixed subtype (subtype B) shared features with both the immune-activating and stroma-proliferating subtypes, whereas upregulated DEGs of these subtypes exhibited no overlap. As it was a blend of the two subtypes, very few genes were unique to the mixed subtype.

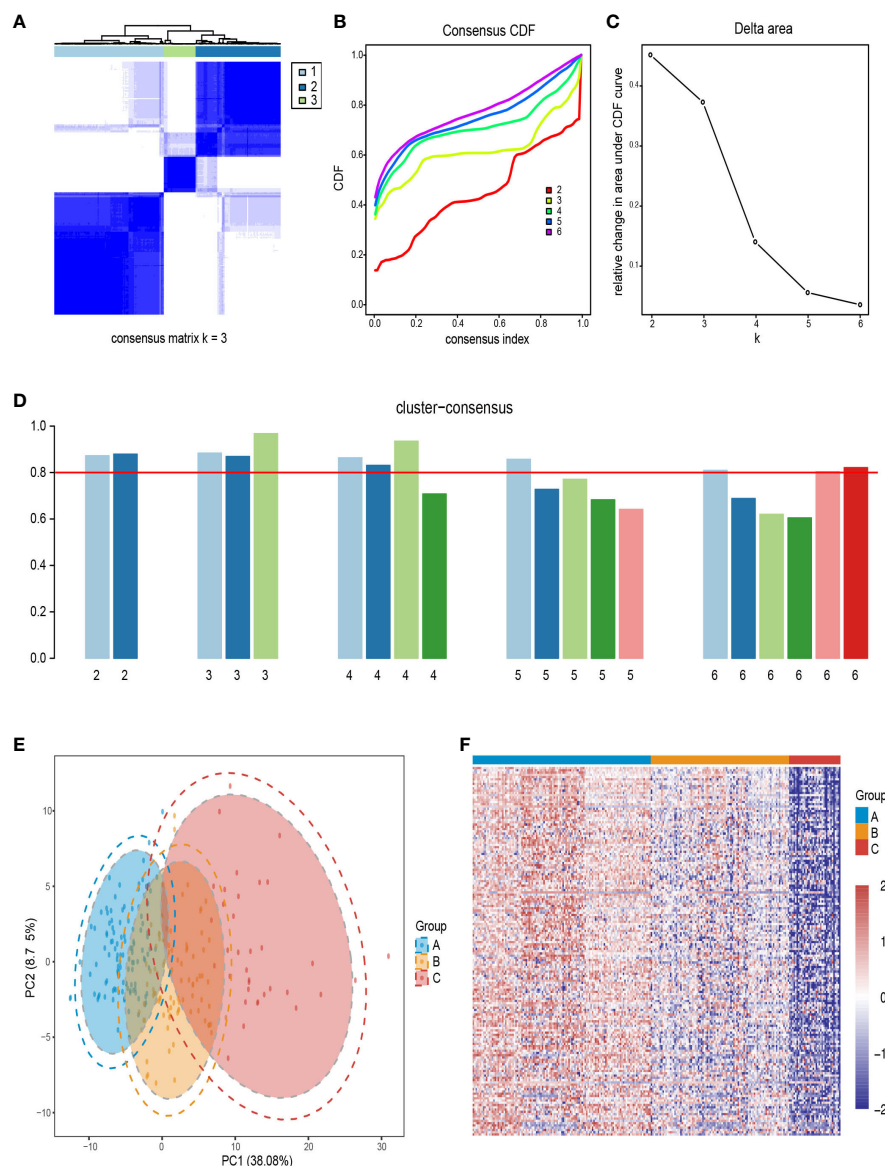


FIGURE 3

Identification of psoriasis skin subtypes based on upregulated DEGs. (A) A recorded consensus matrix at $k=3$ for the psoriasis skin compendium. The values of the consistency matrix from white to dark blue are from 0 to 1. (B) Consensus clustering for the CDF for $k=2-6$. (C) Relative change in area under CDF curve for $k=2-6$. (D) The cluster consistency score for each subgroup of $k=2-6$, and the red horizontal line indicates a cluster consistency score of 0.8. (E) PCA for the DEG expression profiles shows the stability and reliability of the classification. (F) A heatmap of 250 patients with psoriasis with a red gradient illustrating the gene expression distribution for three psoriasis skin subtypes. In each column, patients are grouped based on cluster assignment. Red represents overexpression, while blue represents under-expression. CDF, cumulative distribution function; PCA, Principal components analysis.

3.3 Molecular and cellular characterization of the three skin subtypes

There were three clustered subtypes: A ($n=121$), B ($n=94$), and C ($n=35$). To validate whether immune and stromal characteristics differ among these subtypes, we compared enrichment scores of important psoriasis-associated pathways and cell subsets. Twelve psoriasis-associated pathways or processes were curated from the literature and KEGG and Reactome databases. There were significant differences among these subtypes in enrichment scores for these signaling pathways associated with psoriasis. In subtype A, NLR,

TLR, RLR, p53, IFN- α/β , IFN- γ , IL-17, IL-23, T-cell receptor, and B-cell receptor signaling were strongly activated, indicating immune activation. Subtype C was characterized by stroma proliferation and enrichment in the Wnt and PPAR signaling pathways, whereas all signaling pathways are moderately activated in subtype B (Figure 5A).

The xCell software was used to identify cell types leading to gene expression discrepancy among subtypes, and a machine-learning framework was built to estimate cell type enrichment. All three subgroups showed differential activation of immune and stromal cells, consistent with these expression patterns. Immune cells [including neutrophils, basophils, macrophages, plasmacytoid

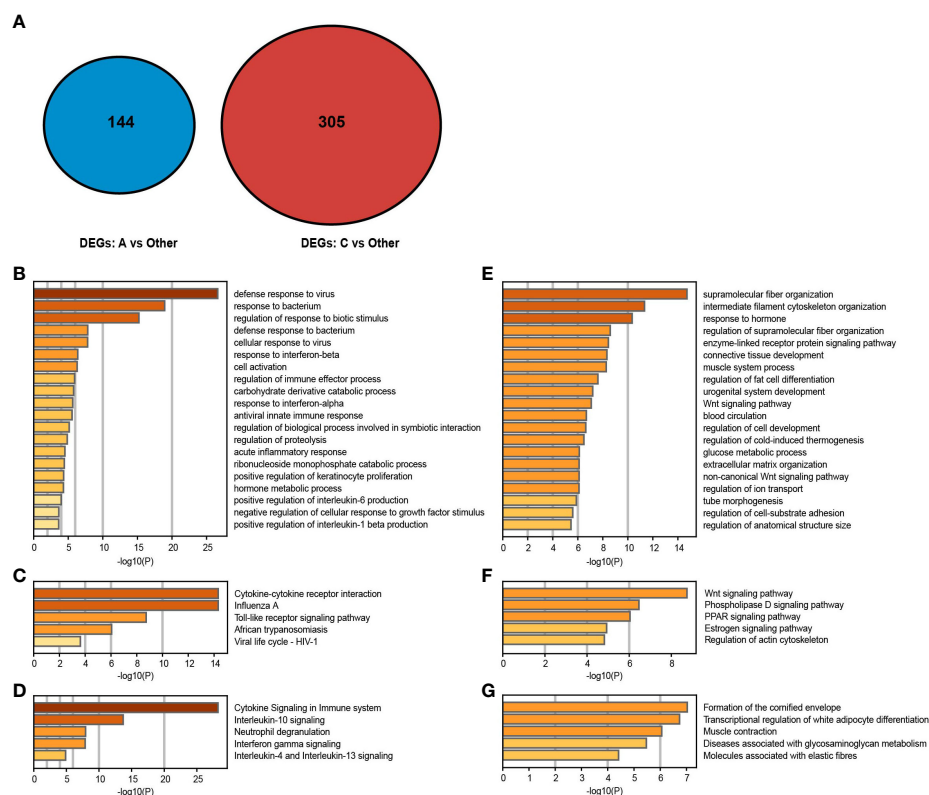


FIGURE 4

Gene expression patterns of psoriasis skin subtypes. (A, B) Molecular pattern distribution of subtypes A and C concerning different biological processes. The top 20 most significant biological processes in the GO-BP database. (C–F) Molecular pattern distribution of subtypes (A, C) concerning different pathways. Top 5 most considerable signaling pathways in the KEGG and Reactome databases. (G) A Venn diagram shows upregulated DEGs in subtypes (A, C).

dendritic cells (pDCs), and monocytes] and stroma cells (including epithelial cells, keratinocytes, and sebocytes) were more activated in subtype A than in subtypes B and C, whereas mast cell activation declined sharply. Moreover, fibroblasts, adipocytes, and endothelial cells were significantly infiltrated in subtype C (Figure 5B). An analysis of functional pathways in subtypes of psoriasis confirmed these results.

3.4 Prediction of the treatment responses of the subgroups

An Xgboost machine learning algorithm developed a 163-gene classifier to verify the psoriasis subtyping scheme. After training the classifier with 176 psoriasis samples, we applied it to the testing set containing 74 psoriasis samples to confirm its practicality. We observed that the AUC of the training set is 0.932, which proves that the model effectively classifies it. Furthermore, the classifier demonstrated robustness by achieving an average classification performance of 0.905 in the testing set. As a consequence, the classifier was highly effective and applicable in assessing psoriasis skin subtypes.

To determine whether the therapeutic response to etanercept, brodalumab, methotrexate (MTX) and ustekinumab were associated with subtypes of psoriasis, we classified patients at baseline using a fitted Xgboost classifier in four datasets. In the etanercept and brodalumab treated groups, the proportion of good responders was

significantly higher in subtype C [9/10 (90.0%) and 10/10 (100.0%)] than in subtypes A [25/39 (64.1%) and 26/33 (78.8%)] and B [11/17 (64.7%) and 25/30 (83.3%); Figures 6A, B]. In contrast, an opposite trend was observed in the MTX-treated group; the proportion of good responders was higher in subtypes A [3/8 (37.5%)] and B [1/3 (33.3%)] than in subtype C [0/4 (0.0%); Figure 6C]. An excellent response to ustekinumab was more frequently observed in subtypes A [32/43 (74.4%)] and B [26/31 (83.9%)] than in subtype C [6/11 (54.5%); Figure 6D]. In spite of this, the differences were not statistically significant, likely due to the limited sample size. In our analysis, subtypes A and B exhibited good responses to MTX and IL-12/23 inhibitors (i.e., ustekinumab) and inadequate responses to TNF- α inhibitors (etanercept) and IL-17A receptor (IL-17RA) inhibitors (i.e., brodalumab) compared with subtype C. Collectively, psoriasis molecular subtyping could have an impact on the benefits of drug treatment. Future clinical studies should integrate this information.

4 Discussion

Studying the molecular features of skin stratification in psoriasis has improved our understanding of its biological complexity and clinical heterogeneity and promoted research on psoriasis. Although previous studies have emphasized that stromal-cell-rich subtypes may respond well to existing biological therapies (9, 10), adequate

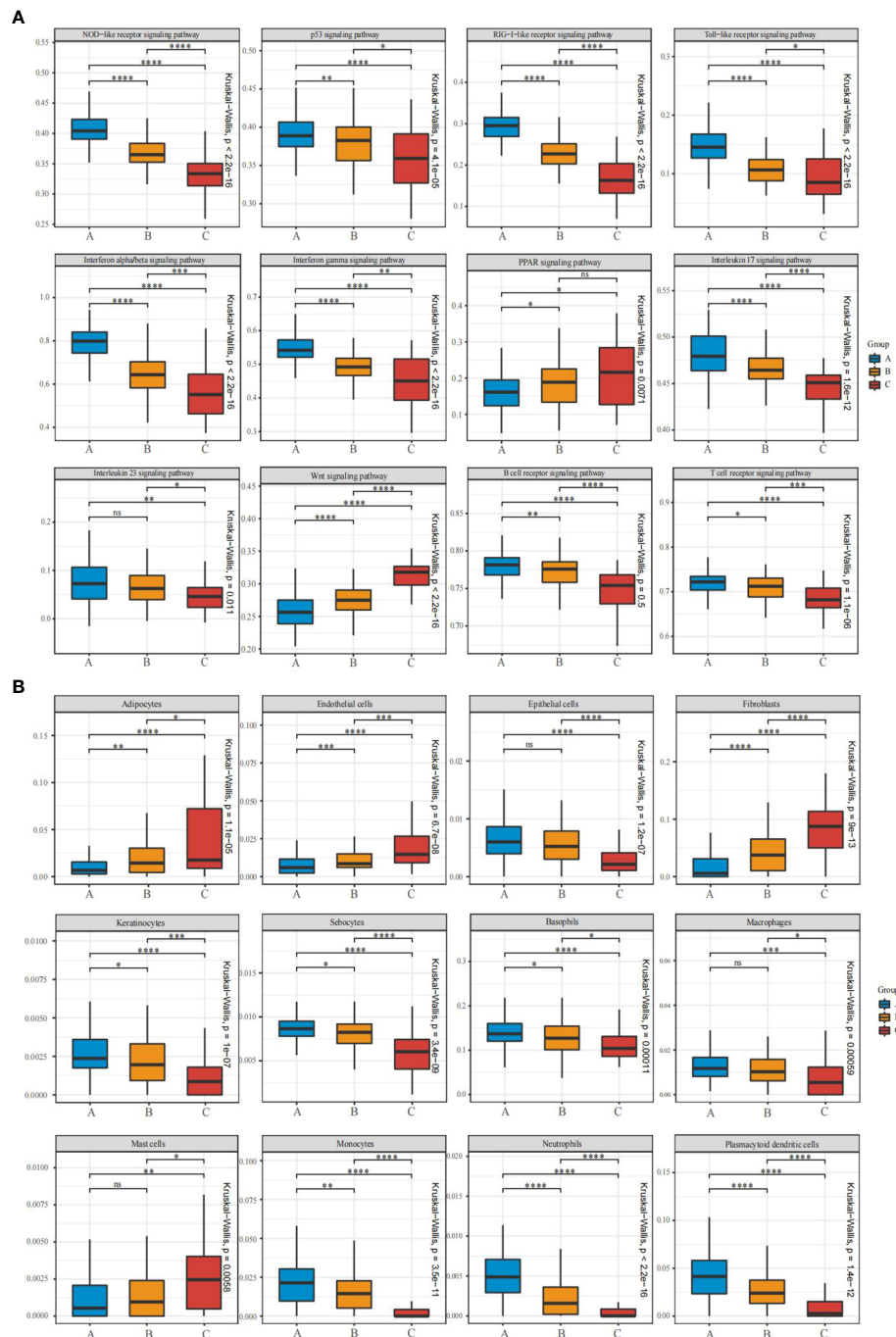


FIGURE 5

Pathway and cell subset-driven characterization of psoriasis skin subtypes. (A, B) Box plots for enrichment scores of pathways and xCell-inferred cell subsets for each psoriasis skin subtype. Differences across the three subtypes were analyzed using the Wilcoxon and Kruskal-Wallis test. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

information on approaching treatment-resistant patients with psoriasis remains lacking. Here, we classified the psoriasis skin tissues based on their molecular signatures using unsupervised cluster analysis (31). We characterized the different features of the three clustered subgroups in cellular components and biological processes and explained the results from therapeutic perspectives. In detail, subtype A (named immune activation subtype) had a transcriptomic signature of immune cells and proinflammatory

activation-related pathways. In contrast, subtype C (designated as the stroma proliferation subtype) exhibited more enriched transcripts in stroma cells (such as fibroblasts and endothelial cells) and tissue proliferative-related pathways, while the features of subtype B (named as mixed subtype) fell somewhere in between. Notably, the therapeutic responses were different among these three subgroups.

Currently, 20-30% of patients do not respond to FDA-approved treatments for psoriasis (34). Therefore, correct disease stratification

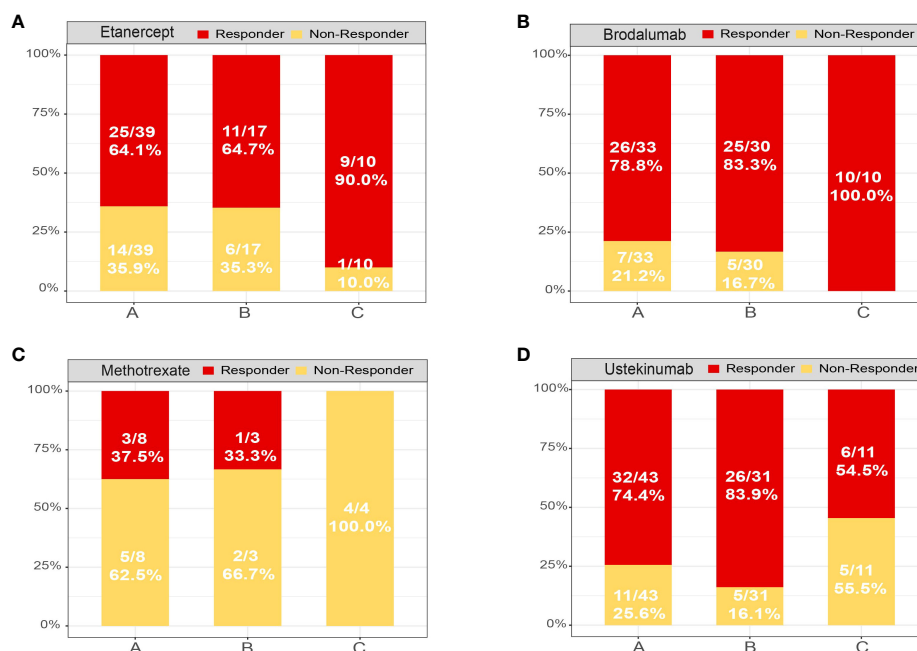


FIGURE 6

Treatment response of each psoriasis skin subtype. Distribution of psoriasis skin subtypes based on the treatment response of patients treated with (A) etanercept, (B) brodalumab, (C) methotrexate and (D) ustekinumab. Response: responded to the biologics; non-response: did not respond to the biologics.

and active exploration of the response of Psoriasis patients to different treatments are the first steps in the precision treatment of Psoriasis. In the past few years, Wang et al. analyzed genome-wide mRNA expression in psoriatic skin biopsies, discovered different immune cell infiltration patterns that distinguish psoriatic lesions from healthy skin, and successfully classified psoriasis into two different immune phenotypes. Among them, the nonlesional phenotype was characterized by overexpression of genes involved in keratinocyte differentiation and referred to as associated with better treatment response of biologics (10). This conclusion is highly consistent with our findings but has not provided an adequate explanation and address on how to approach it. Our results indicate that subtype C was strongly enriched with proliferative tissue pathways, including PPAR and Wnt signaling, and cellular components, such as fibroblasts and endothelial cells, but not for immune cells and proinflammatory signaling pathways. This finding indicates that immune cell activation was not necessarily involved in skin destruction and may provide a reasonable explanation for the conflict between the low level of inflammatory markers and continuous disease progression on the part of patients with psoriasis. Patients with subtype C presented good responses to TNF- α inhibitors (such as etanercept) and IL-17RA inhibitors (brodalumab). Previous studies have shown that a small amount of TNF- α and IL-17 can act on fibroblasts and endothelial cells to stimulate and produce numerous proinflammatory and proliferative cytokines, such as IL-6, IL-8, and CXCL-1, which cause excessive proliferation of psoriatic skin epidermis (35). Notably, Zaba et al. found that the efficacy of TNF- α inhibitors was associated with IL-17RA inhibition, as rapid downregulation of IL-17A pathway-related gene expression was observed in patients who responded to etanercept but not in the non-responders (36). These results suggest that the current

therapeutic strategies targeting TNF- α and IL-17RA could almost wholly inhibit the skin pathology in the good responders. Interestingly, brodalumab's effects on fibroblasts and endothelial cells have been demonstrated in systemic sclerosis (37). Brodalumab reduces fibroblast proliferation and collagen production by maintaining the regulatory T (Treg) cells/T helper (Th) 17 balance (38), resulting in reduced dermal thickness and improvement in modified Rodnan skin score. In addition, Takemichi Fukasawa et al. found that after six months of guselkumab (IL-23 inhibitor) being used to treat patients with psoriasis vulgaris complicated by systemic sclerosis, both Th2 and Th17 cells showed a decline, and the severity of the disease was also significantly improved (39). These findings further confirm that the IL-23/IL-17 axis is an essential pathway for targeted therapy of inflammatory skin diseases.

Activation of the inflammatory profile of subtypes A may account for the positive results of current psoriasis treatment targeting the upstream immune-inflammatory response. Patients with subtypes A were relatively sensitive to disease-modifying anti-rheumatic drugs (DMARDs) (i.e., MTX) and IL-12/23 inhibitors (i.e., ustekinumab). The anti-microbial peptide LL-37 is primarily secreted by keratinocytes and immune cells in the early stages of psoriasis development through direct activation of pDCs and myeloid dendritic cells to secrete IL-12 and IL-23 (40–42). Moreover, IL-12 and IL-23 stimulated TNF- α and IL-17 secretion in Th1 and Th17 cells (43, 44), resulting in a strong skin inflammatory reaction. Ustekinumab was designed to prevent the proliferation of the series of immune cells and the secretion of various proinflammatory cytokines by blocking IL-12/23 (45), an upstream target of the inflammatory signaling pathway enriched in subtypes A and B. The effects of MTX therapy on molecular signatures were largely restricted to proinflammatory pathways and immune cell subsets. In

addition, MTX has recently been discovered to inhibit the JAK/STAT pathway, while many of its side effects are likely to be related to the folate pathway (46). The IL-23 receptor relies on a heterodimer of Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) for signal transduction, thereby highlighting the role of JAKs in the therapeutic potential of JAK inhibitors (47, 48). Moreover, Ishizaki et al. observed that TYK2-deficient mice injected with IL-23 showed significantly reduced ear erythema and epidermal hyperplasia compared to wild-type mice (49). A lack of TYK2 also impaired the infiltration of various immune cells into the skin and the production of IL-17 and IL-22. The JAK/STAT and IL-12/23 pathways have similar contributions to the progression of psoriasis. Thus, it ought to be prudent to research whether the loss of efficacy or delayed treatment resistance can be attributed to the conversion of skin molecular patterns.

Nevertheless, the weak response to TNF- α and IL-17RA inhibitors in patients with subtypes A compared with that for subtype C seemed to contradict their enrichment of numerous inflammatory pathways and underlying pathophysiology. This might be attributed to a paradoxical reaction, in which the disease worsens during treatment with targeted biological drugs. Two main hypotheses have been proposed for this contradictory response (50). One theory is the involvement of TNF- α and IFN- α cross-regulation. Palucka et al. demonstrated that blocking endogenous TNF- α results in increased IFN- γ production by pDC and subsequent T-cell activation resulting in increased TNF- α production (51). The skin lesions in patients with psoriasis induced by TNF- α inhibitors are characterized by IFN- α overexpression compared with those in patients with psoriasis vulgaris. Another hypothesis posits that following TNF- α inhibition, Th17 cells are enhanced and regulatory T cells are downregulated, leading to increased production of the Th17 cytokine IL-22 (52). Both pathways produce cytokines that act on keratinocytes and generate a proinflammatory cycle, leading to suboptimal treatment of psoriasis. This suggests that although targeted therapy for specific cytokines has achieved good efficacy, some people may experience negative feedback after long-term targeted therapy, leading to the loss of treatment effectiveness. Moreover, inhibiting single downstream cytokines (TNF- α and IL-17) to prevent inflammatory response and reverse the disease outcome entirely is challenging. Thus, active intervention in the dysregulation of upstream targets (IL-12/23) is warranted.

Our study has some limitations. First, this study was conducted in different clinical environments from different public datasets; more meta-data is required, but this may prove challenging. Second, owing to the lack of complete annotation for each psoriasis sample, we could not address the association of each psoriasis subtype with clinical factors, such as autoantibody levels. Finally, these differences across responders and non-responders of subtypes were not statistically significant owing to the limited sample size of the clinical response in patients with psoriasis.

5 Conclusions

In summary, we deconvoluted psoriasis skin tissues into pathologically discrete subsets by combining skin transcriptomic

data with machine learning algorithms. We described their different molecular and cellular characteristics regarding treatment response. Our results provide critical insights into distinct and shared mechanistic features of psoriasis to determine the pathobiological approaches for benefiting drug-resistant patients.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Ethics statement

This study used publicly available data from the Gene Expression Omnibus (GEO) database. All subjects in the original studies have provided informed consent, so further ethical approval is not required.

Author contributions

SZ: Writing – original draft. MC: Writing – original draft. LZhe: Writing – original draft. CanW: Writing – review & editing. RZ: Writing – review & editing. SS: Writing – review & editing. JH: Writing – review & editing. LZha: Writing – review & editing. CaiW: Writing – review & editing. XL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1326502/full#supplementary-material>

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Association between systemic immune-inflammation index and psoriasis: a population-based study

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Background: The systemic immune-inflammation index (SII), as measured by lymphocyte, neutrophil and platelet counts in peripheral blood, is regarded as a favorable indicator of both inflammatory state and immune response. Psoriasis is an immune-mediated disease notable for its chronic inflammation of the entire system. Our research sought to explore the latent link between psoriasis and SII.

Methods: We performed a cross-sectional investigation utilizing data extracted from the National Health and Nutrition Examination Survey (NHANES, 2009–2014). Employing multivariate linear regression models and subgroup analysis, we sought to uncover the association between SII and psoriasis.

Results: This study enrolled a total of 17,913 participants as part of its research cohort. Our multivariate linear regression analysis revealed a notable and positive correlation between SII and psoriasis [1.013 (1.000, 1.026)]. As SII tertiles increased, the risk of psoriasis demonstrated an upward trend. The significant dependence on this positive association were maintained in women, BMI (≥ 30 kg/m²), non-stroke and non-cancer subjects in subgroup analysis and interaction tests. Furthermore, we identified a significant association between SII and psoriasis, characterized by two consecutive inverted U-shaped patterns. Notably, the analysis revealed the most prominent inflection point at a specific value of 797.067.

Conclusions: The results indicate a significant correlation between elevated SII levels and the presence of psoriasis. However, to corroborate and strengthen these results, additional large-scale prospective studies are required.

KEYWORDS

systemic immune-inflammation index, psoriasis, PASI, NHANES, stroke

1 Background

Psoriasis, a genetic skin disorder mediated by the immune system, impacts approximately 2–3% of the global population (1, 2). It typically affects the skin, but may also affects different organ systems such as the joints (3). The risk factors of psoriasis can be divided into extrinsic (trauma, drugs, infections, vaccination, lifestyle) and intrinsic (obesity, diabetes mellitus, dyslipidemia, hypertension, mental stress) groups with a view to prevent (4). Innate and adaptive immune responses are involved in the progression of psoriatic inflammation (5). Antimicrobial peptides (AMPs), such as LL-37, β -defensin and S100 protein, trigger and maintain inflammatory pathways in psoriasis (6). In the maintenance phase of psoriasis, the TNF- α /IL-23/IL-17 axis assumes a crucial role (7).

Recent studies have reported that psoriasis shares an underlying chronic inflammatory basis with comorbidities such as metabolic syndrome and cardiovascular disease (8–11). Uncovering the role of adipose inflammation in psoriasis is being intensively investigated (12, 13). Biologics such as efalizumab can delay the onset of systemic inflammatory psoriasis (14, 15). Other studies have suggested that clinical parameters of plasma cytokines and inflammation may serve as novel strategies to monitor the progression of psoriasis (16, 17). Elevated levels of various inflammatory and immune-based indices, such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and monocyte-to-lymphocyte ratio (MLR) have been observed and found to correlate with Psoriasis Area and Severity Index (PASI) scores in individuals with psoriasis (18, 19).

SII has become a prognostic biomarker for a variety of malignant tumors, including gastric cancer (20, 21), non-small cell lung cancer (22, 23), esophageal cancer (24, 25) and colorectal cancer (26). It provides a comprehensive reflection of the body's equilibrium between inflammatory factors and immune responses. Besides, the new score has been established as an effective predictive indicator of cardiovascular events (27). Some recent findings suggest that elevated SII levels are associated with hepatic steatosis and increased urinary albumin excretion (28, 29). The differences in this study are, first, that the study population was a sample of NHANES and that the sample size was increased, as were confounders for psoriasis or general health. Second, SII and the likelihood of psoriasis were plotted as a curve-fitted graph for analysis.

As a result, our objective was to investigate the correlation between the SII and psoriasis within the United States population. To accomplish this, we utilized the NHANES dataset in this study.

2 Methods

2.1 Study population

The NHANES is a comprehensive survey conducted biennially by the National Center for Health Statistics (NCHS), providing cross-sectional data that represents the entire US population. The

survey using the continuous cycle NHANES data set of 2009–2014. Initially, a cohort of 30,468 participants was enrolled for the study. We excluded 5,219 participants with missing SII data (neutrophil, lymphocyte, and platelet count) and 7,336 with missing psoriasis data. The study ultimately included 17,913 eligible participants. Figure 1 presents the visual representation of our sample selection process.

2.2 Definition of systemic immune-inflammation index

The Systemic Immunoinflammatory Index (SII), a new biomarker of local immune response and systemic inflammation throughout the body, has been shown to correlate with the prognosis of cancer patients. Regarded as a continuous variable, The calculation of SII for each participant was performed utilizing the formula:

$$SII = \frac{\text{platelet count} \times \text{neutrophil count}}{\text{lymphocyte count}}$$

Lymphocyte, neutrophil and platelet count using automatic hematology analysis equipment (Coulter[®] DxH analyzer 800) determination of complete blood count, expressed as $\times 10^3$ cells/ml. we designated SII as the exposure variable and psoriasis condition as an outcome variable in this study. Since the effect size was not significant, SII/100 was used to amplify the effect size by a factor of 100.

2.3 Selection of covariates

The covariates considered in this study encompassed various factors, including sex (men/women), age (years), race (Mexican American/other Hispanic/non-Hispanic White/non-Hispanic Black/other races), educational attainment (less than high school, high school or equivalent/above high school), body mass index (BMI, kg/m²), smoking status (yes/no), alcohol consumption (yes/no), presence of coronary artery disease (yes/no), diabetes mellitus (yes/no), congestive heart failure (yes/no), history of stroke (yes/no), and presence of cancer (yes/no). Individuals were categorized into three groups based on their body mass index (BMI): <25 kg/m² indicating normal weight, 25–29.9 kg/m² indicating overweight, and ≥ 30 kg/m² indicating obesity.

2.4 Statistical analysis

The statistical analyses were carried out following the guidelines of the Disease Control and Prevention (CDC), taking into account the appropriate NHANES sampling weights and employing complex multi-stage cohort surveys. Continuous variables are expressed as means with standard deviation (SD) and categorical variables as proportions. We used multivariate logistic regression analysis between SII and psoriasis to construct multivariate tests

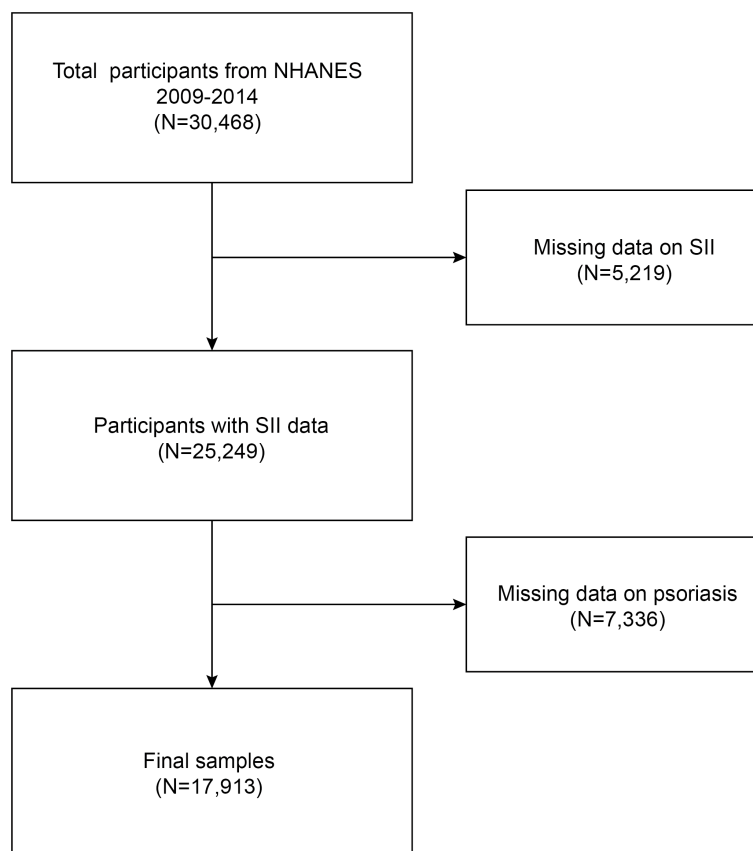


FIGURE 1

Flow chart of participants selection. NHANES, National Health and Nutrition Examination Survey.

that resulted in beta values and 95% confidence intervals. The multivariate test involved three models: model 1 (unadjusted), model 2 (adjusted for gender, age, and race), and model 3 (adjusted for all covariates). Smooth curve fitting was performed simultaneously by adjusting the variables. A threshold effect analysis model was used to investigate the relationship and inflection point between SII and psoriasis. Subgroup analysis of the SII-psoriasis relationship was conducted considering stratification factors including gender (man/woman), BMI (normal weight/overweight/obesity), stroke (yes/no), cancer (yes/no). In the process, we used the statistical calculation and graphics software R (version 4.1.3) and the Irrigation Statistics Software (version 2.0) for statistical research. Significance was established at a threshold of $P < 0.05$.

3 Results

3.1 Baseline characteristics

This study involved 17913 participants with a mean age of (46.02 ± 19.26) years. Of these, 48.67% were men and 51.33% were women. Among these participants, 14.98% were Mexican American, 9.84% were other Hispanic, 41.87% were non-Hispanic white, 22.33% were non-Hispanic black, and 12.10% were from

other races. The mean SII score was (520.20 ± 388.94) for all participants. In comparison to the no history of psoriasis group, the history of psoriasis group is significantly more likely to be older and have comorbidities including stroke, coronary artery disease, diabetes, congestive heart failure and cancer with a higher proportion of non-Hispanic white and education above high school; with higher smoking and alcohol status; and higher levels of BMI and SII. The statistical analysis did not demonstrate a significant difference in gender and stroke between the two groups ($p > 0.05$) (Table 1).

3.2 Association between systemic immune-inflammation index and psoriasis

Our findings suggest that the higher the SII, the greater the likelihood of developing psoriasis. This association remained significant in both our crude model (OR=1.016; 95% CI, 1.003–1.029, $p < 0.05$) and minimally adjusted model (OR=1.013; 95% CI, 1.000–1.026, $p < 0.05$). However, after adjusting all covariates, this significant positive correlation became insignificant in model 3 (OR=1.009; 95% CI, 0.992–1.026, $p > 0.05$). We further transformed SII for sensitivity analysis from continuous variables to categorical variables (quartiles). Participants in the highest quartile (OR=1.645; 95% CI, 1.261–2.145, $p < 0.05$) of SII had a statistically significant

TABLE 1 Baseline characteristics of patients with and without psoriasis.

Variable	History of Psoriasis (n = 474 [2.6%])	No History of Psoriasis (n = 17439 [97.4%])	P Value
Age (years)	50.970 ± 17.662	45.885 ± 19.288	<0.001
Gender (%)			0.802
Men	48.101	48.684	
Women	51.899	51.316	
Race (%)			<0.001
Mexican American	8.650	15.150	
Other Hispanic	9.283	9.851	
Non-Hispanic White	59.072	41.407	
Non-Hispanic Black	10.549	21.504	
Other Races	12.447	12.088	
Education level(%)			0.003
Less than high school	24.262	32.192	
High school or GED	22.363	19.869	
Above high school	53.376	47.830	
Others	0.000	0.109	
Smoking(%)	56.863	43.464	<0.001
Alcohol use(%)	22.432	16.746	0.038
BMI (kg/m2)	29.829 ± 7.058	28.676 ± 7.017	<0.001
Comorbidities(%)			
Diabetes	16.667	11.038	<0.001
Congestive heart failure.	5.470	2.996	<0.001
Coronary artery disease	7.659	3.800	<0.001
Stroke	4.376	3.634	0.603
Cancer	14.880	9.027	<0.001
SII (1,000 cells/μl)	577.261 ± 320.458	518.651 ± 390.534	<0.001

Mean ± SD for continuous variables; the p-value was calculated by weighted linear regression model. % for categorical variables; the p-value was calculated by a weighted chi-square test. BMI, body mass index; SII, systemic immune-inflammation index.

64.5% increased risk of developing psoriasis when contrasted with those situated in the lowest SII quartile. Participants belonging to SII quartile 2 and 3 displayed an increased risk of psoriasis in comparison to the lowest quartile; however, this association did not attain statistical significance (Table 2).

Further subgroup analysis revealed inconsistent associations between SII and psoriasis, as shown in Table 3. In subgroup analyses by gender stratification, our results revealed an

TABLE 2 The association between SII and psoriasis.

	Model 1	Model 2	Model 3
	OR(95% CI) P value	OR(95% CI) P value	OR(95% CI) P value
SII/100	1.016 (1.003, 1.029) 0.01702	1.013 (1.000, 1.026) 0.04364	1.009 (0.992, 1.026) 0.32130
SII quartiles			
Quartile 1	Reference	Reference	Reference
Quartile 2	1.174 (0.881, 1.565) 0.27441	1.084 (0.812, 1.448) 0.58442	1.035 (0.740, 1.448) 0.84087
Quartile 3	1.315 (0.993, 1.741) 0.05616	1.159 (0.873, 1.539) 0.30870	1.078 (0.777, 1.494) 0.65331
Quartile 4	1.944 (1.497, 2.524) <0.00001	1.645 (1.261, 2.145) 0.00024	1.433 (1.052, 1.952) 0.02263
P for trend	<0.00001	0.00003	0.00750

Model 1, no covariates were adjusted. Model 2, age, sex, and race were adjusted. Model 3, age, sex, race, education level, smoking status, drinking status, BMI, diabetes, congestive heart failure,coronary artery disease, stroke and cancer were adjusted. Multivariate logistic regression analysis was used to calculate beta values and 95% confidence intervals. 95% CI, 95% confidence interval; OR, odds ratio; SII, systemic immunity-inflammation index. p< 0.05 was considered statistically significant.

independent and significantly positive association between SII and psoriasis exclusively among women (OR = 1.031; 95% CI, 1.002–1.061, p<0.05) but not statistically significant in all models for men. When examining subgroups by degree of body mass index, we observed a robust positive correlation between SII and the obese group (≥ 30 kg/m2) in both the unadjusted and partially adjusted models. A significant relationship between SII with psoriasis was detected in non-cancer subjects (OR = 1.014, 95% CI 1.001–1.026, p<0.05). The interaction test indicated that no significant dependence was observed for the positive correlations between sex, age, body mass index, and cancer with SII and psoriasis (p for interaction >0.05).Only in the stroke stratification the association between SII and psoriasis was significantly different.

Because people with SII greater than 2000 are more discrete, data with SII greater than 2000 are removed from the curve fitting. We fitted smoothed curves to characterize the nonlinear relationship between SII and psoriasis (Figure 2). We found two consecutive inverted U-shaped relationships between SII and psoriasis, with the most significant inflection point of 797.067.

4 Discussion

The study sample utilized in our research is representative of the American population at a national level. We observed that the higher the SII participants with the greater the chance of psoriasis. Prominently, two consecutive inverted U-shaped relationships between SII and psoriasis was revealed for the first time.The risk

TABLE 3 Subgroup analysis for the association between SII and psoriasis.

SII/100	Model 1	Model 2	Model 3
	OR (95% CI) P value	OR (95% CI) P value	OR (95% CI) P value
gender			
Men	1.011 (0.998, 1.025) 0.1012	1.009 (0.993, 1.026) 0.2843	1.005 (0.979, 1.031) 0.7196
Women	1.038 (1.010, 1.066) 0.0066	1.031 (1.002, 1.061) 0.0339	1.024 (0.985, 1.065) 0.2262
P for interaction	0.1100	0.2021	0.4107
Race			
Mexican American	0.967 (0.860, 1.088) 0.5814	0.970 (0.863, 1.090) 0.6062	0.968 (0.849, 1.102) 0.6222
Other Hispanic	1.070 (0.980, 1.167) 0.1298	1.070 (0.980, 1.168) 0.1310	1.064 (0.951, 1.192) 0.2800
Non-Hispanic White	1.036 (1.008, 1.064) 0.0110	1.030 (1.002, 1.059) 0.0352	1.026 (0.994, 1.058) 0.1091
Non-Hispanic Black	1.029 (0.976, 1.085) 0.2832	1.024 (0.972, 1.080) 0.3716	1.1 (0.900, 1.114) 1.2 0.9866
Other Races	1.031 (0.966, 1.102) 0.3551	1.031 (0.966, 1.100) 0.3639	1.028 (0.945, 1.117) 0.5202
P for interaction	0.2210	0.3125	0.6347
BMI			
<25	1.023 (0.981, 1.066) 0.2857	1.008 (0.959, 1.059) 0.7635	1.008 (0.955, 1.063) 0.7736
>=25,<30	1.010 (0.995, 1.024) 0.1851	1.011 (0.994, 1.028) 0.2126	1.007 (0.982, 1.032) 0.5833
>=30	1.056 (1.019, 1.094) 0.0024	1.045 (1.007, 1.085) 0.0201	1.027 (0.982, 1.074) 0.2358
P for interaction	0.0857	0.2823	0.7365
STROKE			
YES	0.824 (0.678, 1.002) 0.0518	0.803 (0.659, 0.978) 0.0290	0.830 (0.669, 1.031) 0.0920
NO	1.017 (1.003, 1.031) 0.0172	1.015 (1.003, 1.028) 0.0183	1.011 (0.996, 1.026) 0.1634
P for interaction	0.0111	0.0048	0.0339

(Continued)

TABLE 3 Continued

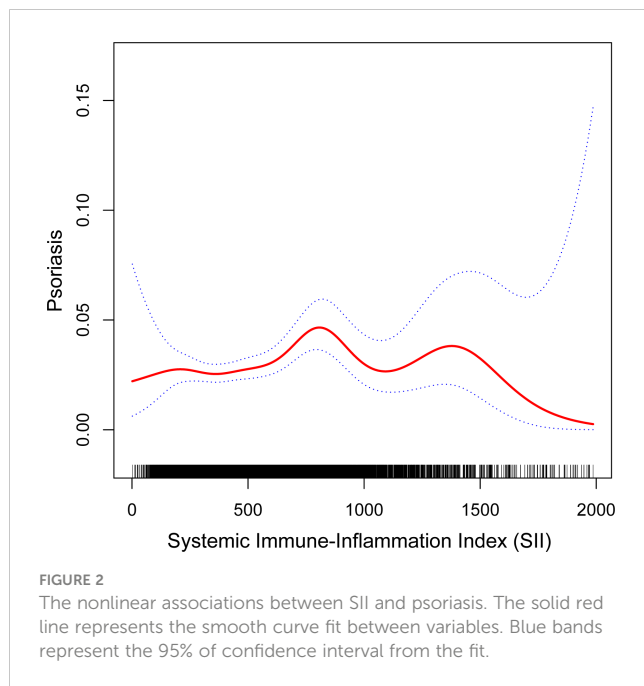
SII/100	Model 1	Model 2	Model 3
	OR (95% CI) P value	OR (95% CI) P value	OR (95% CI) P value
CANCER			
YES	1.007 (0.943, 1.075) 0.8366	1.1 (0.937, 1.072) 1.2 0.9503	0.996 (0.921, 1.077) 0.9187
NO	1.015 (1.002, 1.028) 0.0227	1.014 (1.001, 1.026) 0.0351	1.000 (1.000, 1.000) 0.2222
P for interaction	0.8075	0.7380	0.7286

Model 1: no covariates were adjusted. Model 2: age, gender, and race were adjusted. Model 3: age, sex, race, education level, smoking status, drinking status, BMI, diabetes, congestive heart failure,coronary artery disease, stroke and cancer were adjusted.
In the subgroup analysis stratified by gender and age, the model is not adjusted for sex and age, respectively.
Multivariate logistic regression analysis was used to calculate beta values and 95% confidence intervals.
95% CI, 95% confidence interval; OR, odds ratio; SII, systemic immunity-inflammation index; BMI, body mass index.
p< 0.05 was considered statistically significant.

was highest when SII was 797.067. Interestingly, the significant positive correlation between SII and psoriasis was only observed among non-Hispanic whites. Additionally, gender or weight status may have played a moderating role in the association between psoriasis and SII. There was an association in women, but not in men. The association was observed only in the obese group. In fact, Model 3 was built based on a comprehensive adjustment for all possible relevant variables, which included various factors that may influence inflammatory status, such as age, BMI, co-morbidities, and so on. If adjustments for these variables are able to attenuate or eliminate differences in SII among psoriasis patients, then the results may become less significant.

As far as we know, it is the first NHANES-based investigation of the relationship between SII and psoriasis. The link here reported regarding SII and psoriasis is similar to those previously reported, with one study from Turkey (30) showing that SII of psoriasis patients was considerably greater than that of control group and patients with moderate/severe psoriasis had higher SII than with mild psoriasis. More recently, Sugimoto et al. also demonstrated a positive correlation between SII and PASI scores and found that patients with higher SII scores had lower persistence of treatment with conventional systemic medications (31). Dincer Rota et al. reported that significantly elevated SII values were observed in both the subgroup with PASI ≥ 4.5 and among patients experiencing nail and genital involvement (32). Moreover, it has been discovered that the mean SII was lower in the patients receiving biologic treatment compared to the untreated patients (33, 34). According to an observational retrospective study, SII was positively correlated with disease severity and was an independent prognostic factor for mild and moderate psoriasis (35).

Our findings are closely related to the evidence that psoriasis is a systemic inflammatory disease (36–40). Recent research has revealed that the pathogenesis of psoriasis and its complications are related to



systemic inflammatory pathways. Observations from the ^{18}F -fluorodeoxyglucose positron emission tomography/computed Tomography study also confirmed the hypothesis that psoriasis is a systemic inflammatory disease (41). In multiple studies, in addition to marked increases in systemic arterial and subcutaneous inflammation, subclinical inflammation was also observed in the liver, joints, and tendons for individuals with moderate-to-severe psoriasis, and in the aorta of patients with mild psoriasis (42). Furthermore, insulin resistance may be more helpful in predicting subclinical atherosclerosis than traditional cardiovascular disease risk factors, and femoral artery ultrasonography better detects subclinical atherosclerosis in patients with moderate-to-severe psoriasis (43, 44). Serum levels of several proinflammatory cytokines, including $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, IL-6, IL-8, IL-12, IL-17A and IL-18 were elevated in individuals diagnosed with psoriasis compared to healthy controls, suggesting that psoriasis develops systemically (45).

Furthermore, the positive correlation between SII and psoriasis exhibits significant variations in relation to stroke occurrence. Extensive research has demonstrated that individuals with psoriasis face an elevated risk for cerebrovascular disease (stroke) which is attributed to the severity of their psoriasis condition (46–49). In addition, the incidence of ischemic heart disease and cerebrovascular disease increased in patients with psoriasis combined with depression (50). Numerous studies have sought to establish a causal link between psoriasis and cardiovascular disease. Cardiovascular risk factors such as dyslipidemia, diabetes mellitus, hypertension, metabolic syndrome and obesity have also increased in psoriasis patients (51). Atherosclerosis is the main pathologic change that precedes myocardial infarction and stroke. Notably, individuals with psoriasis have been found to have higher arterial stiffness than healthy controls, with arterial stiffness positively correlating with psoriasis duration (52, 53). Multiple studies measured by imaging techniques have reported a higher prevalence and greater severity of coronary artery calcification and atherosclerosis among individuals

diagnosed with psoriasis in comparison to their healthy counterparts (54–56). The association between psoriasis and cardiometabolic disorders can be attributed to various mechanisms, involving the secretion of adipokines, oxidative stress, insulin resistance, angiogenesis, microparticles, hypercoagulability and common inflammatory pathways (51). Thus, the administration of effective systemic anti-inflammatory drugs may prove beneficial in reducing cardiovascular risk in psoriasis patients (57).

Platelet, neutrophil, and lymphocyte counts constitute SII. The pathogenic role of neutrophils, the most abundant innate immune cells, is associated with chronic inflammatory and autoimmune diseases (58). The abundant presence of neutrophils in psoriatic lesions is a distinctive histopathological characteristic of psoriasis (59). Recent reports have proposed that neutrophils play a role in psoriasis pathophysiology through processes such as respiratory burst, degranulation, and the formation of neutrophil extracellular traps, influencing psoriatic immunity and clinical outcomes (60). In addition, patients with psoriasis continue to consume platelets due to an enhanced coagulation response (61). Platelets play an important role in cardiovascular disease as mediators of hemostasis and thrombotic inflammation (62). Numerous studies have reported elevated levels of platelet activation biomarkers in individuals with psoriasis when compared to healthy controls. These biomarkers include platelet-derived microparticles (PDMPs) and mean platelet volume (MPV). Interestingly, a positive correlation has been observed between the levels of these biomarkers and PASI scores (63). The core pathogenesis of psoriasis involves aberrant function of multiple T-cell subsets, including regulatory T cells (Tregs), T helper (Th)1 cells, Th2 cells, Th17 cells, and Th22 cells, accompanied by aberrant release of associated cytokines, such as $\text{IFN-}\gamma$, tumor necrosis factor ($\text{TNF-}\alpha$), and members of the IL-23 and IL-17 families (45).

In recent years, with the advent of biologics, the effectiveness and safety of psoriasis treatments have greatly improved. A major challenge and limitation is to find better predictors of treatment response to determine which patients are more likely to benefit from specific biologics. SII is positively correlated with PASI scores (30–32), and the correlation with treatment response (31, 33, 34) has been confirmed by some studies. In addition, correlations have been found between SII and metabolic syndrome (64), cardiovascular disease (65), and depression (66) in psoriasis comorbidities. Taken together, SII appears to be a possible biomarker associated with psoriasis disease activity, treatment response, and the development of comorbidities, thus helping patients to develop more personalized and effective treatment strategies.

Our study has several limitations worth noting. Firstly, due to the cross-sectional design of our research, we cannot establish causality. Second, relying on self-reported questionnaire data without clinical assessment by healthcare professionals to determine the presence or absence of psoriasis may lead to misdiagnosis or underestimation of psoriasis cases, especially mild or atypical psoriasis cases. Recall bias may also affect the validity of study results. Moreover, the NHANES database does not differentiate between the various types of psoriasis and fails to obtain information on which period of disease progression (progressive/quiescent/degenerative) a psoriasis patient is in, whereas different types of psoriasis at different stages may interact biologically with SII in different ways. As it is well known that

the inflammatory response is a dynamic process, high (or low) values of SII observed at a single time point may not fully reflect the complexity and dynamics of systemic inflammation. However, the interaction between inflammation and psoriasis is complex. Although we controlled for some confounders, other confounders, such as duration of psoriasis, types of psoriasis complications not included, use of biologics, and history of long-term use of anti-inflammatory medications to treat complications, may still have had an impact on the study results. Because NHANES did not document these factors, we were unable to include them in our analysis. Therefore, our results may not fully reflect the complete picture.

5 Conclusion

Our results establish a connection between elevated SII levels and psoriasis. SII may be a simple, practical, and easily accessible tool for monitoring disease activity and treatment efficacy in patients with psoriasis. To validate our findings, additional extensive prospective studies are warranted.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.cdc.gov/nchs/nhanes/.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

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XZ: Data curation, Writing – original draft, Writing – review & editing. JL: Writing – original draft. XL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Multiple environmental antigens may trigger autoimmunity in psoriasis through T-cell receptor polyspecificity

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Introduction: Psoriasis is a T-cell mediated autoimmune skin disease. *HLA-C*06:02* is the main psoriasis-specific risk gene. Using a $V\alpha 3S1/V\beta 13S1$ T-cell receptor (TCR) from a lesional psoriatic CD8⁺ T-cell clone we had discovered that, as an underlying pathomechanism, *HLA-C*06:02* mediates an autoimmune response against melanocytes in psoriasis, and we had identified an epitope from ADAMTS-like protein 5 (ADAMTSL5) as a melanocyte autoantigen. The conditions activating the psoriatic autoimmune response in genetically predisposed individuals throughout life remain incompletely understood. Here, we aimed to identify environmental antigens that might trigger autoimmunity in psoriasis because of TCR polyspecificity.

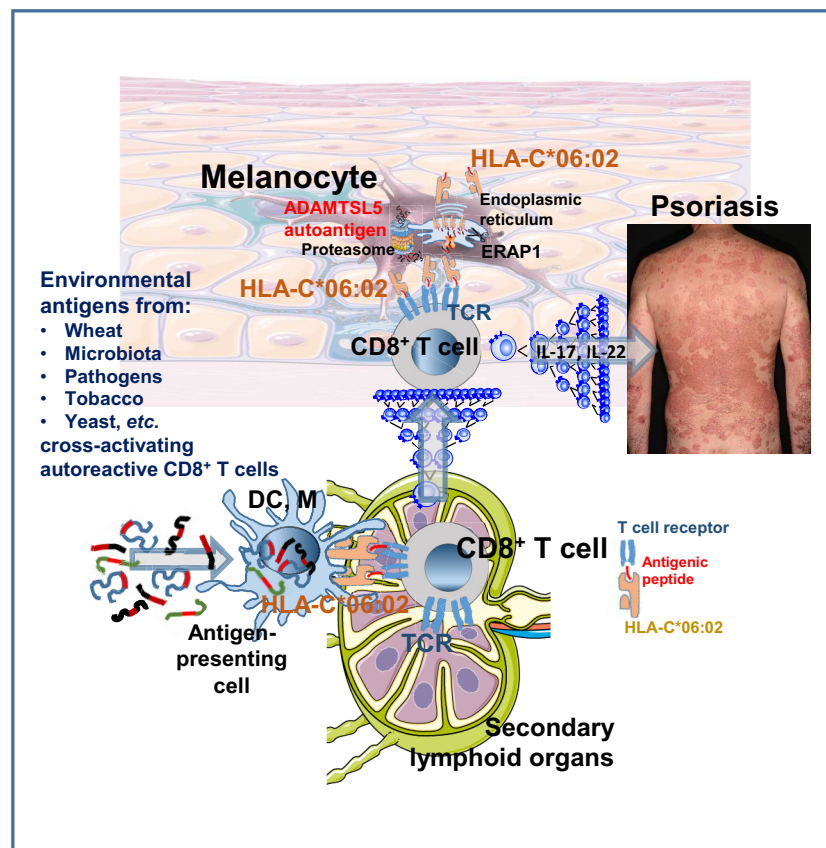
Methods: We screened databases with the peptide recognition motif of the $V\alpha 3S1/V\beta 13S1$ TCR for environmental proteins containing peptides activating this TCR. We investigated the immunogenicity of these peptides for psoriasis patients and healthy controls by lymphocyte stimulation experiments and peptide-loaded *HLA-C*06:02* tetramers.

Results: We identified peptides from wheat, *Saccharomyces cerevisiae*, microbiota, tobacco, and pathogens that activated both the $V\alpha 3S1/V\beta 13S1$ TCR and CD8⁺ T cells from psoriasis patients. Using fluorescent *HLA-C*06:02* tetramers loaded with ADAMTSL5 or wheat peptides, we find that the same CD8⁺ T cells may recognize both autoantigen and environmental antigens. A wheat-free diet could alleviate psoriasis in several patients.

Discussion: Our results show that due to TCR polyspecificity, several environmental antigens corresponding to previously suspected psoriasis risk conditions converge in the reactivity of a pathogenic psoriatic TCR and might thus be able to stimulate the psoriatic autoimmune response against melanocytes. Avoiding the corresponding environmental risk factors could contribute to the management of psoriasis.

KEYWORDS

psoriasis, pathogenic T-cell receptor, T-cell receptor polyspecificity, autoimmune response, environmental antigens, diseases triggers, *HLA-C*06:02*



GRAPHICAL ABSTRACT

Introduction

Psoriasis is a common T-cell mediated autoimmune skin disease frequently accompanied by arthritis (1, 2). Within a complex genetic predisposition (3), *HLA-C*06:02* is the main psoriasis-specific risk gene (4). Psoriatic skin lesions develop upon epidermal infiltration and activation of CD8⁺ T cells that exhibit marked clonality and conserved TCR patterns, indicating that common disease-specific autoantigens may drive autoimmune T-cell activation in psoriasis (5–9).

Despite the genetic predisposition, psoriasis is not a congenital disease. The typical age of onset in the 2nd and 3rd decade of life (10) and the concordance rate in identical twins, which ranges from 20% to 73% (11, 12), support that environmental, infectious and lifestyle factors may be crucial for initiating autoimmune pathology in psoriasis. Identifying antigenic triggers that convert the genetic predisposition into active autoimmune disease by stimulating the psoriatic autoimmune response against melanocytes could enable causally oriented preventive and therapeutic strategies. However, since the initiating circumstances often remain unclear, treatments for psoriasis are primarily directed at the terminal inflammatory phase (1).

By analyzing the reactivity of a TCR from a lesional psoriatic CD8⁺ T-cell clone rearranging the V α 3S1 and V β 13S1 variable region genes characteristic of infiltrating lesional CD8⁺ T cells (V α 3S1/

V β 13S1 TCR) (5, 7), we had previously established as an underlying pathomechanism in psoriasis that *HLA-C*06:02* may mediate an autoimmune response against melanocytes, and we had identified an epitope from ADAMTS-like protein 5 (ADAMTSL5) as a melanocyte autoantigen presented by *HLA-C*06:02* (13, 14). Extensive immunohistological studies further confirmed that the CD8⁺ T cells in lesional psoriatic epidermis indeed react against melanocytes, as indicated by the V α 3S1/V β 13S1 TCR (13). Unlike the melanocyte-depleting cytotoxic autoimmune response causing vitiligo, CD8⁺ T cells activated against melanocytes in psoriasis cause chronic inflammation through the production of IL-17 and IL-22 (15–19).

A suspected mechanism for self-reactive T cells to evade peripheral tolerance mechanisms is through the antigen recognition mode of TCRs. TCRs are polyspecific and ligated by antigenic peptides sharing a certain amino acid motif (20, 21). It is defined by two or three amino acids anchoring the peptides in the discrete pockets of the peptide binding groove of the cognate HLA molecule, and one or two TCR-specific contact residues, while tolerating a broad amino acid diversity at other positions of the antigenic peptides. Therefore, it was postulated that due to the extensive TCR cross-reactivity environmental antigens may induce autoimmune diseases by stimulating potentially autoreactive T cells (22). Accordingly, in ankylosing spondylitis (AS), which is part of the psoriatic arthritis spectrum, TCRs of clonally expanded CD8⁺ T

cells from synovial fluid or uveitis were found to recognize both microbial and self-peptides presented by the AS-specific risk allele HLA-B*27 (23).

To identify environmental peptides that ligate the V α 3S1/V β 13S1 TCR and thus might stimulate the autoimmune response against melanocytes in psoriasis, we searched protein databases for proteins containing the peptide recognition motif of this TCR. We identified several environmental and microbial antigens that correspond to previously suspected psoriasis risk conditions and stimulated the V α 3S1/V β 13S1 TCR and CD8⁺ T cells of psoriasis patients. Thus, our results directly support in patients that due to the polyspecificity of TCRs, different environmental antigens may stimulate autoreactive T cells and promote autoimmunity in psoriasis.

Materials and methods

Study design

The study was designed to identify environmental antigens that can trigger a psoriatic autoimmune response due to TCR polyspecificity. We performed a sequential approach: 1. Precise characterization of the peptide recognition motif of an autoreactive V α 3S1/V β 13S1 TCR from a lesional psoriatic CD8⁺ T cell clone specific for a melanocytic psoriatic autoantigen. 2. Use of the peptide recognition motif to search databases for environmental antigens that activate the autoreactive V α 3S1/V β 13S1 TCR. 3. Verification of the immunogenicity of candidate antigens for CD8⁺ T cells from psoriasis patients. 4. Investigation of whether the same CD8⁺ T cells can recognise both autoantigen and environmental peptides using peptide-loaded HLA-C*06:02 tetramers. 5. Follow-up of the effect of exposure prophylaxis of certain environmental antigens (wheat) on the course of psoriasis.

Patients providing blood samples

41 Patients with type 1 chronic plaque psoriasis (early onset, positive family history and/or HLA-C*06:02 (10)) were randomly recruited from the psoriasis outpatient clinic of the Department of Dermatology and Allergy, University Hospital, Ludwig-Maximilian-University, to represent the normal patient distribution. 12 healthy subjects without a personal or family history of psoriasis or psoriatic arthritis served as controls selected to approximately match age. Median age of Pso was 47.6 y, STDEV 14.43 y; median age of HC was 44.9 y, STDEV 12.75 y; $P = 0.0840$. Patients and healthy controls participated voluntarily and gave their written informed consent.

Patients undergoing a gluten-free diet

Several patients with inadequate response to their current therapy and missing therapeutic alternatives due to contraindications to other drugs (pre-existing malignancies, chronic infections), lack of response to or side effects of previously taken drugs, or concern about these,

opted for a GfD because of our experimental findings. We followed the course of psoriasis at routine patient visits by means of the Psoriasis Area and Severity Index (PASI) and the Physicians Global Assessment (PGA), the standard of care parameters for measuring psoriasis activity, for six months or longer. Patients who showed a substantial improvement in terms of a PASI 75 score or better within three months continued with GfD to maintain the benefit. Dietary errors occurred during holiday periods abroad (Paris, Rome) when patients were unable to adhere to GfD for language reasons. Patient data are summarized in Table 1.

Motif-based searches for naturally occurring peptide ligands of the V α 3S1/V β 13S1TCR

Sequence logos of previously identified mimotopes and self-peptide ligands of the V α 3S1/V β 13S1 TCR were created at WebLogo (<https://weblogo.berkeley.edu/logo.cgi>). Emboss prophecy (<http://emboss.sourceforge.net/apps/release/6.6/emboss/apps/prophecy.html>) was used to create search motifs from positively tested antigenic peptides. The main octamer search motifs were RXXRXXRL, RXXNXRRL, RXXRXRRL, RXXNXLRL, RXXRXLRL, RXXQXLRL, RXXRXLRM, and RXXRXRRM. Unrestricted searches at the UniProtKB/Swiss-Prot databases were carried out using the Expasy ScanProsite tool and the Protein BLAST Basic Local Alignment Search Tool (https://prosite.expasy.org/scanprosite/scanprosite_doc.html, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The list of results was manually curated for amino acids at positions 3-4 and 6 seen in the heat maps and sequence logos of previously identified mimotopes and stimulatory peptides (Supplementary Figure S1A, Supplementary Table S1). In nonamers, amino acids at P1 were supplemented according to the natural protein sequence. Binding to HLA-C*06:02 was verified using the NetMHCpan - 4.1 server (<https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1>).

Generation of plasmid-encoded peptides for V α 3S1/V β 13S1-TCR hybridoma stimulation

For expression of short candidate peptides, forward and reverse oligonucleotides (Biomers) carrying a 5'-CACCATG overhang and a stop codon in 3'-position of the target sequence (Supplementary Table S2) were annealed and ligated into pcDNA3.1D/V5-His-TOPO using pcDNA3.1 Directional TOPO Expression Kit (Invitrogen, Carlsbad, USA) according to manufacturer's instructions as described (13, 14).

Culture conditions and stimulation of the CD8⁺ V α 3S1/V β 13S1-TCR reporter T-hybridoma cell line

Generation and culture conditions of the V α 3S1/V β 13S1-TCR CD8⁺ reporter T hybridoma from the paired $\alpha\beta$ -TCR chains

rearranging the V α 3S1 and the V β 13S1 gene segments of a lesional psoriatic CD8⁺ T-cell clone have been described (7, 13). RPMI 1640 culture medium contained 10% FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 1x MEM non-essential amino acids and 10 μ g/ml ciprofloxacin, supplemented with selection antibiotics G418 (1.5 mg/ml), hygromycin (300 μ g/ml, both Genaxxon), puromycin (1 μ g/ml, Sigma) and blasticidin (3 μ g/ml, PAA). For co-culture experiments, TCR hybridoma cells were pelleted and resuspended in hybridoma growth medium without selection antibiotics before addition to antigen-presenting cells.

To determine the antigenicity of peptide antigens for the V α 3S1/V β 13S1-TCR hybridoma, COS-7 cells were cotransfected with HLA-C*06:02- and peptide-encoding plasmids (250 ng each) using FuGENE HD reagent (Promega). After 24 h, medium was replaced with fresh medium containing V α 3S1/V β 13S1-TCR hybridoma cells as described (13, 14). After 24 h of co-culture, the degree of hybridoma activation was determined on a BD FACSCanto Flow Cytometry System with respect to the percentage of sGFP⁺ hybridoma cells. For evaluation of TCR stimulation, V α 3S1/V β 13S1-TCR hybridoma cells were cultured in culture plates either untreated or pre-coated with CD3 antibody (eBioscience #14-0032-82, 17A2, 2 μ g/ml in PBS). The percentage of hybridoma cells induced to express sGFP directly correlates with the degree of TCR ligation and was therefore used to quantify TCR stimulation (14). The maximal possible yield of 50% to 70% of anti-CD3-stimulated sGFP-positive hybridoma cells reflects their maximum activatability also observed in former experiments that had established this technology (24).

Stimulation of PBMC

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood using Ficoll density gradient centrifugation (Biocoll, Biochrom). They were seeded in 96 well flat bottom plates at 2×10^5 cells/well and cultured in RPMI 1640 medium supplemented with 10% human AB serum. Highly purified synthetic peptides (> 95%, Thermo Fisher) corresponding to the plasmid-encoded environmental antigens stimulating the V α 3S1/V β 13S1 TCR were used for stimulation at a final concentration of 10 μ g/ml. An unrelated peptide without a human protein correlate previously isolated from HLA-C*06:02 of lymphoblastoid B cells (25), VRHDGGNVL, here named “FALK” peptide, served as a comparison. HLA-C*06:02 typing was performed by PCR restriction fragment length polymorphism analysis as described previously (26), or determined by sequence-based typing at the Laboratory for Immunogenetics and Molecular Diagnostics, University Hospital, Ludwig-Maximilian-University of Munich.

Measurement of stimulation-induced [³H]-thymidine incorporation of PBMC and induction of CD137 expression on CD8⁺ T cells

To measure stimulation-induced proliferation, triplicates of PBMC cells were pulsed overnight on day 5 of stimulation with 24,500 bq/well

[³H]-thymidine (Hartmann Analytic (Braunschweig, Germany), and proliferation was assessed according to the incorporated radioactivity. Harvesting and measurement of radioactive decay (counts per minute) were done using an automatic filter counting system (Inotech Biosystems, Derwood, USA).

Peptide-induced activation of CD8⁺ T cells was assessed based on the induction of CD137 expression (27). PBMC were incubated with synthetic peptides and harvested after 24 hours. Cells were stained with CD-137 FITC/PE-Cy7 (Clone 4B4-1, eBioscience Cat. Nr. 11-1379-42, BioLegend, Cat.Nr. 309818) and CD8 perCP/Cy5.5 SK1 (BioLegend, Cat.Nr. 344710) for 30 min on ice. For flow cytometry analysis of CD137 expression, lymphocytes were gated using FSC and SSC. Cells gated for CD8 expression were analyzed for the expression of CD137. Isotype IgG controls and unstimulated cells were included in each experiment. At least 1×10^5 cells per sample were analyzed with a FACSCanto II (Becton Dickinson). FlowJo software v10.7.2 (BD Biosciences USA) was used to analyze the results. A representative analysis is given in **Supplementary Figure S3**.

Peptide-induced activation was expressed as stimulation index (SI), which is the median cpm of PBMC triplicates or percentage of CD137⁺ CD8⁺ T cells of stimulated cells relative to the mean percentage of unstimulated control cells.

Determination of Gliadin antibodies

Gliadin antibodies were determined in undiluted serum samples by the Alegria® system, a quantitative automated indirect enzyme-linked immune reaction (ORGENTEC Diagnostika GmbH, Mainz, Germany) according to the manufacturer's instructions. If samples were positive for IgG and IgA antibodies against deamidated gliadin peptide (DGP) epitopes, anti-DGP IgA was determined in a second step. Antibody titers ≥ 10 U/ml are considered elevated in both tests (https://products.orgentec.com/pdfs/ifu/ORG%20251S_IFU_EN_QM113115_2018-01-02_4.pdf).

Generation of HLA-C*06:2 tetramers

HLA-C*06:02 tetramers were generated essentially as described with a few minor modifications (28). A DNA construct (IDT) encoding the extracellular domain of HLA-C*06:02, with valine replacing alanine at codon 245 (29) and fused with a sequence encoding for the BirA biotinylation motif was inserted in a bacterial expression vector. The HLA-C*06:02 heavy chain and β_2 -microglobulin (β_2 m) were produced separately in *E. coli*. Bacteria were pelleted by centrifugation and lysed in a buffer containing lysozyme and Triton X-100. After several washes purified inclusion bodies were obtained and solubilized in a urea containing buffer. Inclusion bodies of the HLA-C*06:02 heavy chain and β_2 m were mixed with synthetic nonamer peptides for ADAMTSL5 or wheat-1 (LRMRRRCRRM) or wheat-2 (VRAGRVLVRV, see **Supplementary Table S2**) in a folding buffer containing a mix of reduced and oxidized glutathione. Folding reactions took place at 4°C in the dark for 3-5 days. The folded

complexes (or MHC monomers) were concentrated, dialyzed and biotinylated in a solution containing D-biotin and ATP to which the BirA enzyme (Avidity) was added. Biotinylated monomers were dialyzed against an NaCl 150 mM, Tris 10 mM pH 8.0 buffer and purified on a monomeric streptavidin affinity column (ThermoFisher) by elution with D-biotin. The biotinylated complexes were dialyzed against NaCl 150 mM, Tris 10 mM pH 8.0, concentrated and tested by HPLC on a gel filtration column (Cytiva) for determining monomer purity and percentage of free β 2m. Monomers were tetramerized with streptavidin conjugated to phycoerythrin or Brilliant Violet 421 at a 4/1 ratio (28). The HLA-C*06:02 tetramers were produced by MBL International, Woburn, MA, and will become commercially available.

Tetramer staining, gating strategy and flow cytometry analysis

Freshly isolated peripheral blood mononuclear cells (PBMC) (1×10^6 cells/ml) were resuspended in RPMI 1640 medium supplemented with 1% PenStrep, 10 μ g/ml ciprofloxacin and 10% FCS. Cells were incubated with 50 nM dasatinib (Santa Cruz Biotechnology, TX, USA), 50 U/ml IL-2 and 25 mM glucose at 37°C for 30 min (30). Either PE or BV421 labelled HLA-C*06:02 tetramers or both were added at a concentration of 10 μ g/ml each and incubated at 37°C for 25 min, and then stained with the following antibodies at 37°C for 30 min: Anti-human-CD3-FITC (Clone SK7, Invitrogen); Anti-human-CD8-PerCp/Cyanine5.5 (Clone: SK1, Biolegend); Anti-human-CD56-PE-Cy7 (Clone: B159, BD Pharmingen). Cells were washed and resuspended with FACS Buffer (PBS with 1% FBS) and then stained with 4', 6-diamidino-2-phenylindole (DAPI, 0.33 μ M) for 5 min at room temperature. All samples were acquired on BD LSRFortessa (BD Biosciences). Data were analyzed using FlowJo software v10.7.2 (FlowJo LLC). Antigen-specific TCR binding of the HLA-C*06:02 tetramers was verified by tetramer staining of the V α 3S1/V β 13S1 TCR hybridoma and two TCR hybridomas expressing TCRs of unknown specificity (Figure 4). The gating strategy that resulted in cell populations consisting of Live/CD3⁺/CD8⁺/CD56^{neg} HLA-C*06:02 tetramer⁺ cells is shown in Supplementary Figure S4. Lymphocytes were determined by forward (FSC) and side scatter (SSC) gating. To discriminate live from dead cells, the DAPI negative population was gated. Live cells were gated for the CD56-negative population to minimize the impact of Killer cell Ig-like receptors (KIR) of natural killer cells on tetramer binding. Next, live/CD56^{neg} were gated on the CD3⁺CD8⁺ double-positive cells. FALK-HLA-C*06:02 tetramer was used for comparison to determine the cut-off between background and positive staining of ADAMTSL5-HLA-C*06:02 tetramer⁺ cells. Flow cytometry analysis of PBMC stained with the HLA-C*06:02 tetramers and antibodies specific for CD3, CD56 and the anti-human-Killer-cell immunoglobulin-like receptor (KIR)/CD158, KIR2DL1/2DS1, had excluded binding of the HLA-C*06:02 tetramers to CD3⁺ T cells through KIRs.

Statistical analysis

Statistical analyses of the data given in Figures 3, 4 and Supplementary Figure S2 were performed using GraphPad Prism software (ver. 8) and further confirmed by XLSTAT ver2014.5. Microsoft Excel was used to store data and to run XLSTAT. For each value of multiple groups, non-parametric Kruskal-Wallis H test was used for multiple comparisons, and Bonferroni correction was applied ($P < 0.0001$, for [³H]-thymidine incorporation or CD137 expression, HC vs. PV). Two-group comparison was performed using a non-parametric two-sided Mann-Whitney U test for unpaired continuous variables. Two-tailed $P < 0.05$ was considered statistically significant. Age groups of patients and healthy controls were compared using Fisher's exact test. In principle, no data were excluded from analyses. Some stimulation experiments were excluded when the positive control samples with CD3 antibody stimulation from the same samples had failed or seeding cell numbers were inappropriately adjusted. Investigators were not blinded for samples except measurement of [³H]-thymidine incorporation.

Results

The peptide motif ligating the ADAMTSL5-specific psoriatic V α 3S1/V β 13S1 TCR is composed of five amino acids

To identify antigenic psoriasis triggers the HLA-C06:02-restricted psoriatic V α 3S1/V β 13S1 TCR was expressed in a reporter mouse hybridoma cell line which produces super green fluorescent protein (sGFP) upon TCR ligation (24). We employed HLA-C06:02-cotransfected COS-7 cells to present plasmid-encoded candidate peptides in co-culture experiments and analyzed TCR activation by measuring the percentage of sGFP⁺ hybridoma cells that reflects the degree of TCR stimulation (14).

We first precisely characterized the peptide recognition motif of the V α 3S1/V β 13S1 TCR. Alanine scanning mutagenesis (31) revealed that exchange of the amino acids at positions P1, P3, P4, and P6 against Ala had no effect on the antigenicity of the ADAMTSL5 nonamer (Figure 1A). Ala mutations of the amino acids at P 2, 7, or 9, which anchor the peptide in the HLA-C*06:02 binding groove (32–34), or of the amino acids at P5 or P8, which mediate contact of the ADAMTSL5 peptide with the V α 3S1/V β 13S1 TCR (33), completely abolished the ability to stimulate the V α 3S1/V β 13S1 TCR. The antigenicity of the ADAMTSL5 required Arg as HLA-C*06:02 anchor at P2, because it was lost upon exchange against other HLA-C06:02 anchor amino acids at this position (Gly, Thr, Pro, or Tyr) (32–34) (Figure 1B), while it tolerated a replacement against other HLA-C*06:02 anchor amino acids at P9 (Phe, Ile, Met, or Val, Figure 1C).

We integrated the mutagenesis data and previously identified mimotopes and self-peptide ligands of the V α 3S1/V β 13S1 TCR (13) into heat maps of amino acid preferences and sequence logos (Supplementary Table S1, Supplementary Figure S1A). This confirmed that the V α 3S1/V β 13S1 TCR recognition motif required

Arg as HLA-C*06:02 anchor residue at P2 and as TCR contact residue at P8, whereas other positions of the motif showed some variations. P5 was occupied either by Arg, Asn or Gln, while P7 could accommodate Arg or Leu, and P9 was occupied either by Leu, Met, Val, Phe or Ile. All other positions showed greater amino acid diversity. Accordingly, the recognition motif of the V α 3S1/V β 13S1 TCR is based on the five amino acid positions P2, P5, and P7-P9. It determines the cross-reactivity between different peptides exhibiting this motif. In octamers, the NH₂-terminus corresponds to P2.

Motif-based search identifies multiple environmental peptide ligands of the pathogenic psoriatic V α 3S1/V β 13S1 TCR

We searched the NCBI and UniProt protein databases with the aggregate data of the V α 3S1/V β 13S1-TCR recognition motif for environmental proteins containing homologous amino acid sequences that may elicit a cross-reactive autoimmune response against the melanocyte autoantigen. For this, we variably combined the fixed amino acids at P2 and P8 in an octamer search matrix with the other amino acids of the TCR recognition motif. For respective nonamers, P1 was completed according to the natural protein sequence.

After verifying high affinity binding to HLA-C*06:02 by the NetMHCpan 4.1 server (<https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1>), 50 different environmental peptides were selected from the search results (Supplementary Table S2) and used to stimulate the V α 3S1/V β 13S1 TCR as plasmid-encoded octamers or nonamers. 24 of them stimulated the V α 3S1/V β 13S1 TCR hybridoma (Figure 2). They were derived from various sources that included pathogenic bacteria, fungi, yeasts, skin or mucosal microbiota, and crops.

Thus, the TCR recognition motif had allowed us to identify diverse exogenous peptide ligands of the autoreactive psoriatic V α 3S1/V β 13S1 TCR. Stimulation with octamer and nonamer peptides from ADAMTSL5, wheat and *Flavonifractor plautii* revealed that the amino acid at P1 is of less importance for the antigenicity, as both the octamers lacking P1 and the nonamers stimulated the V α 3S1/V β 13S1 TCR, as recently reported (14) (Supplementary Figure S2). Sequence comparison between stimulatory and nonstimulatory ligands furthermore showed that the TCR recognition motif alone did not guarantee TCR ligation, as it was also present in non-stimulatory peptides (Supplementary Figures S1B, C). In particular, Arg at P6 seemed to prevent activation of the V α 3S1/V β 13S1 TCR. This indicates that certain amino acids at otherwise neutral peptide positions may alter the contact interface for the V α 3S1/V β 13S1 TCR.

Environmental peptides stimulating the autoreactive V α 3S1/V β 13S1 TCR are particularly immunogenic for CD8⁺ T cells from psoriasis patients

Exogenous peptides stimulating the autoreactive ADAMTSL5-specific V α 3S1/V β 13S1 TCR are potential environmental triggers of

the psoriatic autoimmune response. To assess their immunogenicity directly in patients, we stimulated peripheral blood mononuclear cells (PBMC) from patients with chronic plaque psoriasis or healthy controls with corresponding synthetic peptides. We focused on peptides originating from conditions directly related to the human environment in terms of nutritional and consumer products, infections, and the microbiota. Since the psoriatic autoimmune response is restricted by HLA-C*06:02 (13) and mediated by CD8⁺ T cells (8), we analyzed the antigen-induced T-cell proliferation of PBMC by [³H]-thymidine in combination with activation-induced CD137 expression on CD8⁺ T cells (Supplementary Figure S3), which are the actual pathogenic T cells in psoriasis (8). CD137 is a member of the TNFR-family. It is induced by functional stimulation on human CD8⁺ T-cells and is a reliable marker for their antigen-specific activation (27). A peptide of unknown origin with the sequence VRHDGGNVL, here referred to as the “FALK” peptide, which is known to bind to HLA-C*06:02 as it was formerly isolated from an HLA-C*06:02⁺ lymphoblastic B cell line (25), served as a reference unrelated to psoriasis pathogenesis.

Measurement of peptide-induced [³H]-thymidine incorporation showed that most environmental antigens had higher immunogenicity for psoriasis patients than for healthy subjects (Figures 3A, C). Besides the ADAMTSL5 octamer and nonamer, the difference was statistically significant for peptides from wheat (*Triticum aestivum*, wheat-1: RCG1B, wheat-2: Hypothetical protein CFC21_050201), *Chlamydia trachomatis*, *Klebsiella pneumoniae*, and *Mycobacterium tuberculosis*. Analysis of CD137 expression (Figures 3B, D) revealed that several peptides induced a significantly higher CD137 expression on CD8⁺ T cells from psoriasis patients than from healthy controls. In addition to ADAMTSL5, they included peptides from wheat, *Actinomyces oris*, *C. trachomatis*, the IQM3-like isoform from *Nicotiana tabacum* (tobacco), *Saccharomyces cerevisiae* as well as *Clostridium* sp. and *Streptococcus agalactiae*, both of which belong to the microbiota. The particular immunogenicity of ADAMTSL5 and wheat peptides for psoriasis patients is highlighted by the coincidence of significantly increased stimulation-induced [³H]-thymidine incorporation and CD137 expression on CD8⁺ T cells (Figures 3A, B).

CD8⁺ T cells may recognize both the autoantigen and wheat peptides

To investigate whether, according to the polyspecificity of the V α 3S1/V β 13S1 TCR, the same CD8⁺ T cells in psoriasis patients can recognize both the ADAMTSL5 peptide and environmental antigens, we employed fluorescent HLA-C*06:02 tetramers loaded either with the ADAMTSL5 or the wheat peptides to stain CD8⁺ T cells from HLA-C*06:02⁺ psoriasis patients and healthy individuals.

The binding specificity of the peptide-loaded tetramers was established by staining of the V α 3S1/V β 13S1-TCR hybridoma with ADAMTSL5 or wheat peptide-loaded but not FALK peptide-loaded HLA-C*06:02 tetramers, which served as disease-unrelated control (Figure 4A). None of these peptide-loaded HLA-C*06:02 tetramers bound to hybridomas carrying unrelated TCRs (data

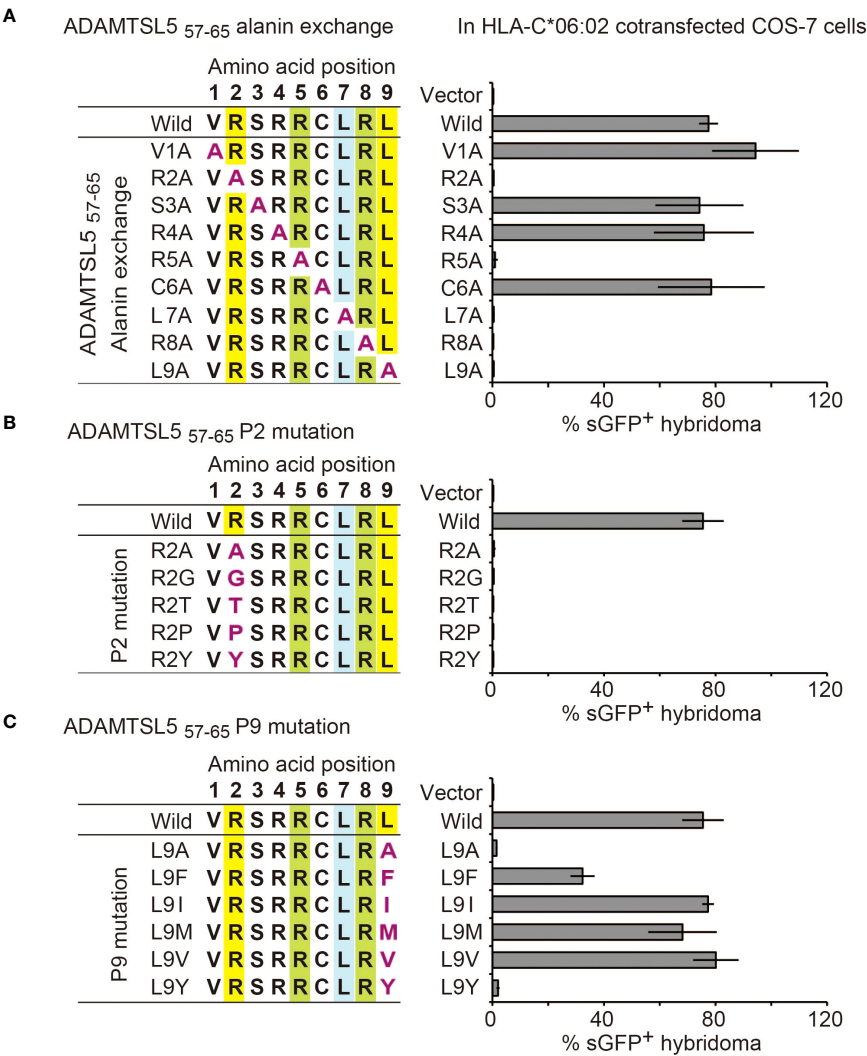


FIGURE 1 Mutagenesis determines the recognition motif of the ADAMTSL5-specific psoriatic V α 3S1/V β 13S1 TCR. Relevance of individual amino acid residues of the ADAMTSL5 nonamer peptide for stimulation of the V α 3S1/V β 13S1-TCR hybridoma as determined by (A) alanine scanning mutagenesis or exchange against other HLA-C*06:02 anchor residues at (B) P2 or (C) P9. Mutated peptides were cloned into plasmids, cotransfected with HLA-C*06:02 in COS-7 cells and used to stimulate the V α 3S1/V β 13S1 TCR hybridoma in co-culture experiments. Percentages of sGFP⁺ hybridoma cells were assessed by flow cytometry after 24h and normalized to CD3 stimulation data in the same experiment. Data are summarized from duplicates (A) or (B, C) triplicates of two independent experiments and shown as mean $\pm$ SEM.

not shown). Strict gating of the flow cytometry analyses on CD3⁺ CD8⁺ T cells further excluded that staining results were confounded by tetramer-binding to CD56⁺ natural killer cells or KIR2DL1/2DS1 receptors.

When PBMC were examined, the ADAMTSL5- and wheat-peptide loaded HLA-C*06:02 tetramers stained significantly more CD8⁺ T cells of HLA-C*06:02⁺ psoriasis patients than of HLA-C*06:02⁺ healthy subjects (Figures 4A–C). Simultaneous staining with the ADAMTSL5- and wheat-peptide loaded HLA-C*06:02 tetramers showed that a proportion of CD8⁺ T cells can recognize both the autoantigen and environmental peptides. Moreover, significantly more CD8⁺ T cells were observed to recognize both ADAMTSL5 and wheat peptides in psoriasis patients than in healthy controls (Figures 4D, E).

Gluten-free diet (GfD) can improve psoriasis in some patients

Our data suggested that wheat antigens may stimulate the psoriatic autoimmune response. As a result, several patients with inadequate response to ongoing systemic psoriasis therapy and lack of therapeutic alternatives opted for a GfD, since gluten-free foods are certified wheat-free. Clinical follow-up showed that seven out of 13 patients experienced a stable improvement in their previously treatment-resistant psoriasis while on a gluten-free diet (Table 1, Figures 5A–C). Two patients with psoriatic arthritis (#8, 10) that was not sufficiently controlled by systemic treatments became symptom-free (Figure 5B). Two patients (#8, 9) experienced a marked psoriasis relapse after dietary errors.

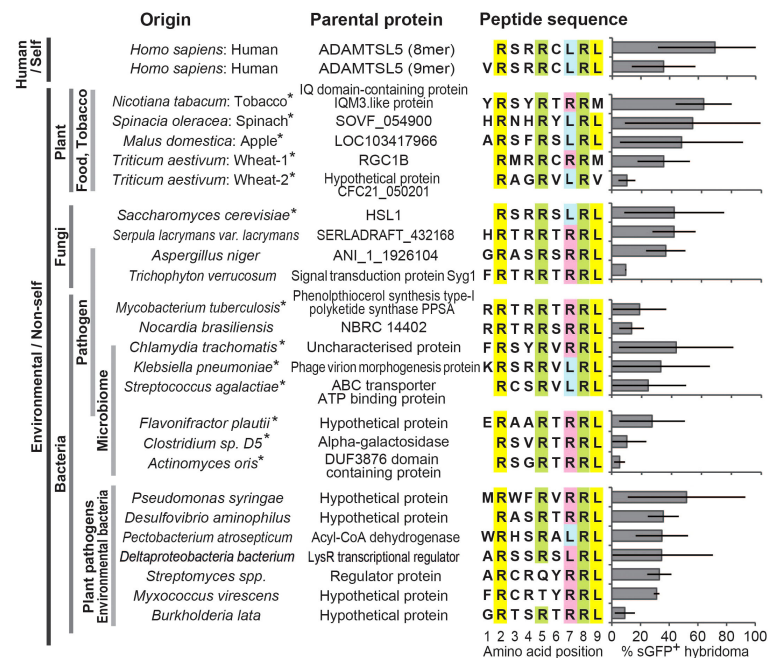


FIGURE 2
Homology search with the TCR recognition motif identifies various environmental peptide ligands of the ADAMTSL5-specific Vα3S1/Vβ13S1 TCR. Response to stimulation of the Vα3S1/Vβ13S1-TCR hybridoma by co-culture with COS-7 cells cotransfected with HLA-C*06:02 and plasmids encoding the ADAMTSL5 epitope or various environmental candidate antigens. Induction of sGFP was determined by flow cytometry analysis after 24h. Results are given as mean relative to CD3 stimulation, bars represent SD. Data are from at least 3 individual analyses or duplicates from two or more independent experiments. Anchor residues for HLA-C*06:02 at P2 and P9 are highlighted in yellow, the auxiliary anchor at position 7 in blue or red and the TCR contact residues of the Vα3S1/Vβ13S1 TCR recognition motif at positions 5 and 8 in green. Asterisks indicate peptides used to stimulate PBMC.

The presence of gliadin serum antibodies, which may be elevated in psoriasis patients (35), did not predict the response to GfD (Table 1), providing further evidence that CD8⁺ T cells, rather than antibodies, mediate the pathogenic effect of environmental antigens in psoriasis. In patient #12, we had measured the frequency of ADAMTSL5- and wheat-specific CD8⁺ T cells in blood during the GfD by peptide-HLA-C*06:02 tetramer staining. The number of ADAMTSL5- as well as wheat-specific CD8⁺ T cells, and of CD8⁺ T cells that recognize both peptides, declined in the course of dietary wheat avoidance (Figure 5D).

Discussion

Analysis of the cross-reactive potential of the pathogenic psoriatic Vα3S1/Vβ13S1 TCR indicated that a variety of environmental antigens may cross-activate the psoriatic autoimmune response against melanocytes due to TCR polyspecificity. Based on its peptide recognition motif, we identified multiple environmental antigens, all of which ligated the Vα3S1/Vβ13S1 TCR. Several of them were particularly immunogenic for CD8⁺ T cells from psoriasis patients. Remarkably, their origin corresponded to previously suspected psoriasis risk conditions.

The environmental antigens stimulating the Vα3S1/Vβ13S1 TCR and CD8⁺ T cells from psoriasis patients included two peptides from wheat. Detection of dual-specific CD8⁺ blood T cells recognizing both the ADAMTSL5 epitope and wheat peptides directly in patients, by

means of peptide-loaded HLA-C*06:02 tetramers, revealed that a cross-reactive T cell response between environmental antigens and self-peptides is a potential pathogenic mechanism in psoriasis. Improvement of psoriasis and psoriatic arthritis observed by us and others (36, 37) in patients under a GfD further supported that wheat peptides may indeed cross-activate the psoriatic autoimmune response. Many wheat peptides resist enzymatic digestion (38), and intact wheat peptides can be detected in blood (39, 40). A T cell-mediated immune response against wheat antigens causes celiac disease and various other pathologic conditions (41), with psoriasis patients being at an increased risk of celiac disease (42). The occasional strikingly high frequency of circulating ADAMTSL5- or wheat peptide-specific CD8⁺ T cells in blood is consistent with other systemic autoimmune diseases such as ANCA vasculitis, which exhibits 6% or more of blood T cells recognizing HLA-tetramer-loaded epitopes of myeloperoxidase, a major ANCA autoantigen (43). In individuals with psoriasis affecting 20% of their body surface area, psoriatic plaques contain approximately 2 x 10¹⁰ T cells (44). The high frequency of circulating ADAMTSL5-specific T cells detected in psoriasis patients may reflect the fact that the large number of pathogenic T cells infiltrating psoriatic skin lesions may require a continuous T cell supply from the circulation. Indeed, preventing the egress of T cells from secondary lymphoid organs by S1P1 agonists or inhibiting T cell recruitment into the inflammatory sites by integrin (CD11a) blockade effectively ameliorates psoriasis (45, 46). Accordingly, measurement of ADAMTSL5 or wheat peptide-specific blood T cells could be a prognostic biomarker for a potential beneficial effect of a wheat-free diet on disease progression.

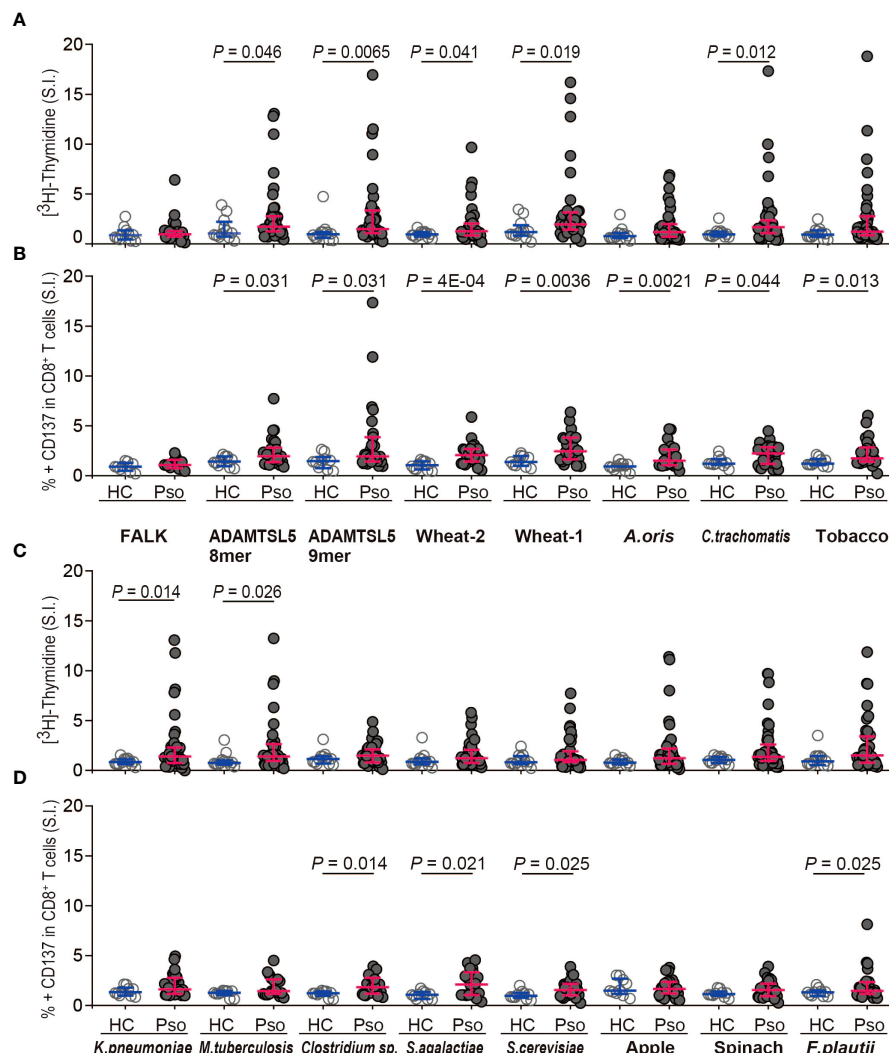


FIGURE 3

Environmental Vα3S1/Vβ13S1 TCR ligands preferentially activate PBMC and CD8⁺ T cells from psoriasis patients. (A, C) [³H]-thymidine incorporation by PBMC from healthy controls (HC, *n* = 12) and psoriasis patients (Pso, *n* = 41) stimulated with synthetic peptides shown in Figure 2 for 5d. The stimulatory response is expressed as stimulation index that represents the fold-increase over the control sample cultured without peptides. FALK peptide served as disease-unrelated control. Each dot represents one individual. Results are given as the median of cpm of triplicates for each sample and compared between groups by non-parametric two-sided Mann-Whitney U test. Bars indicate median and interquartile values. (B, D) Activation of CD8⁺ T cells by stimulation with synthetic peptides for 24h as determined by the induction of CD137 expression on CD8⁺ T cells from HC (*n* = 10) and Pso (*n* = 28). Results are given as stimulation index that is calculated from the mean percentage of CD137⁺ CD8⁺ T cells of duplicates. Two-group comparison was performed using Mann-Whitney U test for unpaired continuous variables. *P*-values < 0.05 are indicated.

The microbiota are thought to play an important role in the development of psoriasis. Firmicutes are significantly overrepresented in skin and gut microbiota of psoriasis patients, and a high intestinal Firmicutes/Bacteroidetes ratio directly correlates with psoriasis severity (47). We identified peptides from *S. agalactiae*, *Clostridium* sp., and *F. plautii* of the bacterial phylum Firmicutes, which activated the ADAMTSL5-specific Vα3S1/Vβ13S1 TCR and stimulated CD8⁺ T cells from psoriasis patients. Another immunogenic peptide was derived from *A. oris*. Periodontitis, associated with a high prevalence of *A. oris* in the oral cavity (48), has been linked to the initiation or propagation of psoriasis and psoriatic arthritis through the immunomodulatory effects of the oral microbiota (49). Continuous exposure to cross-activating microbiota antigens could, therefore,

trigger or maintain activation of the psoriatic autoimmune response in psoriasis patients.

Another immunogenic peptide was derived from the baker's and brewer's yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is part of the intestinal mycobiome (50) and is present in many foods. As it ferments glucose to alcohol, it is contained in alcoholic beverages, the consumption of which is a potential risk factor for psoriasis (51). Yeast antigens prime long-lasting T_H17 responses (52). Therefore, *Saccharomyces* antigens released by autolysis of the yeast cells in the gut or during fermentation in alcoholic beverages (53) could induce a cross-reactive autoimmune response against melanocytes, which differentiates into the pathogenic psoriatic T_H17 phenotype. Remarkably, *S. cerevisiae* antigens drive pathogenic T cell

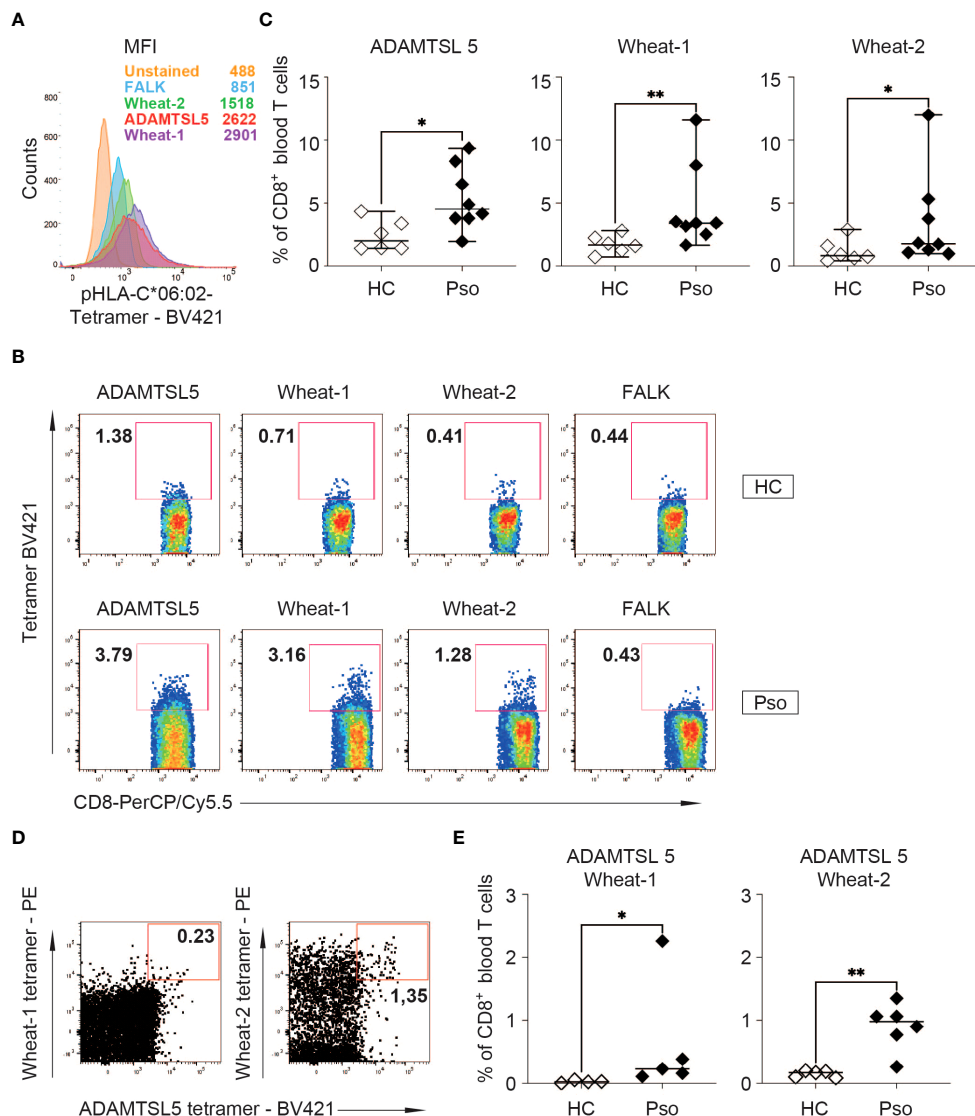


FIGURE 4

Peptide-HLA-C*06:02 tetramer staining of PBMC shows an increased frequency of ADAMTSL5 and wheat-specific CD8⁺ T cells in psoriasis patients. (A) Binding to the V α 3S1/V β 13S1 TCR hybridoma and mean fluorescence staining intensity (MFI) of Brilliant Violet 421 (BV421)-labelled ADAMTSL5, wheat-1, wheat-2 and FALK peptide-HLA-C*06:02 tetramers. According to CD3 antibody staining, approximately 60% of the hybridoma cells express the V α 3S1/V β 13S1 TCR. (B) Representative staining of PBMCs from a HC and a psoriasis patient with the BV421-labelled ADAMTSL5, wheat-1, wheat-2 and FALK peptide-HLA-C*06:02-tetramers. (C) Frequency of CD8⁺ T cells binding ADAMTSL5 or wheat peptide-HLA-C*06:02 tetramers in HLA-C*06:02⁺ healthy controls (HC, $n = 6$) or psoriasis patients (Pso, $n = 8$). (D, E) Representative staining of CD8⁺ T cells from a psoriasis patient and frequency of CD8⁺ T cells double-stained with BV421-labelled ADAMTSL5-HLA-C*06:02 tetramers and PE-labelled wheat-1-HLA-C*06:02 (HC: $n = 4$; Pso: $n = 5$) or wheat-2-HLA-C*06:02 tetramers (HC: $n = 5$; Pso: $n = 6$). The data in (C, E) represent mean values of duplicates. Differences in Live/CD3⁺CD8⁺ tetramer single⁺ or double⁺ cell frequency between HC and Pso (C, E) were assessed by Mann-Whitney U test. P -values < 0.05 are indicated. * P 0.05; ** P 0.01.

responses in Crohn's disease (54), which is associated with psoriasis (55) and shares HLA-C*06:02 as a risk gene (56). Avoiding alcohol intake improved psoriasis (36).

Smoking is another independent risk factor of psoriasis (57), and passive tobacco smoke exposure enhances the risk of psoriasis in childhood (58, 59). The cross-activating peptide from the IQ domain-containing protein isoform (IQM3-like isoform) found in tobacco (*Nicotiana tabacum*) and other crops (Supplementary Table S3) might trigger the autoimmune response against melanocytes in psoriasis through inhalation of incompletely burnt tobacco proteins, which are transferred to smoke aerosols (60).

C. trachomatis infections can induce aberrant autoimmune responses characterized by ankylosing spondylitis, uveitis and inflammatory skin changes resembling psoriasis (61), and psoriatic arthritis patients show an increased prevalence of Chlamydia exposure (62). This attributes a potential pathogenic significance to the chlamydial epitope.

The conditions that either induce or maintain the psoriatic autoimmune response may differ. Infections may break immune tolerance and activate potentially autoreactive naive T cells through strong proinflammatory signals, such as in streptococcal angina, which may precede psoriasis exacerbation (63, 64). Once an

TABLE 1 Patient characteristics and response to gluten-free diet.

Patient #	Sex	Clinical type	Age of disease onset	Gliadin – specific IgG, IgA screen	IgG plus IgA U/ml	IgA U/m	Age at GfD	Ongoing Treatment	PASI at start of GfD	PASI improvement	PsA improvement	Relapse on dietary error
Non-Responders												
#1	M	PsO	23y	pos	11.1	2	43y	Acitretin	15.3	NI	Ø	NR
#2	M	PsO	7y	neg	9.8	3.2	30y	FAE	14	NI	Ø	NR
#3	F	PsO	32y	pos	ND	ND	64y	anti-TNF	25.4	NI	Ø	NR
#4	M	PsO, PsA	51y	pos	12.5	1	55y	anti IL-12/IL-23	17.6	NI	NI	NR
#5	M	PsO	17y	pos	10.6	8	47y	Acitretin	12.2	NI	Ø	NR
#6	M	PsO	11y	pos	12.5	8.5	36y	Acitretin + FAE	26	NI	Ø	NR
Responders												
#7	M	PsO	7y	pos	13.5	12.1	41y	Acitretin	28.5	PASI90	Ø	NR
#8	M	PsO, PsA	9y	neg	neg	neg	55y	Acitretin	22	PASI90	Yes	Yes
#9	M	PsO, PPPP	28y	pos	17.9	17.9	73y	Aprimelast	15.9	PASI75	Ø	Yes
#10	F	PsO, PsA	27y	pos	19.9	17.6	70y	ADA, αIL-12/IL-23, αIL-17 disc.	8.8	PASI75	Yes	NR
#11	M	PsO	26y	neg	neg	neg	48y	Acitretin	18.4	PASI90	Ø	NR
#12	M	PsO	30y	neg	neg	neg	73	MTX, ADA disc.	15.0	PASI90	Ø	NR
#13	M	PsO	16y	neg	neg	neg	39y	Acitretin	14.7	PASI75	Ø	NR

GfD, gluten-free diet; M, male, F, female; y, years; PsO, chronic plaque psoriasis; PsA, psoriatic arthritis; PPPP, palmo-plantar pustular psoriasis; pos, positive; neg, negative; FAE, Fumaric acid esters; MTX, methotrexate; ADA, adalimumab; αIL-12/IL-23, αIL-17, antibodies against IL-12/IL-23 or IL-17; PASI, psoriasis area and severity index; NI, no improvement; PASI75/90, improvement in PASI of ≥75%/≥90%; Ø, not suffering from PsA; ND, not determined; NR, not reported; disc., discontinued. Gliadin IgG and IgA screen before GfD is considered elevated if ≥ 10 U/ml.

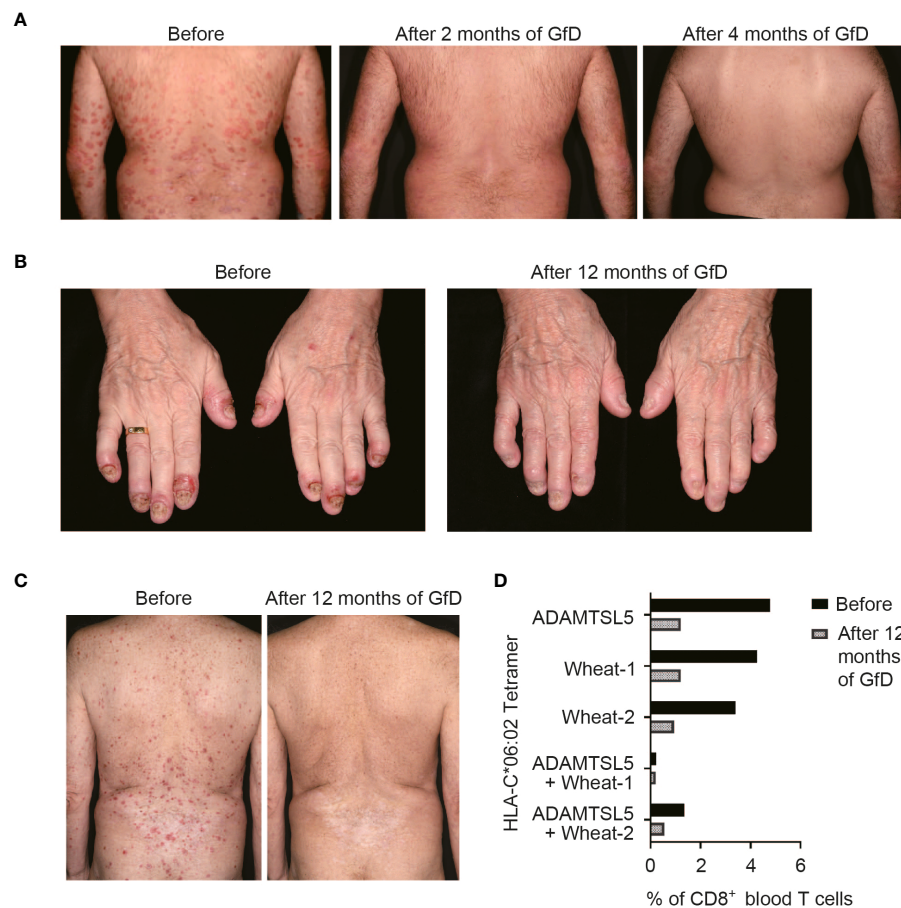


FIGURE 5

Psoriasis may improve upon a gluten-free diet (GfD). (A) Without any change in the therapeutic management, the previously refractory psoriasis cleared within two months upon a GfD in patient #7. (B) The formerly treatment-resistant, highly painful DIP-arthritis in patient #10 resolved completely on a GfD, without the need for further treatment. (C) Stable psoriasis remission following discontinuation of methotrexate and adalimumab treatment during GfD in patient #12. In this patient, live/CD3⁺CD8⁺CD56⁺ single or double tetramer-stained cells were measured by flow cytometry before and 12 months after starting GfD (D). Patient data are given in Table 1.

autoimmune response has been established, stimulation by environmental peptides mimicking the autoantigen may maintain disease activity under less inflammatory conditions. CD8⁺ memory T cells show a much lower activation threshold than naive CD8⁺ T cells (22, 65, 66), and common proinflammatory gene variants associated with psoriasis could provide the necessary costimulatory innate immune signals maintaining T-cell activation by environmental antigens (3). Avoiding stimulatory environmental antigens, such as through a wheat-free diet, may disrupt the constant generation of cross-reactive pathogenic T cells in lymphoid organs, thereby ameliorating psoriasis.

Overall, we show that due to TCR polyspecificity, multiple environmental antigens from different psoriasis risk conditions converge in the reactivity of an autoreactive psoriatic TCR. The various peptide antigens cross-activating the V α 3S1/V β 13S1 TCR provide an explanation for the initiation or perpetuation of the T-cell mediated psoriatic autoimmune response against melanocytes by diet, lifestyle, microbiota, and infections. Identification of the environmental antigens through TCR polyspecificity could thus

help to develop new approaches to treating psoriasis. Given the multitude of potential triggers, improving psoriasis through lifestyle changes may require a multimodal approach tailored to individual patients. Avoiding wheat products, alcohol, and tobacco might benefit a significant proportion of the huge global psoriasis patient population (2). Thus, gaining insights into the specific response pattern of the autoreactive V α 3S1/V β 13S1 TCR against environmental antigens may provide a key to a better pathogenetic understanding and practical management of psoriasis. Our data further support that polyspecificity may be a crucial property of TCRs in the induction of human autoimmune diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Medical Faculty, Ludwig-Maximilian-University of Munich. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

TI: Investigation, Methodology, Visualization, Writing – review & editing. YA: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. SVu: Data curation, Investigation, Methodology, Validation, Writing – review & editing. JS: Data curation, Investigation, Methodology, Validation, Writing – review & editing. SVo: Investigation, Methodology, Writing – review & editing. AG: Investigation, Methodology, Writing – review & editing. KS: Formal analysis, Investigation, Methodology, Writing – review & editing. GR: Investigation, Methodology, Validation, Writing – review & editing. YP: Methodology, Resources, Writing – review & editing. MD: Methodology, Resources, Writing – review & editing. OH: Investigation, Methodology, Writing – review & editing. MH: Data curation, Investigation, Methodology, Writing – review & editing. BS: Investigation, Methodology, Writing – review & editing. RP: Investigation, Methodology, Writing – review & editing. RA: Investigation, Writing – review & editing. KD: Funding acquisition, Methodology, Supervision, Writing – review & editing. AA: Investigation, Methodology, Project administration, Visualization, Writing – review & editing. JP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

YP and MD are employees of MBL International, Woburn, MA, U.S.A. MBL International may market the tetramers for diagnostic purposes.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1374581/full#supplementary-material>

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Transcriptomics in idiopathic pulmonary fibrosis unveiled: a new perspective from differentially expressed genes to therapeutic targets

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Background: The underlying molecular pathways of idiopathic pulmonary fibrosis (IPF), a progressive lung condition with a high death rate, are still mostly unknown. By using microarray datasets, this study aims to identify new genetic targets for IPF and provide light on the genetic factors that contribute to the development of IPF.

Method: We conducted a comprehensive analysis of three independent IPF datasets from the Gene Expression Omnibus (GEO) database, employing R software for data handling and normalization. Our evaluation of the relationships between differentially expressed genes (DEGs) and IPF included differential expression analysis, expression quantitative trait loci (eQTL) analysis, and Mendelian Randomization (MR) analyses. Additionally, we used Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO)/Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to explore the functional roles and pathways of these genes. Finally, we validated the results obtained for the target genes.

Results: We identified 486 highly expressed genes and 468 lowly expressed genes that play important roles in IPF. MR analysis identified six significantly co-expressed genes associated with IPF, specifically C12orf75, SPP1, ZG16B, LIN7A, PPP1R14A, and TLR2. These genes participate in essential biological processes and pathways, including macrophage activation and neural system regulation. Additionally, CIBERSORT analysis indicated a unique immune cell distribution in IPF, emphasized the significance of immunological processes in the disease. The MR analysis was consistent with the results of the analysis of variance in the validation cohort, which strengthens the reliability of our MR findings.

Conclusion: Our findings provide new insights into the molecular basis of IPF and highlight the promise of therapeutic interventions. They emphasize the potential of targeting specific molecular pathways for the treatment of IPF, laying the foundation for further research and clinical work.

KEYWORDS

idiopathic pulmonary fibrosis, differentially expressed genes, microarray data, eQTL analysis, Mendelian randomization, immune cell infiltration

1 Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressively worsening lung disease characterized by increased fibrosis and deterioration of lung function. This deterioration eventually leads to respiratory failure and death. The disease has a grim prognosis, with patients typically surviving only 3–5 years following diagnosis (1). Therefore, there is an urgent need for new effective treatment alternatives. Despite the increasing incidence and prevalence of IPF, knowledge of the disease remains limited, which poses a significant challenge for the treatment of IPF.

In recent years, IPF research has primarily focused on several aspects, notably the emerging cellular and molecular determinants, growth factors, cytokine pathways, genetic susceptibility, cellular senescence, and the potential of anti-aging drugs in enhancing alveolar epithelial cell function to alleviate pulmonary fibrosis (2–5). In addition, recent studies have found that inhibition of the MARCKS-PIP3 pathway delay the evolution of fibrosis and reduce fibroblast activation (6).

The relationship between inflammation and IPF remains a controversial topic. Although inflammation is implicated in the pathogenesis of the disease, the efficacy of anti-inflammatory drugs remains controversial, as highlighted by the unfavorable results of various multicenter clinical trials (7). IPF is primarily a fibrotic condition driven by abnormal activation of alveolar epithelial cells. This activation triggers the formation of scar tissue, resulting in the destruction of lung structure (8). The advent of antifibrotic drugs such as nintedanib and pirfenidone, which have significantly slowed the progression of IPF, has been a major breakthrough. However, the long-term survival benefits of these drugs remain to be finalized (9). Biologic agents might be a more favorable choice for current idiopathic pulmonary fibrosis treatment approaches. Research indicates that, although the efficacy of most biologics in treating IPF is limited, certain therapeutic strategies have demonstrated potential in improving patient quality of life. Further research is required to confirm the safety and effectiveness of these biologics, providing a new direction for future IPF treatment research (10). This research is dedicated to exploring the intricate mechanisms underlying IPF, with a particular focus on cellular and molecular aspects that could unveil novel therapeutic targets. In light of the limited existing treatments and the high mortality associated with IPF, investigating these novel mechanisms is essential for devising more effective treatment approaches.

This study is dedicated to exploring the complex mechanisms of IPF, with a particular focus on cellular and molecular aspects to reveal new therapeutic targets. Given the limited number of available treatments and the high mortality rate associated with IPF,

investigating these novel mechanisms is essential to design more effective therapies.

The main objective of this study was to identify differentially expressed genes (DEGs) in IPF compared to normal samples by analyzing microarray datasets. The study aims to assess the association and causality of these genes with the pathogenesis of IPF through expression quantitative trait loci (eQTL) and MR analysis. In addition, the study will employ Gene Ontology (GO)/Kyoto Encyclopedia of Genomes (KEGG) enrichment analysis and Genome Enrichment Analysis (GSEA) to investigate the potential functional pathways and pathogenesis associated with these DEGs. The study hopes that these approaches will reveal the molecular basis of IPF and lay the foundation for new therapeutic strategies. The study will also suggest areas for future research, emphasizing the importance of a comprehensive understanding of the complex pathobiology of IPF for the development of innovative therapeutic approaches.

2 Materials and methods

2.1 Data collection

Gene expression datasets and clinical phenotype data matching the search terms “idiopathic pulmonary fibrosis”, “Homo sapiens” and “gene expression” were obtained by microarray dataset analysis. All measured gene expression data and corresponding platform probe annotations are downloadable from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). For idiopathic pulmonary fibrosis, dataset filtering criteria included a minimum of eight samples, at least four cases and four controls, samples not chemically treated or genetically modified, and the availability of original data or array gene expression profile analysis in the GEO database.

2.2 Identification of DEGs

R software (version 4.3.2) was used to read and preprocess datasets GSE24206, GSE53845, and GSE195770 for individual dataset correction. The datasets were then merged, and batch correction and differential analysis were performed on 18 normal samples and 61 idiopathic pulmonary fibrosis samples. The “limma” package was used for classical Bayesian data analysis to filter DEGs, with significance criteria set at $P < 0.05$ and $\log\text{FoldChange (LogFC)} > 0.585$. The “pheatmap” package generated volcano plots and heatmaps of DEGs. Gene expression matrices and annotation files downloaded from the GEO database were used for data normalization and standardization. Principal component analysis (PCA) using the “prcomp” function was conducted to eliminate batch effects and facilitate visualization, further assessing and validating key genes distinguishing IPF from healthy control samples.

2.3 eQTL analysis of exposure data

For identifying genetic variants linked to gene expression, eQTL analysis was conducted, leveraging transcriptome and genotype

Abbreviations: IPF, Idiopathic Pulmonary Fibrosis; DEGs, Differentially Expressed Genes; GEO, Gene Expression Omnibus; eQTL, expression Quantitative Trait Loci; MR, Mendelian Randomization; GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, Principal Component Analysis; IVW, Inverse Variance-Weighted; GWAS, Genome-Wide Association Studies; CPI-17, C-kinase-activated PP1 inhibitor, 17kDa; PP1, Protein Phosphatase 1; TLR2, Toll-like Receptor 2; TNF- α , Tumor Necrosis Factor- α .

data from various cohorts. The most extensive meta-analysis of eQTL data to date, conducted by Westra et al., incorporated peripheral blood eQTL data from 5,311 European individuals (11). Summary eQTL data utilized in this study were retrieved from the GWAS Catalog website (<https://gwas.mrcieu.ac.uk/>). The R package “TwoSampleMR” was employed to identify strongly associated SNPs ($p < 5e-08$) as instrumental variables. Linkage disequilibrium parameters were set at $r^2 < 0.001$ and clumping distance = 10,000 kb. SNPs with weak associations or insufficient explanation of phenotypic variance were excluded, applying a filter of “F-test value > 10 ”.

2.4 Determination of outcome data

Outcome data were derived from the genetic association database of the GWAS summary dataset (IEU) (<https://gwas.mrcieu.ac.uk/>). The GWAS ID used was finn-b-IPF, involving 1,028 cases and 196,986 European ancestry controls, including 16,380,413 SNPs. All GWAS summary statistics used in this study are publicly available and free to download. Ethical approval was obtained through the original analysis.

Outcome data were sourced from the genetic association database of the GWAS summary dataset (IEU) available at GWAS Catalog (<https://gwas.mrcieu.ac.uk/>). The specific GWAS ID used was finn-b-IPF, which included 1,028 cases and 196,986 controls of European ancestry, encompassing 16,380,413 SNPs. All GWAS summary statistics referenced in this study are publicly accessible and downloadable. Ethical clearance for this study was granted based on the original analyses conducted.

2.5 MR analysis

MR analysis was performed using the “TwoSampleMR” software package. The inverse variance-weighted (IVW) method was employed to investigate the relationship between specific genes and idiopathic pulmonary fibrosis. Additional sensitivity analyses were carried out using MR-Egger, simple mode, weighted median, and weighted mode methodologies (12, 13). Disease-related genes were identified using a threefold criteria approach: 1. Genes demonstrating a P-value of less than 0.05 in the IVW method were initially selected. 2. Genes were further refined based on the consistency of the direction of MR analysis results (Odds Ratio values) across three different methods. 3. Genes exhibiting signs of pleiotropy with a P-value of less than 0.05 were excluded from the selection.

Following this process, co-expressed genes among these disease-related genes and the DEGs, encompassing both up-regulated and down-regulated genes, were identified through intersection. All intersecting genes were then individually subjected to MR analyses analysis to ascertain their causal links to the disease. This analysis included heterogeneity tests, pleiotropy tests, and leave-one-out sensitivity analyses, to evaluate the robustness and reliability of the results. To visually represent and support these

findings, scatter plots, forest plots, and funnel plots were created and analyzed.

2.6 GO/KEGG enrichment analysis

The “clusterProfiler” R package was used for GO functional annotation and KEGG pathway enrichment analysis of co-expressed genes to understand potential functional pathways and pathogenesis mechanisms. The “clusterProfiler” package is an ontology-based tool for biological term classification and gene cluster enrichment analysis, with the study’s filtering criterion set at $P < 0.05$.

2.7 Immune cell analysis

CIBERSORT analysis was employed to assess the infiltration levels of 22 types of immune cells in idiopathic pulmonary fibrosis (14), exploring the correlation between co-expressed genes in IPF and immune cell infiltration, and further investigating the regulatory mechanisms of IPF co-expressed genes on immune cells.

2.8 GSEA enrichment analysis

GSEA was used to determine whether functions or pathways related to co-expressed genes were enriched at the top or bottom of the ranking, indicating upregulation or downregulation trends, respectively. GSEA enrichment analysis was further employed to explore the activity level of related functions or pathways in the gene expression group. In GSEA, a p-value < 0.05 was considered statistically significant.

2.9 Validation group differential analysis

R software (version 4.3.2) was used to read dataset GSE135065 (preprocessed using the same methods as before) to validate whether co-expressed genes exhibited differences between control and experimental groups, and to compare these findings with the results of our MR analyses analysis.

3 Results

3.1 Overview of the three GEO datasets

This study obtained three IPF microarray datasets from the GEO database as the experimental group. These three datasets together comprise 61 IPF patients and 18 healthy controls. Table 1 provides detailed information about the datasets included.

We corrected and merged the expression values of each gene in its respective dataset using R version 4.3.2, and eliminated batch effects through Principal Component Analysis (PCA). As shown in

TABLE 1 Characteristics of the Three GEO Datasets.

GSE ID	Samples	Tissues	Platform	Experiment type	Last update date
GSE24206	17 cases and 6 controls	lung tissue	GPL570	Array	Mar 25, 2019
GSE53845	40 cases and 8 controls	lung tissue	GPL6480	Array	Jan 23, 2019
GSE195770	4 cases and 4 controls	lung tissue	GPL20844	Array	Jan 03, 2023

Figure 1, the batch effects in the three IPF gene datasets are evident. After correction, as displayed in Figure 2, all samples in the dataset achieved acceptable homogeneity following PCA analysis.

3.2 DEGs identification

In the obtained results, the smaller the p-value, the higher the reliability of gene ranking and differential gene expression. Ultimately, we detected 486 up-regulated DEGs and 468 down-regulated DEGs. Supplementary Table S1 provides detailed information on these significantly differentially expressed genes. The heatmap of DEGs expression in Figure 3 displays the top 50 up-regulated DEGs and the top 50 down-regulated DEGs. The volcano plot of the integrated GEO dataset is shown in Figure 4.

3.3 MR analysis

After screening, we ultimately obtained 26,152 SNPs as instrumental variables, all of which adhered to the three basic assumptions of MR, and all selected SNPs had F-statistics exceeding 10 (Supplementary Table S2 provides detailed information on the included data).

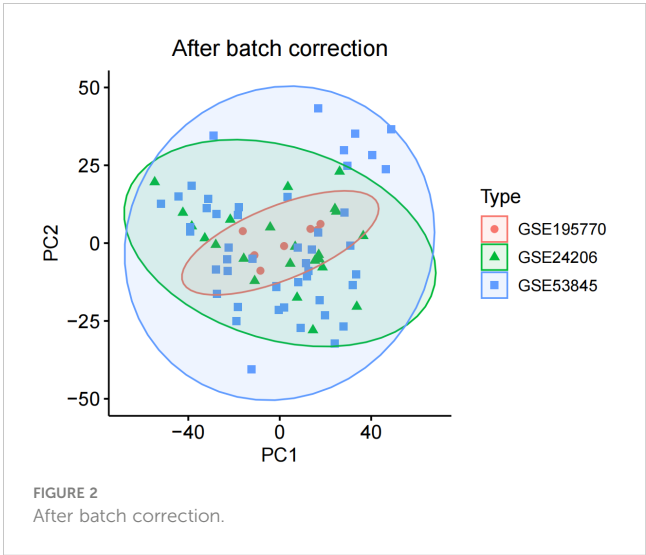
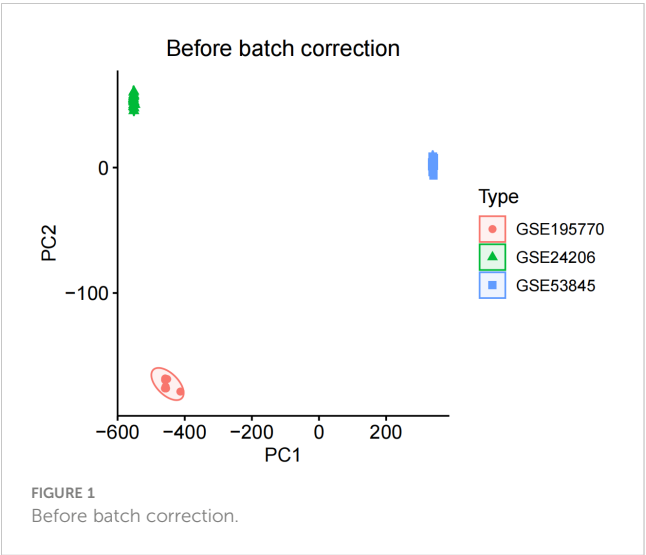
Through the results of MR analysis and the three established filtering criteria, we identified 202 IPF-related genes (Supplementary Table S3 provides detailed information on the data included). Further by intersecting, we obtained co-expressed genes between disease-related genes and DEGs, including 3 up-regulated genes (C12orf75,

SPP1, ZG16B) and 3 down-regulated genes (LIN7A, PPP1R14A, TLR2), as shown in Figures 5A, B.

Subsequently, we conducted MR analyses on these six co-expressed genes with IPF to determine the causal effect of each gene on the disease.

The results revealed that in the MR analysis using the inverse-variance weighted method, all three up-regulated co-expressed genes showed a significant positive causal relationship with IPF. Specifically, C12orf75 (OR=1.162; 95% CI:[1.000 to 1.349]; P = 0.049), SPP1 (OR=1.221; 95% CI:[1.072 to 1.392]; P = 0.003), and ZG16B (OR=1.215; 95% CI:[1.018 to 1.451]; P = 0.031) demonstrated this relationship. Conversely, all three down-regulated co-expressed genes showed a significant negative causal relationship with IPF, namely LIN7A (OR=0.836; 95% CI:[0.701 to 0.997]; P = 0.046), PPP1R14A (OR=0.812; 95% CI:[0.673 to 0.980]; P = 0.030), and TLR2 (OR=0.580; 95% CI:[0.380 to 0.886]; P = 0.012).

In addition to MR-Egger, simple mode, weighted median, and weighted mode were used for further validation. Apart from the simple mode in C12orf75, simple mode and MR Egger in SPP1 and ZG16B, simple mode, MR Egger, and weighted mode in TLR2, and simple mode, MR Egger, weighted mode, and weighted median in PPP1R14A and LIN7A, the other methods had significant effects on these six genes. All methods for the three up-regulated genes consistently indicated an increase in IPF risk (OR > 1), while all methods for the three down-regulated genes consistently indicated a decrease in IPF risk (OR < 1) (Figure 6). It was found that the results of the heterogeneity tests and pleiotropy tests for the co-expressed genes all suggested P > 0.05, indicating no statistical significance and no need to consider the impact of heterogeneity



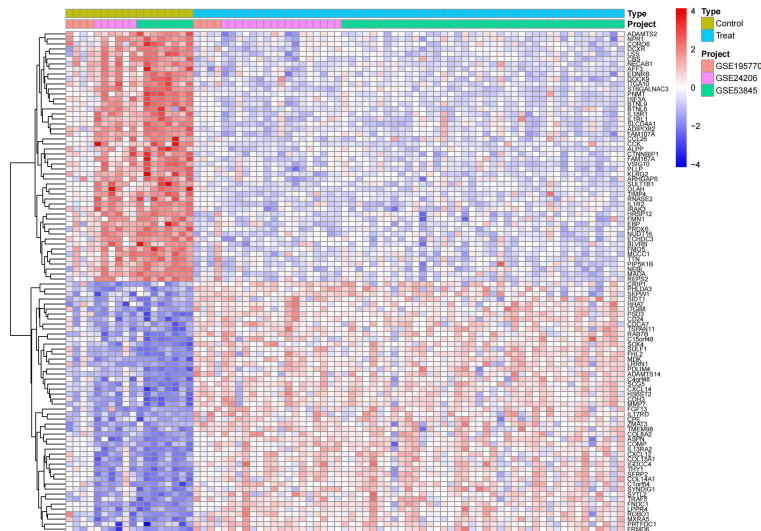


FIGURE 3
Differential gene expression heatmap.

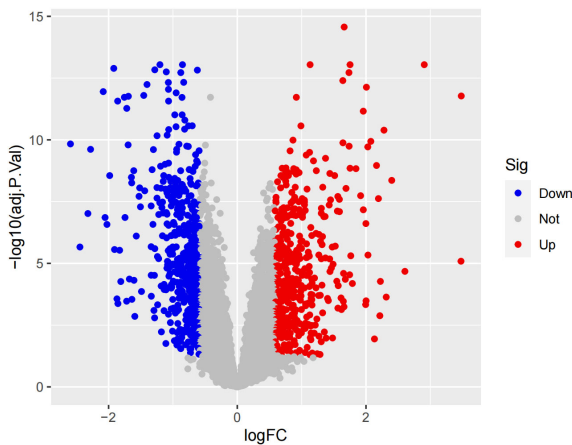


FIGURE 4
Volcano plo.

and pleiotropy on the results. The leave-one-out sensitivity analysis showed that the effect sizes of the included IVs were close to the overall effect size, demonstrating the robustness of the analysis. Detailed information for each gene, including scatter plots, forest plots, funnel plots, and leave-one-out sensitivity analyses, can be found in [Supplementary Figure S1](#). To further clarify the chromosomal distribution of the aforementioned genes, we visualized the co-expressed genes ([Figure 7](#)).

3.4 GO and KEGG enrichment analysis

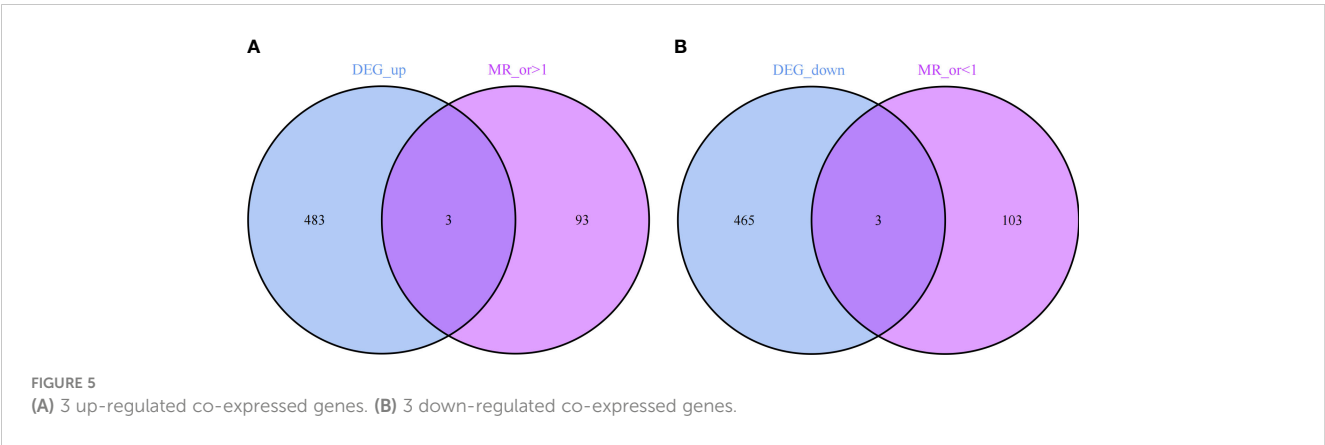
Through GO and KEGG analysis, we further explored the potential roles of these six co-expressed genes ([Figures 8A, B](#)). GO enrichment analysis indicated that the co-expressed genes mainly affect biological functions such as response to macrophage

colony-stimulating factor, negative regulation of nervous system development, response to ketone, tissue homeostasis, anatomical structure homeostasis, and positive regulation of secretion. KEGG enrichment analysis revealed that the co-expressed genes primarily impact the Toll-like receptor signaling pathway and the PI3K-Akt signaling pathway. Detailed data can be found in [Supplementary Table S4](#).

3.5 Assessment of immune cell infiltration in idiopathic pulmonary fibrosis

The functional and pathway analysis of co-expressed genes in IPF shows a close relationship with inflammatory and immune processes. The CIBERSORT algorithm was used to infer immune cell characteristics and to explore the correlation between co-expressed genes in IPF and immune cell infiltration. [Figure 9A](#) displays the proportions of 22 types of immune cells in each sample. We observed a significant difference in a specific immune cell subtype (NK cells resting) between the IPF and control group samples. Specifically, the proportion of NK cells resting was significantly lower in IPF patient samples compared to the control group ([Figure 9B](#)).

Additionally, the correlation analysis with 22 types of immune cells indicates ([Figure 9C](#)) that the co-expressed gene C12orf75 is positively correlated with T cells gamma delta and Mast cells resting, and negatively correlated with NK cells resting, NK cells activated, Monocytes, and Macrophages M1. The co-expressed gene SPP1 is positively correlated with Plasma cells, Tregs, and Macrophages M0, and negatively correlated with T cells CD8, T cells CD4 memory resting, NK cells activated, Monocytes, Macrophages M1, Macrophages M2, and Dendritic cells activated. The co-expressed gene ZG16B is positively correlated with T cells follicular helper and negatively correlated with Monocytes and Dendritic cells resting. The co-expressed gene LIN7A is positively



correlated with T cells CD4 memory resting, NK cells activated, Monocytes, Macrophages M1, and Dendritic cells activated, and negatively correlated with Plasma cells, Tregs, Macrophages M0, and Mast cells resting. The co-expressed gene PPP1R14A is positively correlated with T cells CD8 and Dendritic cells activated, and negatively correlated with Macrophages M0 and Dendritic cells resting. The co-expressed gene TLR2 is positively correlated with Monocytes, Mast cells activated, and Neutrophils, and negatively correlated with Mast cells resting.

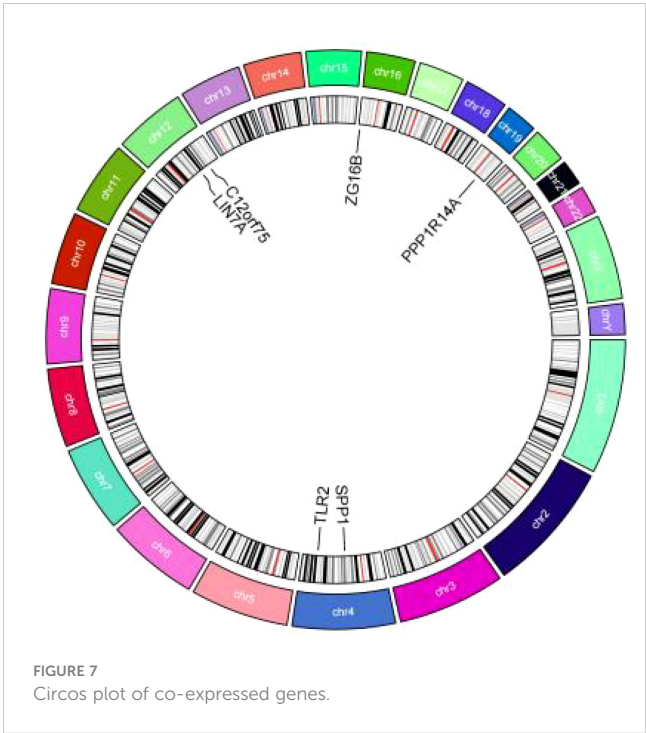
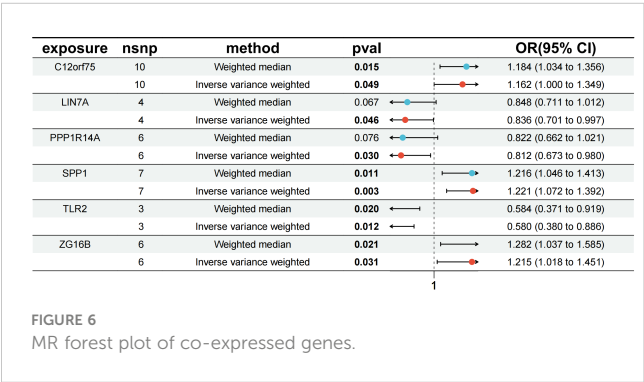
3.6 GSEA enrichment analysis

We found that the co-expressed up-regulated gene C12orf75 has a negative regulatory relationship with the immune cells NK cells resting, and compared to the control group, the proportion of NK cells resting in IPF is lower. Therefore, we further explored the activity level of related functions or pathways in this gene expression group using GSEA enrichment analysis. The results revealed that the top 5 active biological functions in the C12orf75 high expression group are Axoneme Assembly, Cilium Movement, Microtubule Bundle Formation, Ciliary Plasm, and motile cilium (Figure 10A). The top five active biological functions in the C12orf75 low expression group are Myeloid Leukocyte Activation, Phagocytosis, Positive regulation of immune effector process, Positive regulation of Leukocyte Mediated Immunity, and T cell mediated immunity (Figure 10B). The top five active pathways in the C12orf75 low expression group are Allograft

Rejection, Graft Versus Host Disease, Leishmania infection, Lysosome, and Type I Diabetes Mellitus (Figure 10C). No active pathways were found in the C12orf75 high expression group.

3.7 Validation group differential analysis

We confirmed the co-expressed genes found in the MR analysis to have the appropriate levels of expression. The findings revealed that IPF samples had higher expressions of C12orf75, SPP1, and ZG16B than the healthy control group did. SPP1 and ZG16B had significantly higher expressions ($P < 0.05$ and $P < 0.01$, respectively). Meanwhile, the expressions of LIN7A and TLR2 were down-regulated in IPF samples compared to the healthy control group, with significant expression of TLR2 ($P < 0.05$) (Figure 11). Clearly, the expression levels of the three up-regulated and two down-regulated genes are consistent with the results proposed in our MR analysis, lending greater credibility to the MR results.



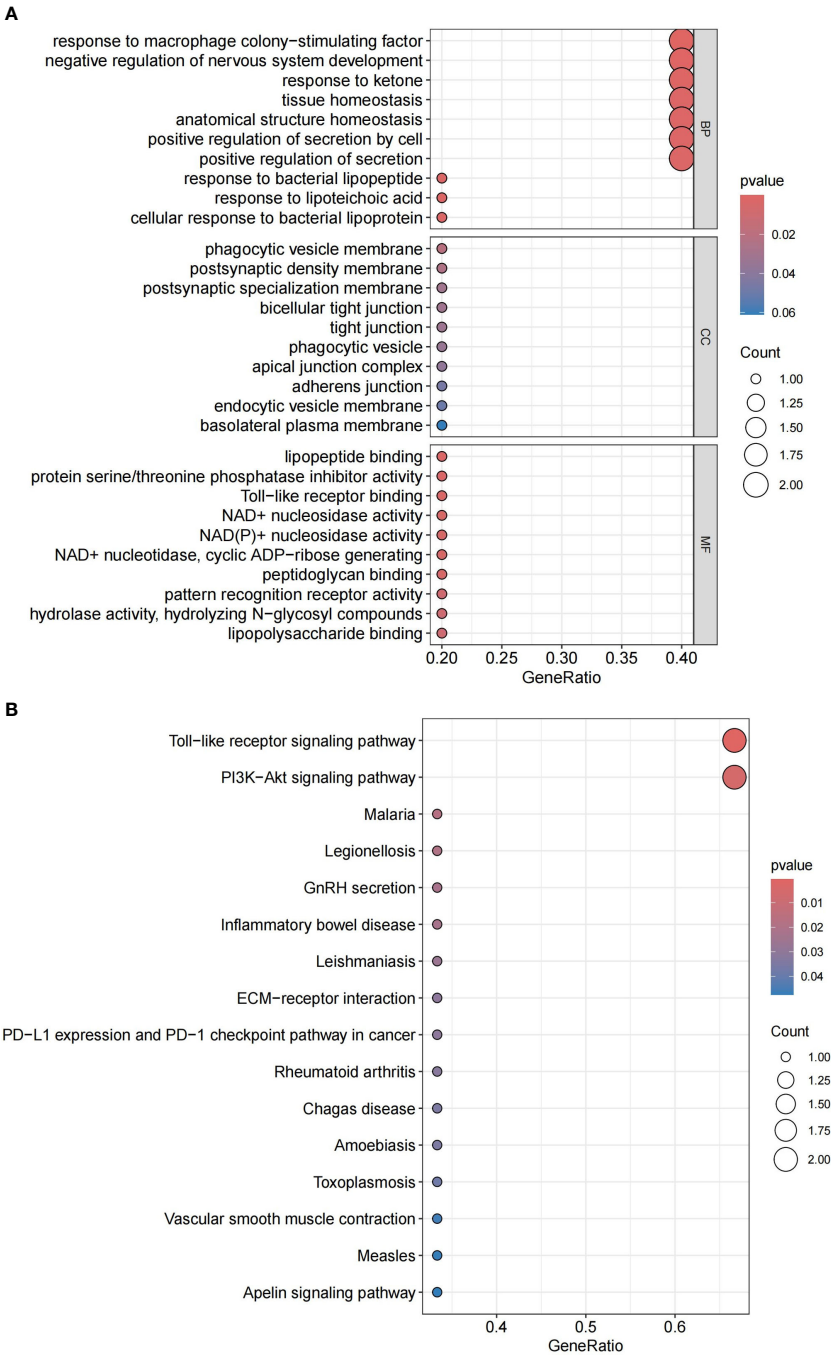


FIGURE 8
(A) GO enrichment analysis of candidate hub genes. (B) KEGG enrichment analysis of candidate hub genes.

4 Discussion

IPF is a chronic, progressive pulmonary disease with an adverse prognosis, rendering it an incurable condition that severely impairs patient quality of life, posing a significant challenge in the current clinical landscape. This study aimed to provide new insights into the molecular mechanisms of IPF through a comprehensive analysis of

datasets from the GEO database, applying MR analysis, and assessing immune cell infiltration, thereby identifying potential targets for future therapeutic strategy development. We extracted three IPF microarray datasets from the GEO database, including 61 IPF patients and 18 healthy controls. Through an in-depth analysis of these datasets, we successfully identified specific DEGs that might be crucial to the pathophysiology of IPF. Our detailed analysis

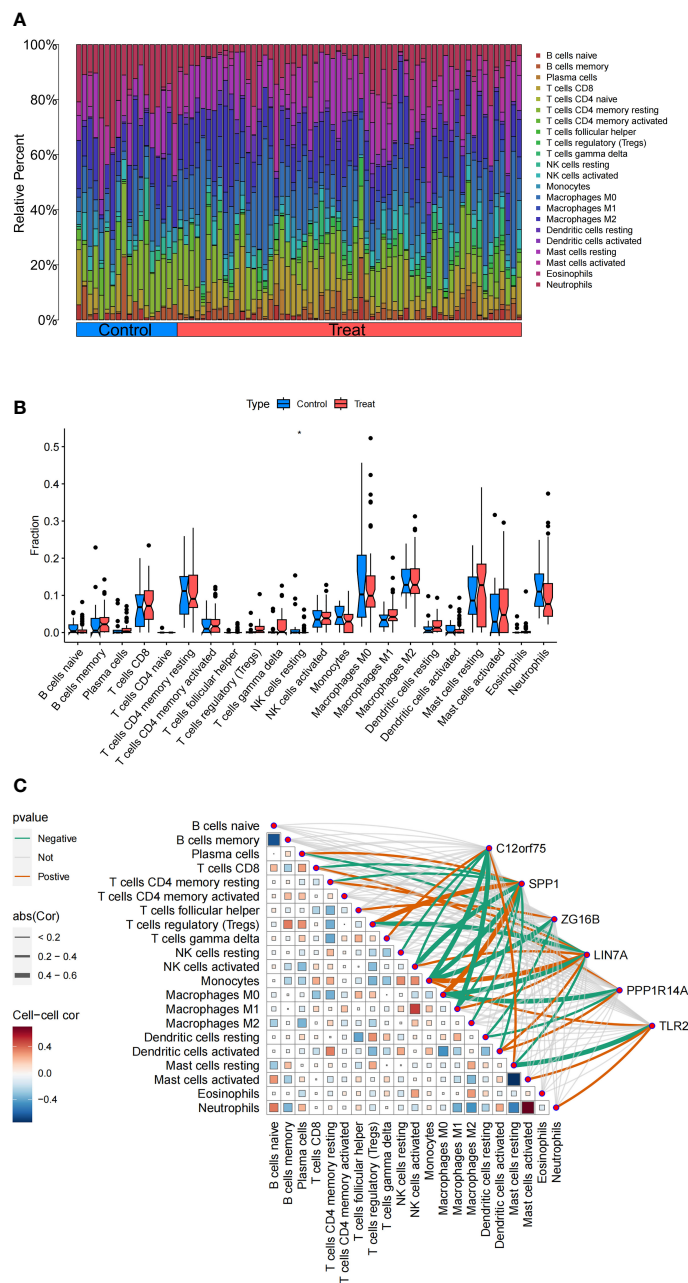


FIGURE 9

Analysis of Immune Cell Infiltration in IPF. (A) Stacked histogram of the proportions of immune cells between the IPF group and the control group. (B) Box plot showing the comparison of 22 types of immune cells between the IPF group and the control group. (C) Heatmap showing the correlation between 22 types of immune cells and co-expressed genes. * $p < 0.05$.

delineated a range of specific DEGs that could be central to the pathogenesis of IPF. This methodology aligns with the research conducted by Dai et al. (15), but extends their work by providing further in-depth MR causal validation for the identified DEGs.

This study intersected 486 up-regulated and 468 down-regulated DEGs with IPF related genes identified through MR analysis, ultimately identifying six co-expressed target genes significantly associated with IPF. This includes three up-regulated genes (C12orf75, SPP1, ZG16B) and three down-regulated genes

(LIN7A, PPP1R14A, TLR2). The expression patterns of these genes provide new insights into the genetic basis of IPF, potentially reflecting key molecular changes in the disease pathology. Further MR analysis confirmed that increased expressions of C12orf75, SPP1, and ZG16B elevate the risk of IPF, while reduced expressions of LIN7A, PPP1R14A, and TLR2 are also associated with an increased disease risk. Our research focuses on these newly identified gene targets, which may play a crucial role in the pathogenesis of IPF.

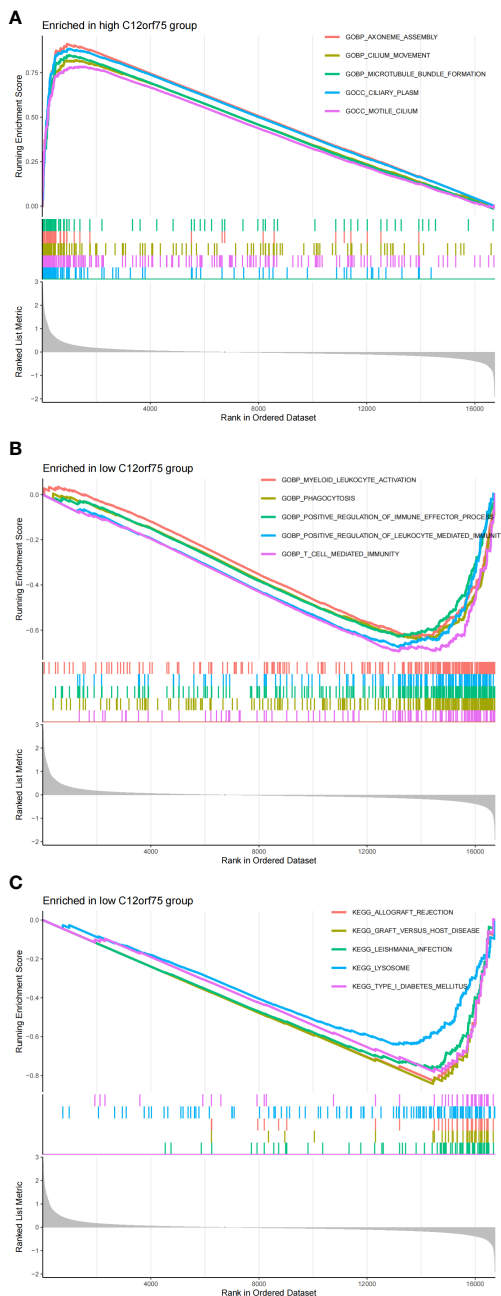


FIGURE 10
Differential impact of C12orf75 expression on biological functions and pathways in IPF. **(A)** The top 5 active biological functions in the C12orf75 high expression group. **(B)** The top 5 active biological functions in the C12orf75 low expression group. **(C)** The top 5 active pathways in the low C12orf75 expression group.

Up-regulated gene

C12orf75: Located on chromosome 12, C12orf75 was found to be significantly up-regulated in IPF patients in our study, suggesting its potential role in the progression of IPF. While current research on the role of C12orf75 in IPF is limited, its expression pattern suggests a possible association with lung tissue repair and fibrotic

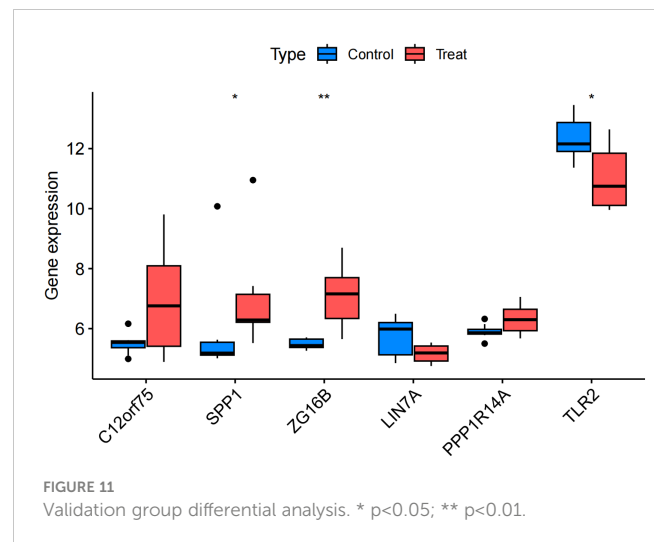


FIGURE 11
Validation group differential analysis. * $p < 0.05$; ** $p < 0.01$.

processes. This aligns with the work of Richeldi et al., who emphasized the importance of targeting specific molecular pathways in IPF treatment (16). The limited current research on C12orf75 in IPF suggests that our findings might unveil a new avenue for investigation.

SPP1 (Osteopontin): Situated on chromosome 4, SPP1 is recognized as an inflammatory and fibrosis regulatory factor. It is extensively expressed in a wide range of cell types and is important for extracellular matrix (ECM) and intercellular communication (17). SPP1's involvement in the pathological processes of IPF is well-documented (18). It is believed to participate in multiple biological functions, including cell adhesion, migration, survival, and immune regulation. In the context of IPF, SPP1's upregulation may exacerbate pulmonary inflammation and fibrosis. Our research corroborates with findings from Zhang et al., indicating a link between SPP1 and heightened immune infiltration in IPF (19). The differential expression patterns observed in IPF, such as the increased expression of SPP1, appear to be critically involved in the disease's pathogenesis. These observations are supported by experimental studies conducted by Morse et al., which identified three distinct macrophage subgroups in the IPF lung, one characterized by high SPP1 expression and pronounced proliferation (20). Macrophages with elevated SPP1 levels may thus play a pivotal role in IPF's pulmonary fibrosis, particularly in the activation of myofibroblasts. SPP1 may be a useful biomarker for IPF patients' diagnosis and prognosis, according to a meta-analysis of 13 studies (21). Given these insights, the specific function of SPP1 in the pathology of IPF warrants further exploration.

ZG16B (Zymogen Granule Protein 16B): Located on chromosome 16, the specific function of the ZG16B gene is not yet fully understood; it may play a role in exocrine tissues. Recent studies have found an upregulation of ZG16B in various cancers (22–24) and it plays a significant role in the development of diseases such as atherosclerosis (25). Mody et al. discovered that ZG16b protein is located in serous and seromucous acinar cells in single-cell RNA sequencing data of healthy human labial minor salivary

glands (26), possibly related to mucosal secretion pathways, cell migration, and homeostatic markers. It may serve as a new biomarker for salivary gland dysfunction (27). However, current research on the direct association of ZG16B with IPF is limited, and our findings may reveal a new research direction.

Down-regulated gene

LIN7A: Located on chromosome 12, LIN7A plays a crucial role in the nervous system and cell polarity. Matsumoto and others have found that LIN7A is key in brain development, and its deficiency may lead to intellectual disabilities and incomplete corpus callosum development (28). Its down-regulation in IPF might similarly impact cell signaling and tissue remodeling. Currently, there's limited research on LIN7A's role in IPF, offering a new perspective in understanding the cellular biology of IPF.

TLR2 (Toll-like Receptor 2): Situated on chromosome 4, TLR2 is essential for initiating innate immune responses because it can identify various molecular patterns linked to microbial pathogens. TLR2's activation is crucial in combating infections through the stimulation of immune cells and promotion of inflammatory responses (29). Research has shown that respiratory epithelial cells exposed to TNF- α or corticosteroids exhibit a notable increase in TLR2 expression (30, 31), suggesting that TLR2 expression may be modulated by changes in the pulmonary cytokine environment due to tissue injury. In cardiovascular diseases, the downregulation of TLR2 expression in myocardial infarction models has demonstrated a protective effect (32). As an integral part of the immune system, TLR2's involvement in IPF could be significant, particularly in terms of inflammation and immune regulation. The activation of TLR2 may facilitate the recruitment and activation of inflammatory cells, influencing IPF development (33). A study by Samara et al. suggests a critical role for TLR2 in IPF's immune regulation process (34), thereby highlighting the need for further research into its specific mechanisms in IPF.

Beyond the gene-specific effects, our research extends to the immunological dimension of IPF. To delve deeper into the modes of action of genes, we further explored the potential roles of these co-expressed genes, especially their importance in macrophage responses, regulation of nervous system development, and maintenance of tissue homeostasis, through GO and KEGG enrichment analysis. These analyses reveal key biological processes and pathways related to IPF, closely associated with inflammation and immune processes.

Using the CIBERSORT algorithm, we evaluated the distribution of various immune cell subgroups in IPF, thus illuminating the contribution of immune cells to the disease's pathogenesis (35). This approach is in line with the growing scholarly interest in analyzing immune cells associated with IPF (36). Building upon these findings, we further investigated the connections between target genes of IPF and immune cell infiltration, and the specific regulatory effects these genes exert on immune cells. Our results align with existing research on IPF, reinforcing the importance of immune cells in the disease's development (33). They also offer

insights into potential future therapeutic avenues, such as modulating specific immune cell activities to decelerate or reverse the progression of IPF (37). This aspect of the research not only corroborates existing knowledge but also opens new doors for targeted treatment strategies in IPF.

PPP1R14A (Protein Phosphatase 1 Regulatory Inhibitor Subunit 14A): Located on chromosome 19, PPP1R14A belongs to the Protein Phosphatase 1 (PP1) inhibitor family and is responsible for encoding CPI-17 (C-kinase-activated PP1 inhibitor, 17kDa). CPI-17 regulates various cellular functions by inhibiting PP1 activity, especially in smooth muscle cell contraction. Past research indicates that PPP1R14A plays a crucial role in the development and progression of some tumors (38–40). Current research on PPP1R14A's role in IPF is limited, necessitating further investigation.

We found that in the co-expressed up-regulated genes, C12orf75 has a negative regulatory relationship with the immune cells NK cells resting, and compared to the control group, the proportion of NK cells resting in IPF is significantly reduced. Therefore, through further GSEA enrichment analysis, we discovered that C12orf75 appears to play multifaceted roles in the pathogenesis of IPF. Its expression levels may be related to changes in immune cell activity and biological functions associated with ciliary processes and autoimmune responses. C12orf75 may play a role in regulating the immune response in IPF, particularly by affecting NK cell activity and leading to an imbalance in the immune environment. The high expression of C12orf75 might be associated with ciliary dysfunction, potentially disrupting normal lung epithelial cell function and leading to the fibrotic process. The active pathways in the low expression group of C12orf75 indicate the presence of autoimmune components in IPF, potentially mediated by the regulatory action of C12orf75.

The consistency of the MR analysis with the validation group's differential analysis results confirms the robustness of our MR findings, enhancing the credibility of these genes' association with IPF disease. The upregulation of SPP1 and ZG16B may promote inflammatory responses in IPF, potentially being key driving factors in the development of IPF. The downregulation of TLR2 may lead to dysregulation of the immune response, thereby promoting the progression of IPF, while the downregulation of LIN7A may affect intercellular signal transmission, leading to abnormal lung tissue repair processes, and thus promoting fibrosis. Certainly, newly identified therapeutic targets in recent research also warrant further attention. For example, research teams have found through basic experiments that MKP-5 expression was increased in lung fibroblasts derived from IPF. Consequently, inhibiting MKP-5 could potentially serve as a novel therapeutic target for IPF treatment (41). Autotaxin (ATX) may represent another potential therapeutic target for idiopathic pulmonary fibrosis. Research teams, through a comprehensive literature review, have discovered that targeting ATX not only offers a novel approach to IPF treatment but also highlights its relevance in the pathogenesis of both IPF and COVID-19 related pulmonary complications. This suggests that ATX could be a potential common therapeutic target, warranting further investigation (42).

However, these hypotheses necessitate further experimental validation to clarify how these genes influence these pathways

and contribute to IPF progression. This includes validation in larger independent cohorts and more comprehensive functional studies before clinical applications are considered. It's also important to recognize potential limitations such as selection bias and the inherent constraints of the statistical methods and bioinformatics tools used in our study.

5 Conclusions

This study provides a detailed analysis of Idiopathic Pulmonary Fibrosis, identifying key genes and pathways using advanced bioinformatics and statistical methods. It emphasizes the importance of immune cells and genetic factors, focusing on genes such as C12orf75, SPP1, ZG16B, LIN7A, PPP1R14A and TLR2. The findings provide insights into the complex molecular mechanisms of IPF and offer avenues for novel therapeutic interventions. However, these results require further validation and consideration of the inherent limitations in data selection and analysis techniques. Overall, the study contributes to a deeper understanding of IPF, indicating the need for targeted molecular and immunological approaches in future treatment and research strategies.

Data availability statement

Summary statistics of genetic instruments can be obtained from the websites listed on the referenced GWAS or GWAS meta-analysis.

Ethics statement

All the GWAS/GWAS meta-analysis from which the summary statistics were extracted from had obtained ethics approval from the respective institutional review board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

WH: Data curation, Software, Visualization, Writing – original draft, Writing – review & editing. YX: Conceptualization, Data curation, Methodology, Supervision, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1375171/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Scatterplots, forest plots, funnel plots and leave-one-out sensitivity analysis plots for six target genes.

SUPPLEMENTARY TABLE SN

Results of MR analysis, heterogeneity and pleiotropy tests for six co-expressed genes.

SUPPLEMENTARY TABLE S1

DEGs identification.

SUPPLEMENTARY TABLE S2

Instrument variables for exposure.

SUPPLEMENTARY TABLE S3

IPF related genes screened by MR.

SUPPLEMENTARY TABLE S4

GO and KEGG enrichment results.

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Causal relationship between inflammatory cytokines and autoimmune thyroid disease: a bidirectional two-sample Mendelian randomization analysis

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Background: Autoimmune thyroid disease (AITD) ranks among the most prevalent thyroid diseases, with inflammatory cytokines playing a decisive role in its pathophysiological process. However, the causal relationship between the inflammatory cytokines and AITD remains elusive.

Methods: A two-sample Mendelian randomization (MR) analysis was performed to elucidate the causal connection between AITD and 41 inflammatory cytokines. Genetic variations associated with inflammatory cytokines were sourced from the FinnGen biobank, whereas a comprehensive meta-analysis of genome-wide association studies (GWASs) yielded data on Graves' disease (GD) and Hashimoto thyroiditis. Regarding the MR analysis, the inverse variance-weighted, MR-Egger, and weighted median methods were utilized. Additionally, sensitivity analysis was conducted using MR-Egger regression, MR-pleiotropy residual sum, and outliers.

Results: Seven causal associations were identified between inflammatory cytokines and AITD. High levels of tumor necrosis factor- β and low levels of stem cell growth factor- β were indicative of a higher risk of GD. In contrast, high levels of interleukin-12p70 (IL-12p70), IL-13, and interferon- γ and low levels of monocyte chemotactic protein-1 (MCP-1) and TNF- α suggested a higher risk of HD. Moreover, 14 causal associations were detected between AITD and inflammatory cytokines. GD increases the levels of macrophage inflammatory protein-1 β , MCP-1, monokine induced by interferon- γ (MIG), interferon γ -induced protein 10 (IP-10), stromal cell-derived factor-1 α , platelet-derived growth factor BB, β -nerve growth factor, IL-2ra, IL-4, and IL-17 in blood, whereas HD increases the levels of MIG, IL-2ra, IP-10, and IL-16 levels.

Conclusion: Our bidirectional MR analysis revealed a causal relationship between inflammatory cytokines and AITD. These findings offer valuable insights into the pathophysiological mechanisms underlying AITD.

KEYWORDS

inflammatory cytokines, autoimmune thyroid disease, causality, GWAS, Mendelian randomization

1 Introduction

Autoimmune thyroid disease (AITD) encompasses a group of organ-specific autoimmune diseases characterized by the production of antithyroid antibodies and infiltration of lymphocytes in the thyroid gland (1). Epidemiological investigations established that its prevalence is approximately 5% in the general population and has steadily increased in recent years (2, 3). Clinically, Graves' Disease (GD) and Hashimoto thyroiditis (HT) are the most common forms of AITD, hallmarked by hyperthyroidism and hypothyroidism, respectively (4). Among them, the former represents a specific autoimmune disease featured by increased thyroid hormone secretion driven by an immune system imbalance that promotes the production of thyroid-stimulating antibodies (5). Additionally, as an AITD, the pathogenesis of HT is similar to that of GD. Specifically, the pathogenesis of AITD involves genetic defects and genetic susceptibility, psychological factors, infections, and stress reactions. Meanwhile, environmental pollution and a high-iodine diet can contribute to or exacerbate autoimmune reactions leading to HT (6). At present, there is an urgent need to investigate the pathogenesis of AITD to develop insights into clinical interventions.

Inflammatory cytokines are a group of proteins or peptides generated by immune cells and exhibit a diverse range of biological activities. Consequently, they play a pivotal role in autoimmune responses (7, 8). Previous studies have documented a close correlation between inflammatory cytokines and the development and progression of AITD (9, 10). On the one hand, cytokine imbalance directly or indirectly induces thyroid epithelial cell growth, abnormal differentiation, and immune dysfunction and can also change the state of immunoreactive cells and participate in the occurrence and development of AITD (11). On the other hand, lymphocytes infiltrating the thyroid in patients with AITD can also contribute to cytokine production. For example, in the helper T cell Th1/Th2 subgroups, Th1 cells, predominantly expressing interleukin-2 (IL-2) and interferon- γ (IFN- γ), stimulate delayed hypersensitivity and mediate cellular immune responses. In contrast, Th2 cells, characterized by expression of IL-4, IL-5, IL-10, and IL-13, modulate antibody production and humoral immune responses (12). At the same time, a growing body of evidence has highlighted the pivotal role of Th17 cells in the development of AITD. Of note, Th17 cells predominantly synthesize IL-17A, IL-17F, IL-21,

and IL-22, along with their associated cellular and secretory components (13). Although an increasing number of studies have documented an association between inflammatory cytokines and AITD, their causal relationship remains to be investigated.

Given that genetic predisposition plays a key role in the development of AITD, it should be explained from the perspective of genetics (14). Mendelian randomization (MR) is a superior analytical approach for drawing etiological conclusions by employing genetic variation as an instrumental variable (IV) for exposure. This approach has a lower susceptibility to confounding factors owing to the random allocation of germline genetic variation during meiosis, allowing it to accurately represent exposure without being influenced by reverse causation (15). Importantly, MR studies can be performed to explore numerous dependable genetic variants owing to the public release of extensive gene-wide association data. Hence, this investigation aimed to examine the causal relationship between inflammatory cytokines and AITD.

2 Materials and methods

2.1 Study population

As depicted in [Figure 1](#), MR relies on three fundamental assumptions: (1) IVs exhibit a robust association with the exposure factor; (2) IVs are not correlated with any confounding factors; and (3) IVs solely impact the results through the exposure and not via any other mechanisms (16). In the current study, pooled data from the published genome-wide association studies (GWASs) of 41 inflammatory cytokines and AITD were utilized. Firstly, genetic variants associated with each inflammatory factor were selected to identify the causal relationship of each inflammatory cytokine with GD and HT. Secondly, AITD-associated genetic variants were employed to infer causality between GD and HT and inflammatory cytokines, respectively.

As detailed in [Table 1](#), the genetic associations of 41 inflammatory cytokines were analyzed using data from 8,293 Finnish participants in the Cardiovascular Risk in Finnish Youth Study and the FINRISK Study (17). The first step involved genetic adjustment for 10 principal genetic components, comprising age, gender, and body mass index, as well as for population stratification and cryptic kin using genomic control.

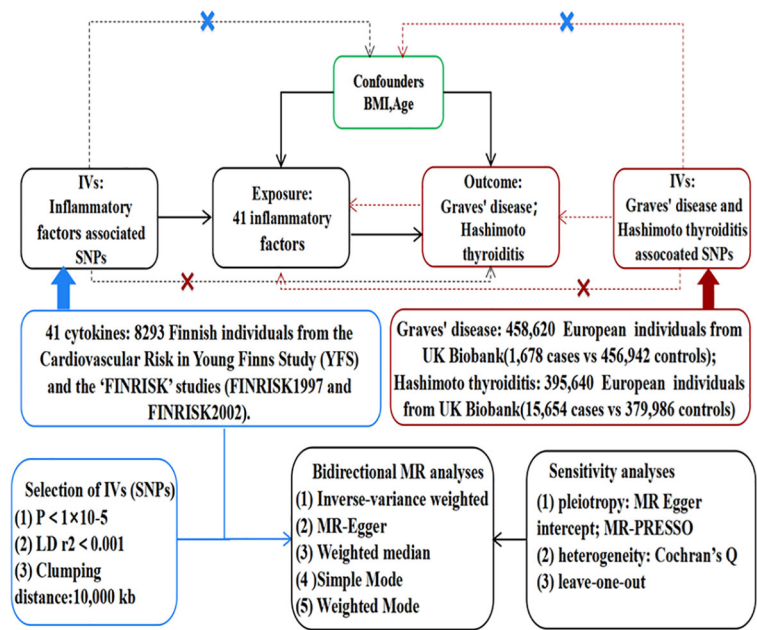


FIGURE 1 Assumptions and study design of the MR study of the associations between 41 inflammatory factors and autoimmune thyroid disease. BMI, body mass index; IVs, instrumental variables; SNPs, single-nucleotide polymorphisms; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier.

TABLE 1 Details of the studies and datasets used in the study.

Variable	Abbreviation	Ancestry	Population	Consortium
Graves' disease	GD	European	458,620	IEU GWAS
Hashimoto thyroiditis	HT	European	395,640	IEU GWAS
Macrophage inflammatory protein-1 α (CCL3)	MIP-1 α	European	3,522	FINRISK 2002 and Young Finns Study
Macrophage inflammatory protein-1 β (CCL4)	MIP-1 β	European	8,243	FINRISK 1997, FINRISK 2002, and Young Finns Study
Eotaxin (CCL11)	Eotaxin	European	8,153	FINRISK 1997, FINRISK 2002, and Young Finns Study
Monocyte chemotactic protein-1 (CCL2)	MCP-1	European	8,293	FINRISK 1997, FINRISK 2002, and Young Finns Study
Monocyte specific chemokine 3 (CCL7)	MCP-3	European	843	FINRISK 2002 and Young Finns Study
Monokine induced by interferon- γ (CXCL9)	MIG	European	3,685	FINRISK 2002 and Young Finns Study
Interferon γ -induced protein 10 (CXCL10)	IP-10	European	3,685	FINRISK 2002 and Young Finns Study
Cutaneous T-cell attracting (CCL27)	CTACK	European	3,631	FINRISK 2002 and Young Finns Study
Regulated on activation, normal T cell expressed and secreted (CCL5)	RANTES	European	3,421	FINRISK 2002 and Young Finns Study
Growth-regulated oncogene- α	GRO α	European	3,505	FINRISK 2002 and Young Finns Study
Stromal cell-derived factor-1 α (CXCL12)	SDF-1 α	European	5,998	FINRISK 1997, FINRISK 2002, and Young Finns Study
Stem cell growth factor- β	SCGF- β	European	3,682	FINRISK 2002 and Young Finns Study
Platelet-derived growth factor BB	PDGFbb	European	8,293	FINRISK 1997, FINRISK 2002, and Young Finns Study

(Continued)

TABLE 1 Continued

Variable	Abbreviation	Ancestry	Population	Consortium
Stem cell factor	SCF	European	8,290	FINRISK 1997, FINRISK 2002, and Young Finns Study
Granulocyte colony-stimulating factor	GCSF	European	7,904	FINRISK 1997, FINRISK 2002, and Young Finns Study
Vascular endothelial growth factor	VEGF	European	7,118	FINRISK 1997, FINRISK 2002, and Young Finns Study
Hepatocyte growth factor	HGF	European	8,292	FINRISK 1997, FINRISK 2002, and Young Finns Study
Macrophage colony-stimulating factor	MCSF	European	839	FINRISK 2002 and Young Finns Study
β -Nerve growth factor	β NGF	European	3,531	FINRISK 2002 and Young Finns Study
Basic fibroblast growth factor	bFGF	European	7,565	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interleukin-1 receptor antagonist	IL-1ra	European	3,638	FINRISK 2002 and Young Finns Study
Interleukin-1 β	IL-1 β	European	3,309	FINRISK 2002 and Young Finns Study
Interleukin-2 receptor, α subunit	IL-2ra	European	3,677	FINRISK 2002 and Young Finns Study
Interleukin-2	IL-2	European	3,475	FINRISK 2002 and Young Finns Study
Interleukin-4	IL-4	European	8,214	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interleukin-5	IL-5	European	3,364	FINRISK 2002 and Young Finns Study
Interleukin-6	IL-6	European	8,189	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interleukin-7	IL-7	European	3,409	FINRISK 2002 and Young Finns Study
Interleukin-8 (CXCL8)	IL-8	European	3,526	FINRISK 2002 and Young Finns Study
Interleukin-9	IL-9	European	3,634	FINRISK 2002 and Young Finns Study
Interleukin-10	IL-10	European	7,681	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interleukin-12p70	IL-12p70	European	8,270	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interleukin-13	IL-13	European	3,557	FINRISK 2002 and Young Finns Study
Interleukin-16	IL-16	European	3,483	FINRISK 2002 and Young Finns Study
Interleukin-17	IL-17	European	7,760	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interleukin-18	IL-18	European	3,636	FINRISK 2002, and Young Finns Study
TNF-related apoptosis inducing ligand	TRAIL	European	8,186	FINRISK 1997, FINRISK 2002, and Young Finns Study
Interferon- γ	IFN- γ	European	7,701	FINRISK 1997, FINRISK 2002, and Young Finns Study
Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	European	3494	FINRISK 2002 and Young Finns Study
Tumor necrosis factor- α	TNF- α	European	3,454	FINRISK 2002 and Young Finns Study
Tumor necrosis factor- β	TNF- β	European	1,559	FINRISK 2002 and Young Finns Study

The GWAS dataset associated with GD and HD was derived from the UK Biobank Project (18). In order to expand the genetic association map beyond European populations, 220 in-depth GWASs were conducted using data from the Biobank in Japan,

integrating text mining of past medical histories and electronic medical records. A meta-analysis involving the UK Biobank and FinnGen yielded approximately 5,000 new loci, thereby enhancing the resolution of the human trait genome map. Finally, a statistical

decomposition of the overall phenomenon's summary statistical matrix, coupled with the identification of potential genetic components, assisted in identifying relevant variants and biological mechanisms associated with current disease classifications in the population.

2.2 Single-nucleotide polymorphism selection

In the present study, single-nucleotide polymorphisms (SNPs) that are significantly associated with the relative abundance of 41 inflammatory cytokines were selected as IVs. Prior investigations have established that incorporating multiple IVs enhances the interpretation of exposure variation, thereby enhancing the accuracy and reliability of the results. Consequently, the 41 inflammatory cytokines were analyzed on the basis of the outcomes of a significant association analysis with $P < 1 \times 10^{-5}$. The linkage disequilibrium criteria were set at $r^2 < 0.001$, whereas the genetic distance was established as 10,000 kb. To ensure the independence of the included SNPs, highly correlated SNPs were excluded. Subsequently, SNPs associated with the relative abundance of the 41 inflammatory cytokines were integrated into the GWAS data of AITD, and corresponding statistical parameters were extracted. The data were harmonized by comparing the statistical parameters at the same sites in the GWAS results of inflammatory cytokines relative abundance and AITD, which facilitated the alignment of the effect values of exposure and outcome with the same effect allele.

2.3 Statistical analysis

In the present study, the inverse variance-weighted (IVW), MR-Egger, and weighted median (WME) methods were employed to comprehensively assess causal effects. The IVW approach assumes the validity of all genetic variants. The causal effect value of each individual IV is calculated by IVs using the ratio method. Afterward, each estimate is meticulously summarized for weighted linear regression, yielding the total effect value (19). Regarding regression, the MR-Egger method and the IVW method primarily diverge in their consideration of the intercept term (20). The WME approach utilizes the intermediate impacts of every accessible genetic variation. Estimates were acquired by weighing the inverse variance of each SNP's association with the outcome (21).

The IVW method is superior to the other two MR methods. Therefore, the IVW method was selected as the preferred method for estimating causal effects. In order to mitigate the risk of false positives, the Benjamini-Hochberg (BH) method was used to control the false discovery rate (FDR). For a clearer explanation of the findings, the impact of the 41 inflammatory cytokines on GD and HT was present as odds ratios (ORs) for each 1 standard deviation genetic predicted change in cytokine levels. In contrast, the impact of GD and HT on the levels of inflammatory cytokines was expressed as β coefficient and 95% confidence intervals (95%

CI). Considering that the connection between effect estimates and testing causation might be influenced by weak instrumental biases, the F-statistic was employed to assess the strength of the IVs using the following formula: $F = R^2(n - K - 1)/k(1 - R^2)$ (22), where R^2 represents the variance (per circulating cytokine) explained by the independent variable (IV), and n represents the sample size. R^2 was estimated on the basis of the minor allele frequency (MAF) and b -value, calculated using the equation: $R^2 = 2 \times \text{MAF} \times (1 - \text{MAF}) \times b^2$ (23).

Furthermore, to ensure the stability and reliability of the findings, quality control consisting of sensitivity analysis, heterogeneity test, and gene pleiotropy test were carried out at every level. To assess the impact of each SNP on the outcome heterogeneity test, sensitivity analysis was employed using the leave-one-out method, and the combined effect size of the remaining SNPs was determined through sequential deletion of individual SNPs. In the case of significant heterogeneity between IVs ($Q_{\text{pval}} < 0.05$), the MR effect size was estimated using random-effect IVW. Otherwise, fixed-effect IVW was adopted (24). In order to examine the strength of the findings, additional sensitivity analyses were performed, including MR-Egger regression, MR-pleiotropy residual sum, and outliers (MR-PRESSO) tests. Specifically, WME accurately estimates the causal impact even with less than half of the data originating from the unreliable IV (21). The P-value of the intercept term in MR-Egger regression can serve as an indication of directional pleiotropy (25). Concerning the MR-PRESSO test, the multi-effect effect was corrected by excluding the outlier. In this study, MR analysis and quality control were conducted using R version 4.0.3 and TwoSampleMR version 0.5.6.

3 Results

3.1 Two-sample MR analysis

The flow chart of 41 inflammatory cytokines and AITD is shown in Figure 1. The calculated F-values ranged from 11.156 to 788.955, all meeting the threshold of greater than 10, indicating that weak instrumental bias was unlikely (Supplementary Tables 1–4). Finally, we identified 21 causal associations between inflammatory cytokines and AITD (Tables 2, 3).

3.2 Inflammatory cytokines and autoimmune thyroid disease

In the MR analysis, the level of TNF- β was positively correlated with the risk of GD (OR, 1.115; 95% CI, 1.024–1.215; $P = 0.013$). However, the level of stem cell growth factor- β (SCGF- β) was negatively correlated with the risk of GD (OR, 0.913; 95% CI, 0.839–0.994; $P = 0.035$). Interestingly, no correlation was noted between the levels of inflammatory cytokines and GD following BH correction (Figure 2; Table 2).

Furthermore, the WME method did not detect a significant correlation between the level of tumor necrosis factor- β (TNF- β)

TABLE 2 Effects of the relationship between meaningful inflammatory cytokines and autoimmune thyroid disease in MR analysis.

Exposure	Outcome	SNPs	Methods	OR (95% CI)	P-value	P _{FDR}
TNF-β	GD					
		8	MR-Egger	1.183 (0.982–1.424)	0.127	
		8	Weighted median	1.096 (0.981–1.225)	0.104	
		8	IVW	1.115 (1.024–1.215)	0.013	0.533
SCGF-β	GD					
		24	MR-Egger	0.921 (0.770–1.102)	0.379	
		24	Weighted median	0.989 (0.872–1.121)	0.857	
		24	IVW	0.913 (0.839–0.994)	0.035	0.718
IL-12p70	HD					
		18	MR-Egger	1.117 (0.997–1.251)	0.075	
		18	Weighted median	1.073 (0.990–1.162)	0.086	
		18	IVW	1.088 (1.019–1.162)	0.012	0.342
IL-13	HD					
		18	MR-Egger	1.024 (0.925–1.134)	0.652	
		18	Weighted median	1.059 (0.986–1.136)	0.115	
		18	IVW	1.055 (1.002–1.111)	0.041	0.385
IFN-γ	HD					
		12	MR-Egger	1.111 (0.871–1.416)	0.416	
		12	Weighted median	1.106 (0.935–1.308)	0.239	
		12	IVW	1.150 (1.018–1.299)	0.025	0.342
MCP-1	HD					
		27	MR-Egger	1.087 (0.924–1.278)	0.322	
		27	Weighted median	0.917 (0.823–1.021)	0.113	
		27	IVW	0.930 (0.866–0.999)	0.047	0.385
TNF-α	HD					
		10	MR-Egger	0.763 (0.640–0.911)	0.017	
		10	Weighted median	0.887 (0.785–1.003)	0.056	
		10	IVW	0.892 (0.812–0.981)	0.018	0.342

MR, Mendelian randomization analysis; SNPs, number of single-nucleotide polymorphism; CI, confidence interval; OR, odds ratio; 95% CI, 95% confidence interval; P_{FDR}, P-value was calculated by the Benjamini–Hochberg method; GD, Graves’ disease; HT, Hashimoto thyroiditis; IVW, inverse variance-weighted.

(OR, 1.096; 95% CI, 0.981–1.225; P = 0.104) and SCGF-β (OR, 0.989; 95% CI, 0.872–1.121; P = 0.857) on the risk of GD, but the direction of the effect was consistent with that of IVW (Table 2; Supplementary Figure 1). As anticipated, the MR-Egger regression intercept showed no evidence of pleiotropy between TNF-β, SCGF-β, and GD (intercept P = 0.511 for TNF-β and intercept P = 0.915 for SCGF-β). No outliers were detected by MR-PRESSO regression. Heterogeneity and sensitivity analysis results corroborated the accuracy of the results (Table 4). Lastly, the leave-one-out method further validated the robustness of the data (Supplementary Figure 5).

At the same time, high levels of IL-12p70 (OR, 1.088; 95% CI, 1.019–1.162, P = 0.012), IL-13 (OR, 1.055; 95% CI, 1.002–1.111, P =

0.041), and IFN-γ (OR, 1.150; 95% CI, 1.018–1.299; P = 0.025) were positively associated with the risk of HD. Whereas, high levels of monocyte chemotactic protein-1 (MCP-1) (OR, 0.930; 95% CI, 0.866–0.999; P = 0.047) and TNF-α (OR, 0.892; 95% CI, 0.812–0.981; P = 0.018) were negatively associated with the risk of HD, and no correlation between inflammatory cytokines and HD was found after BH correction (Figure 3; Table 2).

WME method did not detect IL-12p70 (OR, 1.073; 95% CI, 0.990–1.162; P = 0.086), IL-13 (OR, 1.059; 95% CI, 0.986–1.136, P = 0.115), IFN-γ (OR, 1.106; 95% CI, 0.935–1.308; P = 0.239), MCP-1 (OR, 0.917; 95% CI, 0.823–1.021; P = 0.113), TNF-α (OR, 0.887; 95% CI, 0.785–1.003; P = 0.056) had a significant effect on the risk of HD, but the direction of effect was consistent with that of

TABLE 3 Effects of the relationship between autoimmune thyroid disease and meaningful inflammatory cytokines in reverse MR analysis.

Exposure	Outcome	SNPs	Methods	Beta (95% CI)	P-value	P _{FDR}
GD	MIP-1β					
		22	MR-Egger	0.079 (−0.032–0.191)	0.177	
		22	Weighted median	0.055 (−0.001–0.111)	0.054	
		22	IVW	0.049 (0.010–0.088)	0.014	0.131
GD	MCP-1					
		22	MR-Egger	0.055 (−0.049–0.159)	0.316	
		22	Weighted median	0.063 (0.011–0.114)	0.017	
		22	IVW	0.055 (0.019–0.092)	0.003	0.082
GD	MIG					
		22	MR-Egger	0.250 (0.076–0.424)	0.011	
		22	Weighted median	0.048 (−0.032–0.128)	0.242	
		22	IVW	0.080 (0.014–0.146)	0.018	0.131
GD	IP-10					
		22	MR-Egger	0.180 (−0.011–0.371)	0.080	
		22	Weighted median	0.049 (−0.038–0.136)	0.269	
		22	IVW	0.076 (0.008–0.145)	0.028	0.131
GD	SDF-1α					
		22	MR-Egger	0.021 (−0.089–0.130)	0.716	
		22	Weighted median	0.060 (0.006–0.113)	0.029	
		22	IVW	0.042 (0.004–0.080)	0.030	0.131
GD	PDGFbb					
		22	MR-Egger	−0.005 (−0.109–0.099)	0.927	
		22	Weighted median	0.047 (−0.006–0.099)	0.081	
		22	IVW	0.052 (0.015–0.089)	0.006	0.082
GD	βNGF					
		22	MR-Egger	0.105 (−0.068–0.279)	0.249	
		22	Weighted median	0.140 (0.058–0.222)	0.001	
		22	IVW	0.066 (0.006–0.126)	0.032	0.131
GD	IL-2ra					
		22	MR-Egger	0.134 (−0.021–0.289)	0.106	
		22	Weighted median	0.045 (−0.031–0.122)	0.247	
		22	IVW	0.062 (0.007–0.117)	0.026	0.131
GD	IL-4					
		22	MR-Egger	0.057 (−0.049–0.162)	0.304	
		22	Weighted median	0.068 (0.017–0.119)	0.009	
		22	IVW	0.054 (0.017–0.092)	0.004	0.082
GD	IL-17					
		22	MR-Egger	0.050 (−0.061–0.160)	0.389	
		22	Weighted median	0.056 (0.001–0.110)	0.045	

(Continued)

TABLE 3 Continued

Exposure	Outcome	SNPs	Methods	Beta (95% CI)	P-value	P _{FDR}
		22	IVW	0.044 (0.006–0.082)	0.025	0.131
HD	MIG					
		22	MR-Egger	0.393 (0.052–0.735)	0.050	
		22	Weighted median	0.152 (0.010–0.295)	0.037	
		22	IVW	0.234 (0.109–0.359)	2.35E-4	0.010
HD	IL-2ra					
		22	MR-Egger	0.214 (–0.070–0.498)	0.175	
		22	Weighted median	0.100 (–0.033–0.234)	0.141	
		22	IVW	0.136 (0.035–0.236)	0.008	0.085
HD	IP-10					
		22	MR-Egger	0.100 (–0.273–0.472)	0.613	
		22	Weighted median	0.150 (0.010–0.290)	0.036	
		22	IVW	0.183 (0.052–0.315)	0.006	0.085
HD	IL-16					
		22	MR-Egger	0.181 (–0.066–0.428)	0.185	
		22	Weighted median	0.134 (0.009–0.259)	0.036	
		22	IVW	0.124 (0.034–0.213)	0.007	0.085

MR, Mendelian randomization analysis; SNPs, number of single-nucleotide polymorphism; CI, confidence interval; 95% CI, 95% confidence interval; P_{FDR}, P-value was calculated by the Benjamini–Hochberg method; GD, Graves’ disease; HT, Hashimoto thyroiditis; IVW, inverse variance-weighted.

IVW (Table 2; Supplementary Figure 2). The MR-Egger regression intercept showed no evidence of pleiotropy between these inflammatory cytokines and AITD (intercept P = 0.656 for IL-12p70, intercept P = 0.759 for IL-13, intercept P = 0.533 for IFN- γ , intercept P = 0.330 for MCP-1, and intercept P = 0.444 for TNF- α). No outliers were detected by MR-PRESSO regression. Heterogeneity analysis results confirmed the accuracy of the results (Table 4). At the same time, leave-one-out method further verified the robustness of the data (Supplementary Figure 6).

3.3 Autoimmune thyroid diseases and inflammatory cytokines

In reverse MR analysis, IVW method revealed that GD is associated with higher levels of macrophage inflammatory protein-1 β (MIP-1 β) [β , 0.049; 95% CI, 0.010–0.088; P = 0.014], MCP-1 (β , 0.055; 95% CI, 0.019–0.092; P = 0.003), monokine induced by interferon- γ (MIG) (β , 0.080; 95% CI, 0.014–0.146; P = 0.018), interferon γ -induced protein 10 (IP-10) (β , 0.076; 95% CI, 0.008–0.145; P = 0.028), stromal cell-derived factor-1 α (SDF-1 α) (β , 0.042; 95% CI, 0.004–0.080; P = 0.030), platelet-derived growth factor BB (PDGFbb) (β , 0.052; 95% CI, 0.015–0.089; P = 0.006), β -nerve growth factor (β NGF) (β , 0.066; 95% CI, 0.006–0.126; P = 0.032), IL-2ra (β , 0.062; 95% CI, 0.007–0.117; P = 0.026), IL-4 (β , 0.054; 95% CI, 0.017–0.092; P = 0.004), and IL-17 (β , 0.044; 95% CI, 0.006–0.082; P = 0.025). On the other

hand, no correlation was found between GD and inflammatory cytokines after BH correction (Figure 4; Table 3).

WME exposed that the levels of MIP-1 β (β , 0.055; 95% CI, 0.001–0.111; P = 0.054), MIG (β , 0.048; 95% CI, 0.032–0.128; P = 0.242), IP-10 (β , 0.049; 95% CI, 0.038–0.136; P = 0.269), PDGFbb (β , 0.047; 95% CI, 0.006–0.099; P = 0.081), and IL-2ra (β , 0.045; 95% CI, –0.031–0.122; P = 0.247) were significantly associated with the risk of GD. Similarly, there was a positive correlation between the risk and GD and the levels of MCP-1 (β , 0.063; 95% CI, 0.011–0.114; P = 0.017), SDF-1 α (β , 0.060; 95% CI, 0.006–0.113; P = 0.029), β NGF (β , 0.140; 95% CI, 0.058–0.222; P = 0.001), IL-4 (β , 0.068; 95% CI, 0.017–0.119, P = 0.009), and IL-17 (β , 0.056; 95% CI = 0.001–0.110, P = 0.045) (Table 3; Supplementary Figure 3). At the same time, MR-Egger regression analysis displayed no evidence of directional pleiotropy (intercept P = 0.570 for MIP-1 β , intercept P = 0.986 for MCP-1, intercept P = 0.054 for MIG, intercept P = 0.270 for IP-10, intercept P = 0.684 for SDF-1 α , intercept P = 0.986 for MCP-1, intercept P = 0.054 for MIG, intercept P = 0.270 for IP-10, intercept P = 0.533 for PDGFbb, intercept P = 0.330 for β NGF, intercept P = 0.533 for IL-2ra, intercept P = 0.330 for IL-4, and intercept P = 0.444 for IL-17). No outliers were detected by MR-PRESSO regression. Heterogeneity analysis results confirmed the accuracy of the results (Table 5), whereas the leave-one-out method verified the robustness of the data (Supplementary Figure 7).

In addition, we found that HD induced MIG (β , 0.234; 95% CI, 0.109–0.359; P = 2.35E-4), IL-2ra (β , 0.136; 95% CI, 0.035–0.236; P = 0.008), IP-10 (β , 0.183; 95% CI, 0.052–0.315; P = 0.006), and IL-

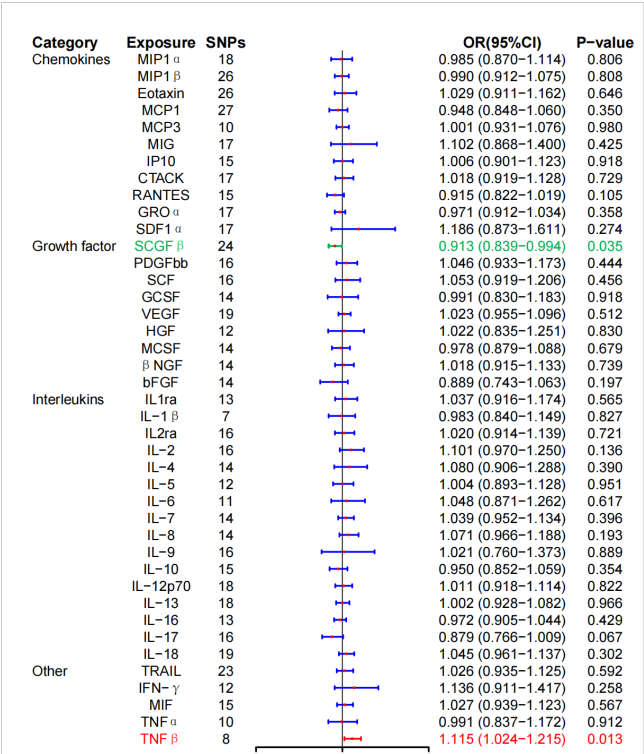


FIGURE 2 Associations between genetically predicted inflammatory cytokines and Graves' disease. SNPs, single-nucleotide polymorphisms; OR, odds ratio; 95% CI, 95% confidence interval; red means positive correlation; green means negative correlation.

16 (β , 0.124; 95% CI, 0.034–0.213; $P = 0.007$) increased levels. However, after BH correction, there was a significant positive correlation between HD risk and MIG expression level ($P_{FDR} = 0.010$) (Figure 5; Table 3).

WME method showed that the risk of HD was associated with serum MIG (β , 0.152; 95% CI, 0.010–0.295; $P = 0.037$), IP-10 (β , 0.150; 95% CI, 0.010–0.290; $P = 0.036$), and IL-16 (β , 0.134; 95% CI, 0.009–0.259, $P = 0.036$) expression level was positively correlated with HD. No correlation was detected between HD risk and IL-2ra (β , 0.100; 95% CI, –0.033–0.234; $P = 0.141$) expression levels (Table 3; Supplementary Figure 4). In addition, the MR-Egger regression intercept showed no evidence of pleiotropy between HD and these circulating cytokines (intercept $P = 0.353$ for MIG, intercept $P = 0.576$ for IL-2ra, intercept $P = 0.647$ for IP-10, and intercept $P = 0.640$ for IL-16). No outliers were detected by MR-PRESSO regression. Heterogeneity analysis results confirmed the accuracy of the results (Table 5). At the same time, the remaining result further verified the robustness of the data (Supplementary Figure 8).

4 Discussion

This study utilized MR to investigate the causal connection between AITD and inflammatory cytokines. To the best of our knowledge, this is the first study to uncover a causal association between the levels of circulating cytokines and AITD. Specifically, high levels of circulating TNF- β and low levels of SCGF- β were

TABLE 4 Heterogeneity and sensitivity analysis of meaningful inflammatory cytokines and risk of autoimmune thyroid disease.

Exposure	Outcome	Methods	Q	P	Intercept	P	MR-PRESSO
TNF- β	GD						
		IVW	5.795	0.564	–0.015	0.511	0.650
		MR-Egger	5.307	0.505			
SCGF- β	GD						
		IVW	25.219	0.339	–0.002	0.915	0.308
		MR-Egger	25.206	0.287			
IL-12p70	HT						
		IVW	6.863	0.976	–0.024	0.656	0.554
		MR-Egger	6.657	0.966			
IL-13	HT						
		IVW	19.306	0.253	0.023	0.759	0.903
		MR-Egger	19.182	0.206			
IFN- γ	HT						
		IVW	16.508	0.086	0.084	0.533	0.759
		MR-Egger	15.771	0.072			
MCP-1	HT						

(Continued)

TABLE 4 Continued

Exposure	Outcome	Methods	Q	P	Intercept	P	MR-PRESSO
TNF-α	HT	IVW	24.352	0.499	0.067	0.330	0.856
		MR-Egger	23.363	0.498			
		IVW	8.307	0.504	-0.067	0.444	0.389
		MR-Egger	7.658	0.468			

MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier; GD, Graves' disease; HT, Hashimoto thyroiditis; IVW, inverse variance-weighted.

associated with a higher risk of GD. TGF-β belongs to the growth and transformational growth family (26) and plays a key role in the pathogenesis of GD (27, 28). In patients with GD, the TGF-β-stimulated humoral response of Th2 cells to infiltrate the thyroid gland leads to the synthesis of thyroid-stimulating hormone receptor (TSH) autoantibodies (TRAb), thereby promoting the development of GD (29–31). SCGF-β plays a vital role in promoting hematopoiesis and hematopoietic recovery. In tissues and organs beyond the bone marrow, SCGF-β can rapidly activate dormant stem cells and stimulate their growth, simultaneously regulating the internal microenvironment to create favorable growth conditions for stem cells (32, 33). Therefore, we postulate that the decrease in the level of SCGF-β may be associated with the inhibition of thyroid stem cell activity in patients with GD. Concurrently, patients with high circulating levels of IL-12p70,

IL-13, and IFN-γ and low levels of MCP-1 and TNF-α are at a higher risk of developing HD. IL-12p70, also referred to as natural killer (NK) cell-stimulating factor, serves as a ligand for IL-12 (34). Its primary immunomodulatory function lies in inducing the differentiation of early helper T cells into Th1 cells, which, in turn, generate pro-inflammatory proteins that trigger macrophage activation, ultimately leading to the initiation of cytotoxic effects (35). Earlier studies have established that IL-12 can exacerbate cytotoxic effects in mice with autoimmune thyroiditis by eliciting a Th1 immune response (36). IL-13, a member of the interleukin family, is largely secreted by activated Th2 cells and plays a critical role in allergic inflammation (37). It promotes M2 polarization. Furthermore, IL-13 actively contributes to the survival, activation, and recruitment of eosinophils (38). Presently, studies investigating the association between IL-13 and AITD are scarce. Therefore, it is essential to undertake comprehensive studies in the future to

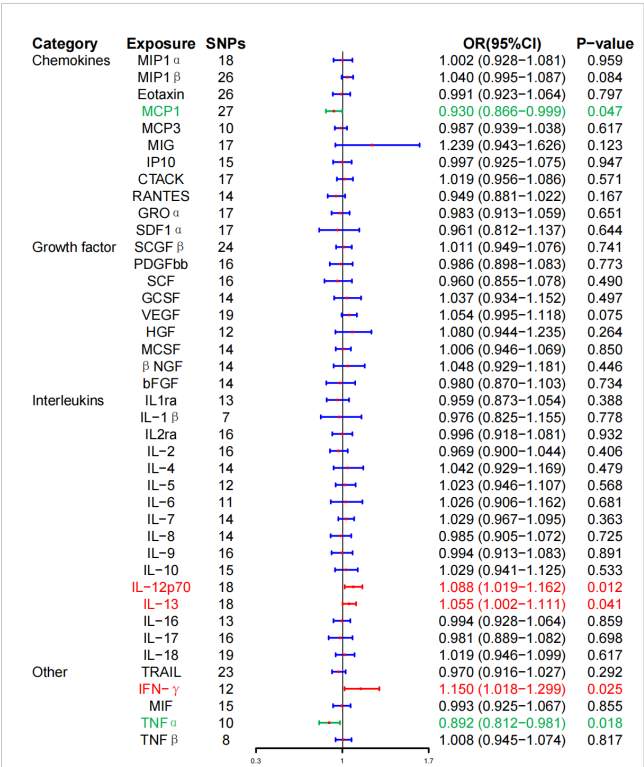


FIGURE 3 Associations between genetically predicted inflammatory cytokines and Hashimoto thyroiditis. SNPs, single-nucleotide polymorphisms; OR, odds ratio; 95% CI, 95% confidence interval; red means positive correlation; green means negative correlation.

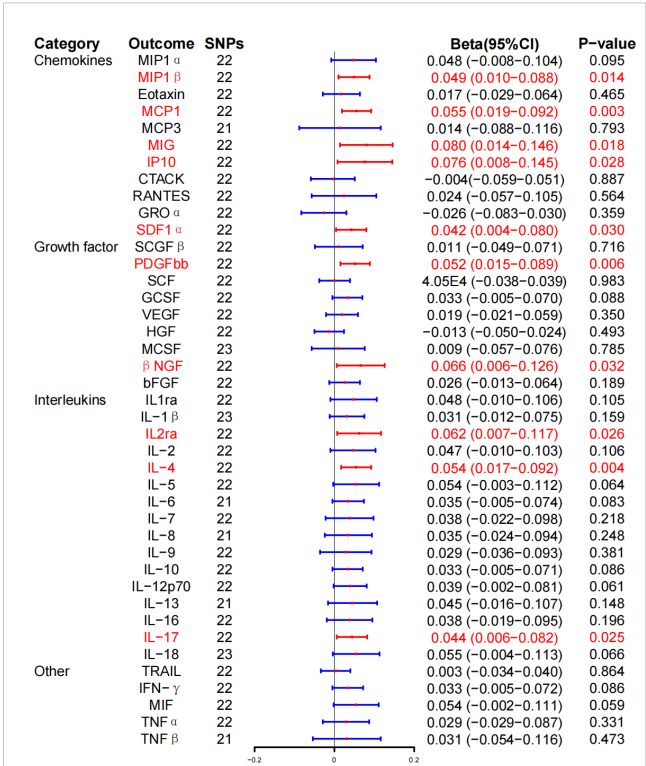


FIGURE 4 Associations between genetically predicted Graves' disease and inflammatory cytokines. SNPs, single-nucleotide polymorphisms; 95% CI, 95% confidence interval; red means positive correlation.

TABLE 5 Heterogeneity and sensitivity analysis of autoimmune thyroid disease with meaningful inflammatory cytokines.

Exposure	Outcome	Methods	Q	P	Intercept	P	MR-PRESSO
GD	MIP-1β						
		IVW	23.261	0.330	−0.007	0.570	0.340
		MR-Egger	22.880	0.295			
GD	MCP-1						
		IVW	16.927	0.716	1.98E-4	0.986	0.650
		MR-Egger	16.926	0.658			
GD	MIG						
		IVW	30.558	0.081	−0.038	0.054	0.050
		MR-Egger	25.248	0.192			
GD	IP-10						
		IVW	32.345	0.054	−0.023	0.270	0.070
		MR-Egger	30.391	0.064			
GD	SDF-1α						
		IVW	20.976	0.460	0.005	0.684	0.510
		MR-Egger	20.799	0.409			
GD	PDGFbb						
		IVW	14.509	0.847	0.013	0.266	0.800
		MR-Egger	13.198	0.869			
GD	βNGF						
		IVW	24.186	0.284	−0.009	0.642	0.200
		MR-Egger	23.920	0.246			
GD	IL-2ra						
		IVW	19.698	0.540	−0.016	0.345	0.560
		MR-Egger	18.764	0.537			
GD	IL-4						
		IVW	15.716	0.785	−0.001	0.965	0.830
		MR-Egger	15.714	0.734			
GD	IL-17						
		IVW	21.125	0.451	−0.001	0.911	0.460
		MR-Egger	21.112	0.391			
HT	MIG						
		IVW	20.587	0.024	−0.030	0.353	0.089
		MR-Egger	18.603	0.029			
HT	IL-2ra						
		IVW	13.292	0.208	−0.015	0.576	0.160
		MR-Egger	12.814	0.171			
HT	IP-10						
		IVW	22.664	0.012	0.016	0.647	0.510
		MR-Egger	22.113	0.009			

(Continued)

TABLE 5 Continued

Exposure	Outcome	Methods	Q	P	Intercept	P	MR-PRESSO
HT	IL-16						
		IVW	9.443	0.491	−0.011	0.640	0.440
		MR-Egger	9.203	0.419			

MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier; GD, Graves' disease; HT, Hashimoto thyroiditis; IVW, inverse variance-weighted.

elucidate this relationship. IFN- γ is the sole member of type II interferons and is produced not only by T cells, macrophages, and NK cells but also by thyroid cells (39). Its immunomodulatory effects include increased phagocytosis of macrophages, NK cell activation, enhancing the expression level of its antigens, and the toxicity of sensitized lymphocytes on target cells (9). Animal studies have documented that IFN- γ -induced apoptosis of thyroid cells is implicated in the pathogenesis of HT (40). Clinical studies further unveiled a significant rise in the number of IFN- γ + cells in patients with HT (41).

MCP-1 belongs to the CC chemokine family and plays a fundamental role in the inflammatory process (42). Currently, numerous clinical studies have observed an elevated expression level of MCP-1 in both thyroid tissue and serum of patients with AITD (43, 44). Importantly, our study found that low levels of MCP-1 were associated with a higher risk of HD, whereas reverse MR analysis revealed up-regulated MCP-1 expression in patients

with GD. TNF- α plays an instrumental role in modulating the inflammatory response, apoptosis, and immune cell activity. Additionally, it triggers the transcription and expression of a diverse array of cytokines, thereby facilitating their synthesis and release (45). Given its central role in the inflammatory response and immune regulation, researchers have developed numerous drugs targeting TNF- α for conditions such as rheumatoid arthritis (46) and Crohn's disease (47). Notably, our study detected a negative association between TNF- α levels and HD. Further investigations with more extensive databases are warranted to validate this correlation.

Reverse MR analysis showed that GD can increase the blood levels of MIP-1 β , MCP-1, MIG, IP-10, SDF-1 α , PDGFbb, β NGF, IL-2ra, IL-4, and IL-17, whereas HD can increase the blood levels of MIG, IL-2ra, IP-10, and IL-16. After BH correction, a strong positive correlation was identified between the risk of HD and the expression level of MIG. MIP-1 β is a CC chemokine that selectively binds to the CCR5 receptor. It chemotaxes NK cells, monocytes, and various other immune cells and can be generated by mast cells, endothelial cells, macrophages, and CD8+ T cells (48). Kemp et al. (46) observed an increased expression of MIP-1 β in the thyroid tissue of patients with AITD, thereby confirming the significant role of MIP-1 in pro-inflammatory processes. MIG belongs to the glutamic acid-leucine-arginine (ELR)-negative CXC chemokine subfamily and can be triggered by IFN- γ (49). CXCL9 plays an instrumental role in modulating the immune system and promoting inflammation. Of note, the recruitment, transport, and maintenance of particular subgroups of activated lymphocytes are essential for initiating and sustaining AITD (50). The secretion of chemokines that bind to CXCR3 by thyroid cells is stimulated by IFN- γ , subsequently attracting Th1 lymphocytes that express CXCR3 and secreting IFN- γ (51). Disrupting MIG could alleviate inflammatory reactions. Therefore, we postulate that targeting MIG may constitute a therapeutic target for the treatment of AITD. IP-10 is a member of the CXC chemokine family (52) and shares receptors such as CXCR3 with MIG. Inflammatory cells, attracted by IP-10, include chemotactic T lymphocytes that infiltrate and proliferate, thereby mediating antibody-specific autoimmune responses and resulting in gland destruction (53). Romagnani et al. (54) documented that the expression of IP-10, MIG, and CXCR3 was downregulated or absent in healthy thyroid tissue, whereas chemokines and their receptors were abundant in the thyroid glands of the majority of patients with GD. IP-10 and MIG were localized in infiltrating lymphocytes, macrophages, and resident epithelial follicular cells, whereas CXCR3 was predominantly localized in infiltrating inflammatory cells. SDF-1 α belongs to the α subfamily of chemokines. Acting as a ligand, it binds to the

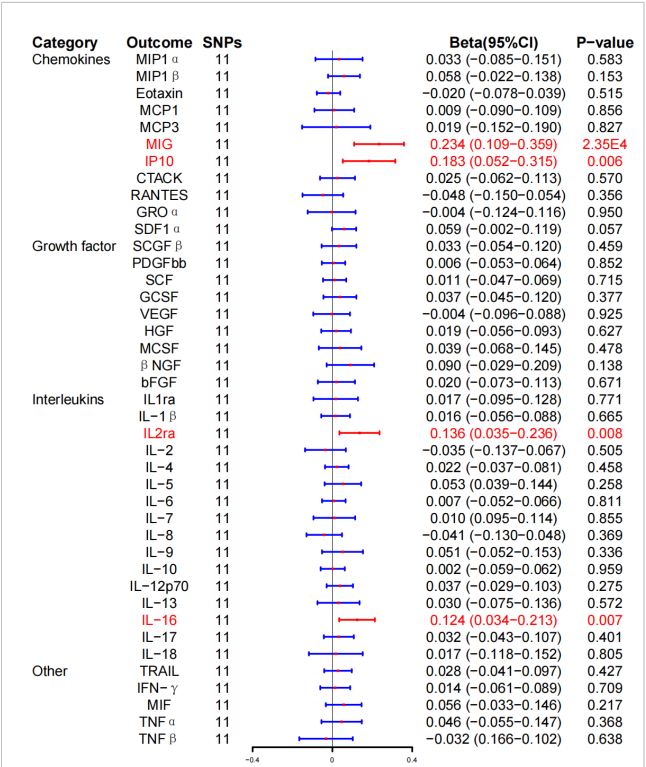


FIGURE 5 Associations between genetically predicted Hashimoto thyroiditis and inflammatory cytokines. SNPs, single-nucleotide polymorphisms; 95% CI, 95% confidence interval; red means positive correlation.

CXCR4 receptor. The SDF-1 α /CXCR4 signaling pathway plays an essential role in various physiological processes, including cell transport, angiogenesis, embryogenesis, tumor invasion, and metastasis (55, 56). Consequently, existing studies predominantly focused on investigating the value of SDF-1 α as a therapeutic target in thyroid malignancies. However, research addressing its role in AITD is limited (57, 58). PDGFbb participates in regulating cell growth and division. Following injury, platelets secrete PDGFbb, which facilitates the infiltration of neutrophils and macrophages. Simultaneously, PDGFbb promotes the secretion of a new extracellular matrix by fibroblasts and conduces to Insulin-like growth factor (IGF)-1-mediated reepithelialization (59, 60). Previous investigations have evinced that PDGFbb possesses the capability to induce orbital fibroblast proliferation, as well as the production of hyaluronic acid and cytokines in the orbital tissue of patients with Graves' ophthalmopathy (61). β NGF primarily stimulates the growth, development, differentiation, and maturation of both central and peripheral neurons, concurrently upholding the physiological functioning of the nervous system (62). Simultaneously, it is involved in immunomodulation, directly influencing the innate and adaptive immune responses of B and T cells. Importantly, it induces the release of neuropeptides and neurotransmitters, thereby modulating immune system activation within inflammatory tissues (63). It is worthwhile mentioning that elevated levels of autoantibodies against β NGF were noted in the blood and tissues of individuals with AITD. Moreover, it has the potential to activate mast cells, triggering the release of inflammatory mediators, exacerbating the inflammatory state, and contributing to tissue damage in AITD (64, 65). The expression of IL-2ra is paramount for the proliferation and growth of T cells (66). Recent research has identified the occurrence of polymorphisms in this gene among patients with AITD (14), whereas Nakanishi et al. (67) observed elevated serum IL-2ra levels in patients with AITD. IL-4 is crucial for the development and function of helper Th2 cells (68). According to a previous study, the proportion of IL-4+ cells was increased within the thyroid tissues of HT patients (66), consistent with the results of our study. IL-17 serves as a primary effector of Th17 cells, playing a vital role in promoting their activation (69). Xue et al. (70) pointed out that the expression level of CD4+, IL-17+, and Th17 cells in the blood of untreated patients with AITD was higher than that of healthy individuals. Additionally, El-Zawawy et al. (71) reported a significant elevation in the expression level of serum IL-17A in patients with AITD compared to the healthy control group. IL-16 is a pro-inflammatory cytokine that exerts chemotactic effects on CD4 T lymphocytes, monocytes, and eosinophils, playing a role in both the inflammatory response and tumor development. Ongoing research has provided evidence of the expression of the IL-16 protein in the thyroid glands of patients diagnosed with GD and HT (72). Furthermore, existing evidence suggests that the expression levels of IL-16 in the serum could be a candidate marker for assessing disease activity and the severity of AITD (73).

To sum up, cytokines are involved in the pathogenesis of AITD (74). Their secretion profile can be either pro-inflammatory or anti-inflammatory and either pro-apoptotic or anti-apoptotic (75).

These cytokines can participate in governing the immune system and the differentiation, growth, and secretory function of thyroid cells through their endocrine, autocrine, and paracrine modes. By modulating TSH levels, thyroid cells induce the abnormal expression of major histocompatibility complex-II antigens, which are transformed into antigen-presenting cells and resulting in autoimmune pathological damage and the development of AITD (76). Th1 lymphocytes generate pro-inflammatory proteins, including IFN- γ and IL-2, driving macrophage activation and inducing cytotoxic effects. On the other hand, Th2 lymphocytes generate anti-inflammatory proteins that suppress the production of Th1 cytokines. Additionally, they primarily stimulate B cells to generate antibodies and trigger the activation of anti-apoptotic molecules (77). Th17 lymphocytes release pro-inflammatory cytokines such as IL17 and exert a significant impact on persistent inflammation (78, 79).

While this study established a causal relationship and identified candidate targets for subsequent functional studies, it also has limitations that should not be overlooked. (1) This study used European population GWAS data for MR analysis, necessitating further studies in other populations. (2) Although MR is a highly efficient method for causality analysis, future animal tests are warranted to corroborate our findings. (3) The relationship between inflammatory cytokines and AITD is multifaceted, and elucidating the etiology and pathogenesis of circulating cytokines requires exploration from multiple aspects. (4) Ascribed to the complex etiology of AITD, circulating cytokines cannot fully explain the pathogenesis, necessitating more comprehensive data for further investigation.

In summary, the present study utilized the MR approach to investigate the causal association between inflammatory cytokines and AITD. Our results collectively unveiled that high levels of TNF- β and low levels of SCGF- β were associated with a high risk of developing GD. At the same time, high levels of IL-12p70, IL-13, and IFN- γ and low levels of MCP-1 and TNF- α suggest a higher risk of developing HD. Moreover, GD can increase the blood levels of MIP-1 β , MCP-1, MIG, IP-10, SDF-1 α , PDGFbb, β NGF, IL-2ra, IL-4, and IL-17, whereas HD can lead to elevated levels of MIG, IL-2ra, IP-10, and IL-16 levels. These cytokines hold significant implications for noninvasive early diagnosis of AITD. Finally, these cytokines may represent novel targets for the prevention, treatment, and long-term management of AITD.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Ethics statement

Since the data used are publicly available in the database, no additional ethical approval was needed in this case.

Author contributions

ZY: Investigation, Methodology, Project administration, Resources, Funding acquisition, Writing – review & editing. FG: Software, Supervision, Validation, Visualization, Writing – original draft. YT: Conceptualization, Methodology, Resources, Validation, Writing – original draft. YZ: Project administration, Resources, Software, Supervision, Writing – review & editing. YG: Conceptualization, Data curation, Project administration, Writing – original draft. GY: Data curation, Investigation, Project administration, Visualization, Writing – review & editing. SW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1334772/full#supplementary-material>

SUPPLEMENTARY TABLE 1

The F number and R2 detect the intensity of the IVs between 41 Inflammatory cytokines and GD in MR analyses. GD, Graves' Disease; IVs, instrumental variables.

SUPPLEMENTARY TABLE 2

The F number and R2 detect the intensity of the IVs between GD and 41 Inflammatory cytokines in MR analyses. GD, Graves' Disease; IVs, instrumental variables.

SUPPLEMENTARY TABLE 3

The F number and R2 detect the intensity of the IVs between 41 Inflammatory cytokines and HT in MR analyses. HT, Hashimoto thyroiditis; IVs, instrumental variables.

SUPPLEMENTARY TABLE 4

The F number and R2 detect the intensity of the IVs between HT and 41 Inflammatory cytokines in MR analyses. HT, Hashimoto thyroiditis; IVs, instrumental variables.

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Predictive factors of psychiatric syndrome in patients with systemic lupus erythematosus

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Introduction: Early detection of neuropsychiatric systemic lupus erythematosus (NPSLE) remains a challenge in clinical settings. Previous studies have found different autoantibodies as markers for NPSLE. This study aimed to describe the distribution of psychiatric syndromes in a group of patients with systemic lupus erythematosus (SLE) and to investigate the association between psychiatric syndromes and specific autoantibodies.

Methods: This retrospective study was conducted at a single medical center in China. We reviewed medical records of hospitalized patients with SLE who were consulted by psychiatrists due to potential mental disorders. Results of serum autoantibodies and general laboratory tests were collected. The correlation between clinical variables was examined. Binary logistic regression analyses were used to determine factors related to NPSLE and different psychiatric diagnoses.

Results: Among the 171 psychiatric manifestations in 160 patients, 141 (82.4%) were attributed to SLE. Acute confusional state (ACS) had the highest prevalence (57.4%). Anti-cardiolipin (ACL) antibody ($\chi^2 = 142.261$, $p < 0.001$) and anti- $\beta 2$ glycoprotein I (- $\beta 2$ GP1) antibody ($\chi^2 = 139.818$, $p < 0.001$) varied significantly between groups, with the highest positive rate found in patients with mood disorders (27.3% and 18.2%). SLE disease activity index – 2000 (SLEDAI-2K) score excluding item ACS and item psychosis was a predictor of NPSLE (OR 1.172 [95% CI 1.105 – 1.243]).

Conclusions: Disease activity reflected by SLEDAI-2K score is a predictor for NPSLE. Antiphospholipid antibodies are associated with mood disorders in SLE. Further separate investigation of neuropsychiatric disorders is needed in order to better comprehend NPSLE's pathological mechanism.

KEYWORDS

systemic lupus erythematosus, neuropsychiatric systemic lupus erythematosus, mental disorders, biomarkers, referral consultation

1 Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that affects multiple organs or systems, characterized by excessive production of pathogenic autoantibodies (autoabs) against a range of autoantigens (1). Neuropsychiatric lupus (NPSLE) refers to SLE involving the nervous system, including 19 clinical syndromes such as epilepsy, psychosis, and acute confusional state, which are divided into two categories: central nervous system involvement and peripheral nervous system involvement (1). Due to the variety of clinical manifestations from mild to severe, early detection of NPSLE remains a challenge in clinical settings and frequently necessitates multidisciplinary collaboration (2, 3). As a sign of critical illness, NPSLE also predicts an increase in disability and mortality (2, 4). Hence, it is important to identify the risk of neuropsychiatric (NP) involvement in the early stages of the disease in order to achieve an optimal outcome.

The attribution of neuropsychiatric manifestations has been a major challenge in clinical settings, possibly due to the unelucidated etiopathogenesis of NPSLE. This complexity is also mirrored in the study outcomes, as the prevalence of NPSLE drastically span from 14% to 95% (5). Based on the American College of Rheumatology (ACR) Nomenclature of 19 NPSLE syndromes (6), Ainiala et al. distinguished between minor and major manifestations (7). Later attribution models also took into account chronological association of NP symptoms and SLE, in addition to confounding factors, such as the attribution model proposed by the Italian Study Group (8). Until this day, clinical diagnosis remains the gold standard for NPSLE.

Psychiatric syndromes in SLE include acute confusional state (ACS), anxiety disorders, cognitive dysfunction (CD), mood disorders, and psychosis, among which ACS and psychosis are considered of the most diagnostic or “organic” significance of NPSLE (6, 9), or major manifestations by Ainiala et al. (7). Anxiety disorders, mood disorders, and CD are common in patients with SLE, but their contributing factors are more complex. For instance, anxiety disorders and mood disorders may be related to psychosocial factors, while CD may be secondary to cerebrovascular disease (2, 10).

Vasculopathy, autoabs-mediated tissue and neuronal damage, inflammatory mediators, blood-brain barrier (BBB) dysfunction, and others are some of the suggested pathogenetic mechanisms for NPSLE (10, 11). Ho et al. proposed that autoabs such as anti-ribosomal P protein (-ribP) antibodies (abs), and anti-phospholipid (APL) abs may point to more “organic” causes, while patients with negative autoabs, and no positive results from head MRI and EEG results, point to “functional” causes. They also found that among 19 NP syndromes, the positivity of APL abs (serum anti-cardiolipin (ACL) abs, lupus anticoagulants (LA), anti- β 2GPI) and anti-ribP abs were specifically significantly associated with the manifestations of mood disorder, psychosis, ACS, and CD (12). NP involvement might be aided by serum anti-Smith (-Sm) abs permeating BBB in patients with NPSLE (13, 14). Similar to the widely varying prevalence of NPSLE, biomarkers including autoabs varied from

study to study, possibly as a result of different diagnostic criteria for NPSLE (15, 16).

The purpose of this study was to describe the distribution of psychiatric syndromes in patients with SLE and to investigate the association between psychiatric syndromes and specific autoabs and general laboratory results.

2 Methods

2.1 Subjects

We conducted this single-center retrospective cross-sectional study at Peking Union Medical College Hospital (PUMCH), a tertiary general hospital in Beijing, China. A review of the medical records of patients hospitalized at PUMCH between April 2013 and July 2020 was conducted by combining the efforts of both the Departments of Rheumatology and Clinical Immunology and Psychological Medicine.

The inclusion criteria of this study were: (a) Diagnosed as SLE by treating rheumatologists according to the 2012 Systemic Lupus Collaborating Clinics classification criteria for SLE (17); (b) Received psychiatric consultations due to potential mental disorders. Exclusion criteria were: (a) Comorbidity of other rheumatic diseases; (b) Incomplete information on psychiatric consultations or diagnoses. Psychiatric diagnoses were established through a full consideration of history, findings in mental status examination, and investigations. The diagnostic criteria for psychiatric syndromes in SLE, including ACS, psychosis, anxiety disorder, and mood disorder, were established upon the integration of the International Classification of Diseases Tenth Edition (ICD-10) (18) and the 1999 ACR NPSLE nomenclature (6). CD in NPSLE typically necessitates neuropsychological tests, such as the ACR neuropsychological battery for SLE. The standard procedure of mental status examination executed by consulting psychiatrists at PUMCH include general assessment of cognitive function, normally including tests of attention, memory, executive function and motor performance, verbal function and language skills, and concept formation and reasoning. Should the patient demonstrate dysfunction in a single domain, it is classified as mild CD. Should the patient exhibit cognitive decline in 2-3 domains, it is recognized as moderate CD. If the patient demonstrates impairments in 4-5 domains, it equates to severe CD. In this study, we defined CD related to SLE as the subject exhibiting impairment in at least two of the five cognitive domains.

The attribution of each patient’s psychiatric syndromes was determined by the attending rheumatologist, viewed as the gold standard. The primary basis for diagnosis and differential diagnosis is ACR Nomenclature for NPSLE (6) and EULAR recommendations for the management of SLE with neuropsychiatric manifestations (19). The information typically considered encompasses historical recollection, characteristics of symptoms, laboratory results, autoimmune antibodies, radiological and EEG observations, confounding factors that necessitate exclusion, and opinion from psychiatric consultation.

The study was conducted in accordance with the Helsinki Declaration of the World Medical Association. The design of the study was reviewed and approved by the Ethics Committee of PUMCH, and informed consent was waived due to observational/non-interventional design.

2.2 Data collection

General demographic information, medical features such as SLE disease activity index – 2000 (SLEDAI-2K) scores (20), psychiatric diagnoses, and usage of glucocorticoids (GC) and immunosuppressants (IS) were collected from medical records. Results of the following tests were also collected: complete hemogram, C3 and C4 levels, serum immunoglobulin (Ig), cerebrospinal fluid (CSF) analyses, anti-double stranded DNA (-dsDNA) abs, anti-Sm abs, anti-ribP abs, and the APL abs (LA, anti- β 2 glycoprotein I (- β 2GPI) abs, ACL abs). All abs were tested by enzyme-linked immunosorbent assay, and range of values to be considered negative were shown in [Supplementary Material 1](#). Results of head MRI were also collected.

2.3 Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics 21.0.0.0. (IBM Corp., Armonk, NY, USA). Categorical variables are described as frequencies (percentages). Quantitative variables are described as mean $\pm$ standard deviation or median (interquartile range [IQR]) based on the normality of distribution. Fisher's 1-way ANOVA and Bonferroni correction or the Kruskal–Wallis test were used for among-group comparisons of continuous normally and non-normally distributed variables, respectively. The Chi-square test or Fisher's exact test was used for among-group comparisons of categorical variables. The correlation between clinical variables and psychiatric diagnoses was examined using Spearman's correlation test. Subsequently, binary logistic regression analyses were used to determine factors related to NPSLE and different psychiatric diagnoses, with age and gender incorporated into the model. A two-tailed *p* value < 0.05 was considered statistically significant.

3 Results

A total of 160 patients with SLE were included. Demographic information, clinical characteristics, and laboratory data of participants are summarized in [Table 1](#); [Supplementary Material 2](#). There were 145 women (90.6%), with a median age of 28 (IQR 23 - 39), and a median course of disease of 1.5 years (IQR 0.3 - 6). Seventy-five percentage of patients manifested multi-organ or -system involvement. The mean SLEDAI-2K score was 20.2 (SD 8.2). One hundred and forty-eight patients (92.5%) were diagnosed with at least one psychiatric condition. Of the 171 psychiatric manifestations, 141 (82.4%) were attributed to SLE. The highest to lowest prevalence was observed in ACS (81, 57.4%), CD (35, 24.8%), psychosis (14, 9.9%), and mood disorder (11, 7.8%), respectively.

Compared between groups, there were no significant differences in sex ratio, age, age of onset, or course of disease. [Tables 1, 2](#) depict that the mean SLEDAI-2K score demonstrated a significant variance amongst diagnostic groups ($F = 7.526$, $p < 0.001$). Removal of items pertaining to ACS and psychosis, revealed no significant differences in the remainder of the SLEDAI-2K score between groups ($F = 1.164$, $P = 0.330$). Patients presenting with bipolar disorder secondary to SLE displayed the lowest mean SLEDAI-2K score of 9.3 ± 4.2 . Seven patients (4.3%) were diagnosed concurrently with antiphospholipid syndrome (APS). Three patients experienced ACS, three presented with CD, and one exhibited psychosis.

All patients were prescribed GC as standardized therapy, and cortisol pulse therapy was implemented in 89 patients (55.6%). The most to least frequently administered IS were cyclophosphamide (66.3%), hydroxychloroquine (61.3%), mycophenolate mofetil (18.1%), tacrolimus (8.8%), cyclosporine A (4.4%), belimumab (1.9%), leflunomide (1.9%), and thalidomide (1.9%).

In relation to specific autoantibodies in various diagnostic cohorts, more patients carried positive ACL and anti- β 2GPI abs in the group of mood disorders (36.4% and 27.3). No notable differences in the positivity rates of anti-dsDNA abs, anti-Sm abs, and anti-ribP abs existed across groups. Additionally, there was no significant difference in hemoglobin, white blood cell count, platelet, plasma Igs, C3, C4, LA, and CSF analyses among diagnostic groups ([Supplementary Material 2](#)).

Of the 132 patients who underwent head MRI, abnormal results were noted in 85 patients (64.4%). These included 14 presenting with ischemic lesions, 37 with inflammatory lesions, 5 exhibiting both ischemic and inflammatory lesions, and 29 demonstrating other abnormalities. Notably, the findings of head MRI outcomes did not significantly differ across patients with disparate psychiatric diagnoses, as indicated in [Table 3](#). Of the seven patients with secondary APS, four had ischemic lesions, two demonstrated both ischemic and inflammatory lesions, while the remaining patient did not undergo MRI.

Correlation analysis revealed that bipolar disorder exhibited a weak correlation with the SLEDAI-2K score excluding the ACS and psychosis item ($r = -0.167$, $p = 0.039$), course of disease ($r = 0.170$, $p = 0.035$), and anti-Sm ab ($r = 0.185$, $p = 0.023$). Psychosis demonstrated a weak correlation with hemoglobin ($r = 0.187$, $p = 0.020$). ACS and CD were not significantly correlated with any clinical features presented. The SLEDAI-2K score excluding the ACS and psychosis item demonstrated a moderate correlation with NPSLE ($r = 0.421$, $p < 0.001$). Binary logistic regression revealed that the SLEDAI-2K score excluding the ACS and psychosis item was a predictor of NPSLE (OR 1.172 [95% CI 1.105 - 1.243]). With the available data, we did not identify distinct indicators for each specific psychiatric syndrome.

4 Discussion

In this research, we analyzed the correlation between clinical variables and NPSLE, concentrating predominantly on psychiatric disorders. Identifying biomarkers associated with the manifestation

TABLE 1 Intergroup comparisons of clinical features and autoantibodies.

	All patients N=160	ACS N=81	Psychosis N=14	Mood Disorders N=11		Cognitive Dysfunction N=35	None N=12	F/ χ^2	p
				Depressive N=1	Bipolar N=10				
Female, n (%)	145 (90.6)	75 (92.6)	12 (85.7)	Female	8 (80.0)	32 (91.4)	11 (91.7)	2.281	0.809
Age, median (IQR)	28 (23, 39)	28 (24, 41)	29 (23, 33)	47	26 (22, 41)	28 (23, 43)	26 (21, 30)	0.993	0.424
Disease course/yr	1.5 (0.3, 6)	0.5 (0.2, 4.5)	2.8 (0.8, 6.0)	9	6.0 (2.7, 9.3)	1.5 (0.3, 7)	1.8 (0.2, 5.8)	1.569	0.172
SLEDAI-2K, mean $\pm$ SD	20.2 $\pm$ 8.2	21.4 $\pm$ 7.3	18.3 $\pm$ 9.3	22	9.3 $\pm$ 4.2	14.9 $\pm$ 7.4	14.5 $\pm$ 8.2	7.526	<0.001
SLEDAI-2K excluding ACS and psychosis, mean $\pm$ SD	13.6 $\pm$ 7.5	13.8 $\pm$ 7.0	11.4 $\pm$ 10.8	22	9.3 $\pm$ 4.2	14.9 $\pm$ 7.4	14.5 $\pm$ 8.2	1.164	0.330
APS n (%)	7 (4.3)	3 (3.7)	1 (7.1)	0 (0)	0 (0)	3 (8.6)	0 (0)	2.735	0.741
anti-dsDNA ab positivity, n (%)	61 (53.0)	47 (58.0)	7 (50.0)	Negative	7 (70.0)	16 (45.7)	8 (66.7)	2.885	0.718
anti-Smith ab positivity, n (%)	42 (29.0)	20 (24.7)	5 (35.7)	Negative	6 (60.0)	11 (31.4)	1 (11.1)	7.656	0.176
anti-ribP ab positivity, n (%)	50 (34.0)	30 (37.0)	5 (35.7)	Negative	5 (50.0)	15 (42.9)	5 (55.6)	2.513	0.775
ACL positivity, n (%)	23 (15.8)	10 (12.3)	1/12 (8.3)	Positive	3 (30.0)	3 (8.6)	1 (10.0)	142.261	<0.001
anti- β 2GP1 ab positivity, n (%)	21 (14.6)	6 (7.4)	1/12 (8.3)	Positive	2 (20.0)	2 (5.7)	0 (0.0)	139.818	<0.001
LA/seconds, median (IQR)	1.2 (1.0, 1.2)	1.0 (0.9, 1.1)	1.1 (1.0, 1.3)	/	1.1 (1.0, 1.2)	1.0 (0.9, 1.2)	1.1 (1.0,1.1)	0.879	0.479

ACS, acute confusional state. APS, antiphospholipid syndrome. anti-dsDNA, anti-double stranded DNA antibodies. anti-Smith, anti Smith antibodies. anti-ribP, anti-ribosomal P, protein antibodies. ACL, anti-cardiolipin antibodies. anti- β 2GP1, anti- β 2 glycoprotein I antibodies. LA, lupus anticoagulants.
p values suggesting statistical significance ($p < 0.05$) was in bold font.
"/" stands for not applicable (N/A).

of diverse syndromes may foster comprehension of the pathogenesis of NPSLE. Psychiatric disorders in SLE, as secondary psychiatric disorders, suggest involvement of the CNS. Comparable to primary mental disorders, whilst there may be common pathological mechanisms among diverse types of syndromes, such as those of NPSLE, it should likewise exist distinct pathological mechanisms, such as those exclusive to psychosis. Presently, a definitive “gold standard” is lacking, and previous endeavors to delineate biomarkers for NPSLE have yielded conflicting results (21). Differences in diagnostic criteria and the absence of standardized reporting may be two possible explanations (15, 22). For instance, from a psychiatric perspective, mood disorders are two types of mental disorders of diverse nature, as both depressive and bipolar disorders consist of several subtypes. Consequently, markedly heterogeneous conclusions are likely to emerge from various studies concerning mood disorders in SLE. Moreover, the exploration of the genesis of primary psychiatric disorders necessitates consideration of the influence of social and psychological factors. Regrettably, in the majority of research pertaining to NPSLE, inclusive of this study, psychometric

evaluation data have been omitted or incomplete (5). This deficiency should be rectified in subsequent investigations.

Our study demonstrated that the SLEDAI-2K score exhibits distinctions in disease activity among patients with varied psychiatric syndromes. As ACS and mental disorders are constituents of the SLEDAI-2K score, the SLEDAI-2K global score of patients experiencing ACS or psychosis may surpass other patients who do not fulfill these two classifications. Following the exclusion of these two items, it was determined that there exists no notable disparity in disease activity amongst different diagnostic cohorts. Moreover, there weren’t noteworthy intergroup discrepancies in the majority of investigations, potentially due to the minimal sample size within certain subgroups. The correlation between the SLEDAI-2K score and NPSLE is predictable, as NPSLE is often a sign of critical illness (4). Upon our attempt to remove the ACS and psychosis items, it was evident that the remaining SLEDAI-2K score retained its capability of predicting NPSLE, providing evidence for a strong correlation between disease activity and the manifestation of psychiatric symptoms in SLE patients.

TABLE 2 SLEDAI-2K results.

Item n (%)	All patients N=160	ACS N=81	Psychosis N=14	Mood Disorders N=11		Cognitive Dysfunction N=35	None N=12
				Depressive N=1	Bipolar N=10		
Seizure	29 (18.1)	14 (17.3)	1 (7.1)	1	1 (10.0)	5 (14.3)	2 (16.7)
Psychosis	14 (8.8)	0 (0)	14 (100.0)	0	0 (0)	0 (0)	0 (0)
Organic brain syndrome	81 (50.6)	81 (100.0)	0 (0)	0	0 (0)	0 (0)	0 (0)
Visual disturbance	6 (3.8)	4 (4.9)	1 (7.1)	0	0 (0)	2 (5.7)	1 (8.3)
Cranial nerve disorder	2 (1.3)	2 (2.5)	0 (0)	0	0 (0)	1 (2.9)	0 (0)
Lupus headache	13 (8.1)	6 (7.4)	0 (0)	1	1 (10.0)	4 (11.4)	2 (16.7)
CVA	6 (3.8)	1 (1.2)	0 (0)	0	0 (0)	1 (2.9)	0 (0)
Vasculitis	11 (6.9)	6 (7.4)	1 (7.4)	0	0 (0)	3 (8.6)	1 (8.3)
Arthritis	28 (17.5)	14 (17.3)	3 (21.4)	0	0 (0)	5 (14.3)	2 (16.7)
Myositis	10 (6.3)	6 (7.4)	1 (7.4)	0	0 (0)	5 (14.3)	1 (8.3)
Urinary casts	7 (4.4)	3 (3.7)	0 (0)	0	0 (0)	3 (8.6)	1 (8.3)
Hematuria	39 (24.4)	23 (28.4)	2 (14.3)	0	2 (20.0)	10 (28.6)	3 (25.0)
Proteinuria	72 (45.0)	43 (53.1)	3 (21.4)	0	4 (40.0)	18 (51.4)	7 (58.3)
Pyuria	4 (2.5)	3 (3.7)	0 (0)	0	0 (0)	2 (5.7)	0 (0)
New rash	57 (35.6)	31 (38.3)	6 (42.9)	0	3 (30.0)	17 (48.6)	6 (50.0)
Alopecia	28 (17.5)	16 (19.8)	3 (21.4)	0	2 (20.0)	6 (17.1)	3 (25.0)
Mucosal ulcers	28 (17.5)	15 (18.5)	2 (14.3)	0	1 (10.0)	10 (28.6)	3 (25.0)
Pleurisy	32 (20.0)	17 (21.0)	1 (7.4)	1	2 (20.0)	6 (17.1)	2 (16.7)
Pericarditis	22 (13.8)	14 (17.3)	0 (0)	1	2 (20.0)	4 (11.4)	0 (0)
Low complement	105 (65.6)	57 (70.4)	10 (71.4)	1	6 (60.0)	26 (74.3)	8 (66.7)
Increased DNA binding	84 (52.5)	40 (49.4)	9 (64.3)	0	5 (50.0)	15 (42.9)	8 (66.7)
Fever	53 (33.1)	26 (32.1)	5 (35.7)	0	2 (20.0)	10 (28.6)	7 (58.3)
Thrombocytopenia	69 (43.1)	39 (48.1)	5 (35.7)	1	3 (30.0)	18 (51.4)	4 (33.3)
Leukopenia	64 (40.0)	30 (37.0)	4 (28.6)	1	4 (40.0)	20 (57.1)	7 (58.3)

ACS, acute confusional state. CVA, cerebrovascular accident.

Thus far, research has focused on the association of autoabs with NP conditions in SLE (4). Anti-dsDNA ab, one of the hallmark markers of SLE, is associated with overall disease activity and renal involvement. However, its correlation with neuropsychiatric manifestations was not remarkable (23). Research on anti-Sm ab, another diagnostic marker of SLE, revealed a significant increase in anti-Sm ab in patients with ACS compared to those with focal NPSLE and the fact that it plays a significant role in disrupting BBB in NPSLE (13, 14). Despite the controversy surrounding the conclusion, anti-ribP ab has become known as a highly specific biomarker for the diagnosis of SLE and is frequently associated with NPSLE (24). The presence of positive anti-ribP antibodies at baseline was associated with a greater proportion of neurological involvement (adjusted HR = 3.8, 95% CI 2.7-57) and cumulative neuropsychiatric damage during follow-up in our colleagues' recent study, which focused on NPSLE (25). In a recent cross-sectional study by Chessa et al., anti-ribP serum level was independently

associated with depressive symptoms (26). In our present study, however, the aforementioned abs did not differ significantly between psychiatric diagnostic groups. Hanly et al. discovered that impairment in BBB function is an important contributor to cognitive dysfunction, regardless of circulating SLE-related autoantibodies (27). The correlation between autoantibodies and NPSLE may comprise other mediating factors, necessitating future investigation.

The presence of APL abs is commonly associated with thrombosis (28). In NPSLE, APL ab-mediated thrombosis is considered the etiology of cerebrovascular disease and a possible mechanism for CD (21, 29). Owing primarily to the limited number of patients exhibiting APS in our research, no discernible difference was detected in the prevalence of APS across distinct psychiatric diagnoses. Nonetheless, we did discover that ACL and anti-β2GPI1 ab were more frequently observed in patients with mood disorders, a finding consistent with some prior studies. In a previous meta-

TABLE 3 Intergroup comparisons of head MRI results.

MRI abnormality	All patients N=132	ACS N=55	Psychosis N=12	Mood Disorders N=10		Cognitive Dysfunction N=33	None N=10	χ^2	p
				Depressive N=1	Bipolar N=9				
Ischemic	37 (28.0)	18 (32.7)	1 (8.3)	1	1 (11.1)	4 (12.1)	2 (20.0)	5.999	0.306
Inflammatory	14 (10.6)	6 (10.9)	5 (41.7)	/	3 (33.3)	6 (18.2)	4 (40.0)	1.507	0.912
Inflammatory + ischemic	5 (3.8)	3 (5.6)	0 (0)	/	0 (0)	2 (6.1)	0 (0)	1.961	0.854
Other	29 (22.0)	13 (23.6)	0 (0)	/	1 (11.1)	10 (30.3)	2 (20.0)	6.796	0.236
Normal	47 (35.6)	26 (47.3)	6 (50.0)	/	4 (44.4)	11 (33.3)	2 (20.0)	2.824	0.827

ACS, acute confusional state.
"/" stands for not applicable (N/A).

analysis, anti- β 2GP1 ab was found to be significantly associated with mood disorders (OR=6.27 [95% CI, 1.22-32.12]) (12). According to findings from a study focusing on perinatal women with APS, even after pregnancy, women with pure obstetric APS are at increased risk of venous and arterial thrombosis over time, and they also appear to develop more mood disorders (30). Early theoretical models postulated that mood disorders are due to malfunctioning of several neurotransmitters, while recent research has uncovered more neurobiological mechanisms, including vascular dysfunction (31, 32). Phenomena of cerebral hypoperfusion were observed in both depressive and bipolar disorders (33, 34). In order to better elucidate the pathological mechanism of APL abs in mood disorders in SLE, it is important to investigate depressive and bipolar disorders separately in future studies with larger sample sizes.

The main strength of our study lies in that we focused on psychiatric syndrome of patients with SLE, which maximizes the accuracy of diagnoses. Meanwhile, several limitations should not be overlooked. First, as a result of the small sample size of each diagnostic group, certain intergroup differences in autoabs and clinical features may not be fully reflected. Second, the study was conducted at a single medical center in Beijing, China, which may limit the generalizability of the results. Third, due to the retrospective design, data on some clinical confounding factors may have been missed. Validation of these findings requires large-scale prospective multi-center studies, as well as more in-depth research to elucidate the pathological mechanisms underlying NPSLE.

5 Conclusions

Disease activity reflected by SLEDAI-2K score is a predictor for NPSLE. Antiphospholipid antibodies are associated with mood disorders in SLE. A detailed exploration of neuropsychiatric disorders, ideally inclusive of psychosocial evaluations, is imperative to elaborate on the pathogenic mechanism of NPSLE.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics committee of Peking Union Medical College Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because of observational/non-interventional design.

Author contributions

WG: Writing – review & editing, Writing – original draft, Data curation. SZ: Writing – review & editing, Writing – original draft, Methodology. JC: Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. XH: Writing – review & editing, Supervision. YD: Writing – review & editing. YJ: Writing – review & editing, Project administration, Methodology. JW: Writing – review & editing, Supervision, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1323209/full#supplementary-material>

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Clinical characteristics of patients with autoimmune nodopathy with anti-neurofascin155 antibodies

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Background: According to the latest guidelines on chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), patients with CIDP with anti-neurofascin 155 (NF155) antibodies are referred to as autoimmune nodopathy (AN), an autoimmune disorder distinct from CIDP. We aimed to compare the clinical data of patients with AN with anti-NF155 antibodies with those of anti-NF155 antibodies-negative patients with CIDP, and to summarize the clinical characteristics of patients with AN with anti-NF155 antibodies.

Methods: Nine patients with AN with anti-NF155 antibodies and 28 serologically negative patients with CIDP were included in this study. Diagnosis was made according to the diagnostic criteria in the European Academy of Neurology (EAN)/Peripheral Nerve Society (PNS) guidelines on CIDP published in 2021. Demographics, clinical manifestations, electrophysiological examination, cerebrospinal fluid (CSF) tests, and response to treatment were retrospectively analyzed.

Results: Compared with serologically negative patients with CIDP, those patients with AN with anti-NF155 antibodies were younger ($p=0.007$), had a younger onset age ($p=0.009$), more frequent ataxia ($p=0.019$), higher CSF protein levels ($p=0.001$), and more frequent axon damage in electrophysiology ($p=0.025$). The main characteristics of patients with AN with anti-NF155 antibodies include younger age and onset age, limb weakness, sensory disturbance, ataxia, multiple motor-sensory peripheral neuropathies with demyelination and axonal damage on electrophysiological examination, markedly elevated CSF protein levels, and varying degrees of response to immunotherapy.

Conclusions: Patients with AN with anti-NF155 antibodies differed from serologically negative patients with CIDP in terms of clinical characteristics. When AN is suspected, testing for antibodies associated with the nodes of Ranvier is essential for early diagnosis and to guide treatment.

KEYWORDS

chronic inflammatory demyelinating polyradiculoneuropathy, autoimmune nodopathy, nodes of Ranvier, anti-neurofascin155 antibodies, clinical characteristics

1 Introduction

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is an autoimmune neuropathy characterized by demyelination of the peripheral nervous system that progresses for more than 8 weeks with a remission–relapse course (1). Typical clinical symptoms include bilateral symmetrical limb weakness with sensory deficits. The disease is most commonly accompanied by albuminocytologic dissociation in cerebrospinal fluid (CSF). Electrophysiological examination shows demyelinating changes, such as slowing of peripheral nerve conduction velocity (CV), prolongation of distal motor latency (DML), conduction block (CB), temporal dispersion (TD), and prolongation of F-wave latency. Patients can benefit from immunotherapy (2).

In recent years, advances in CIDP research have focused on autoantibodies against the nodal/paranodal protein of nodes of Ranvier (NR) (Figure 1). These antibodies are primarily raised against neurofascin 155 (NF155), contactin 1 (CNTN1), or contactin-associated protein 1 (Caspr1) (3). They mediate a unique neuropathy distinct from typical CIDP. In 2021, the most recent guidelines on the diagnosis and treatment of CIDP named this type of neuropathy as autoimmune nodopathy (AN) and recognized it as a relatively separate category of disease entity (2, 4). Pathogenicity has been demonstrated for these antibodies, which have been identified as specific biomarkers for the diagnosis and treatment guidance of AN (5, 6).

Patients with AN have specific clinical characteristics that vary with positive antibodies. The anti-NF155 antibody is the most common nodal/paranodal antibody detected in AN (5). In this study, we compared and analyzed the clinical data of patients with AN with anti-NF155 antibodies and serologically negative patients with CIDP, and summarized the demographics, clinical manifestations, electrophysiological characteristics, CSF tests, and the treatment of patients with AN with anti-NF155 antibodies to improve the clinicians' understanding of the disease for early recognition, diagnosis, and therapy.

2 Methods

2.1 Patients selection

Clinical data were retrospectively collected from 37 patients with CIDP who were diagnosed in the Department of Neurology of the First Affiliated Hospital of Zhengzhou University from August 2019 to August 2023. Nine patients were positive for anti-NF155

antibodies and 28 patients were serologically negative. The inclusion criteria for all patients met the diagnostic criteria for CIDP according to the latest EFNS/PNS 2021 guidelines. We exclude patients with peripheral neuropathies due to other etiologies such as poisoning, trauma, malignant tumors, and diabetes. The following parameters were planned to be assessed during electrophysiological examination: DML, motor conduction velocity (MCV), compound muscle action potential (CMAP) amplitude, F-wave latency of the median, ulnar, peroneal, and tibial nerves, and sensory nerve action potential (SNAP) amplitude and sensory conduction velocity (SCV) of the median, ulnar, superficial peroneal, and sural nerves. The efficacy of treatment was assessed according to change in the Hughes Disability Scale score, i.e., Δ Hughes (Hughes score at discharge–Hughes score at admission), and the presence or absence of clinical improvement: Δ Hughes < 0 was defined as effective, Δ Hughes = 0 with subjective or objective improvement was defined as partially effective, and Δ Hughes $\geq$ 0 without any improvement was defined as ineffective. This study was approved by the Ethics Committee of our hospital.

2.2 Cell-based assay

The serum samples of the patients were obtained before treatment and sent to the Neurology Laboratory of the First Affiliated Hospital of Zhengzhou University or Zhengzhou jinyu Clinical Laboratory Center. Fixed CBA was used for antibody detection at both institutes.

The specific steps of the CBA approach include the following: HEK 293 T cells were cultured in DMEM medium containing 10% fetal bovine serum. Cells were transfected with a green fluorescent protein (GFP)-marked expression vector containing cDNA encoding human NF155 NM_ (001160331) in the presence of lipo2000. After 4 hours, the transfection solution was replaced with DMEM medium containing 10% fetal bovine serum and the culture was continued for 24–36 hours. The cells expressing GFP-NF155 were fixed, permeabilized, and blocked before incubation with diluted sera (1:50) in PBS for 2 h at 37°C. The cells were subsequently treated for 45 min with Alexa Fluor 594-goat anti-human IgG Fc γ (1,1000) and rinsed with PBS three times. Finally, they were observed under a fluorescence microscope.

2.3 Statistical analyses

Statistical analysis was performed using the SPSS 25.0 software and drawings were performed using Adobe Illustrator 2020 software. Measurement data are presented as the mean $\pm$ standard deviation if normally distributed or as the median with interquartile range if not conforming to normal distribution. Enumeration data are presented as frequencies (percentages). According to their normality, measurement data were analyzed using the t-test or Mann–Whitney U-test. Enumeration data were analyzed using the Fisher's exact test. $P < 0.05$ was considered to indicate statistically significant differences.

Abbreviations: AN, autoimmune nodopathy; NF155, neurofascin 155; CNTN1, contactin 1; Caspr1, contactin-associated protein 1; CIDP, Chronic inflammatory demyelinating polyradiculoneuropathy; CSF, cerebrospinal fluid; EAN/PNS, European Academy of Neurology/Peripheral Nerve Society; IVIg, intravenous immunoglobulin; NR, nodes of Ranvier; RTX, Rituximab; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; CMAP, compound motor action potential; SNAP, sensory nerve action potential; DML, distal motor latency.

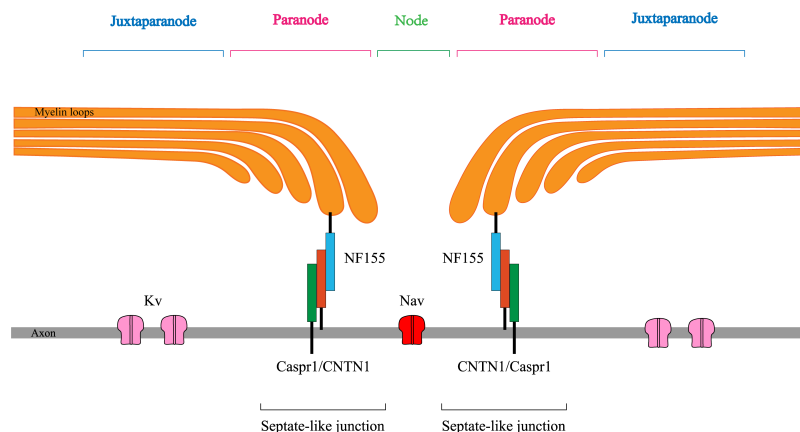


FIGURE 1

Sketchy anatomical map of the node of Ranvier. NF155, neurofascin155; CNTN1, contactin1; Caspr1, contactin-associated protein1; Kv, Voltage-gated K⁺ channel; Nav, Voltage-gated Na⁺ channel.

3 Results

The clinical data of patients with AN with anti-NF155 antibodies and serologically negative patients with CIDP are presented in [Table 1](#). We noticed a significantly younger age (33.8 ± 16.5 vs 51.3 ± 15.7 , $p=0.007$) and onset age in patients with AN with anti-NF155 antibodies than in the serologically negative patients (33.0 ± 16.3 vs 49.8 ± 15.8 ; $p=0.009$) and the former group presented with ataxia more frequently (88.9% vs 42.9%, respectively; $p=0.019$). No differences were found between the two groups in terms of sex, onset characteristics, or disease course. None of the nine patients with anti-NF155 antibodies experienced pain. The frequencies of weakness ($p=0.444$), numbness ($p=0.377$), tremor ($p=0.244$), sensory deficiency ($p=0.216$), pain ($p=0.568$), and cranial nerve involvement ($p=0.543$) were not significantly different between the two groups.

All nine patients with AN with anti-NF155 antibodies exhibited multiple motor–sensory peripheral nerve neuropathy with myelin and axonal involvement ([Supplementary Table 1](#)). Axonal damage was more frequently seen in patients with AN with anti-NF155 antibodies than in serologically negative patients (100% vs 60.7%, respectively; $p=0.025$).

Most patients with CIDP exhibited elevated CSF protein levels. The mean CSF protein levels were significantly higher in patients with AN with anti-NF155 antibodies than in serologically negative patients (3.37 [$1.71, 3.47$] vs 0.79 [$0.56, 1.64$], respectively; $p=0.001$), but the leukocyte counts were not statistically different. Albuminocytologic dissociation was present in most patients with AN with anti-NF155 antibodies.

There was no statistically significant difference in the efficacy of treatment regimens between the two groups of patients. Most of the serologically negative patients with CIDP were effectively treated with corticosteroids and intravenous immunoglobulin (IVIg) therapy. One patient with AN with anti-NF155 antibodies was treated with IVIg but did not respond to it. The other patients with AN with experienced varying degrees of symptomatic relief after

receiving immunotherapies. Multiple treatment regimens were often administered to the same patient ([Table 2](#)).

4 Discussion

The anti-NF155 antibody is the most prevalent antibody in AN. Previous studies have reported that patients with AN with anti-NF155 antibodies present with a specific clinical phenotype that is mainly characterized by young onset age, acute or subacute disease onset, limb weakness, sensory deficiency, ataxia, tremor, cranial nerve involvement, and significantly elevated CSF protein levels, and poor response to IVIg treatment ([4, 5](#)). The clinical characteristics of patients with AN with anti-NF155 antibodies in this study differed from those of previously reported patients, mainly in terms of electrophysiologic axonal damage. The obvious absence of tremor, pain, or cranial nerve involvement may suggest that the disease may have a broad spectrum of phenotypic variations and heterogeneity.

We compared data from nine patients with AN with anti-NF155 antibodies with those from 28 serologically negative patients with CIDP. The former group featured a younger age and onset age, more frequent ataxia, higher CSF protein levels, and more frequent axonal damage on electrophysiological examination. We did not find any other significant differences between the two groups of patients.

In our study, the mean onset age was 33 years in patients with AN and 50 years in the serologically negative patients with CIDP. Zhang et al. found mean onset age of 33 and 47 years, respectively, similar to our findings ([7](#)). The mean age at onset in the Japanese patients with CIDP reported by Devaux et al. was 31 and 48 years, respectively, and another study of Japanese patients with CIDP yielded similar results ([6, 8](#)). By comparison, two studies from Europe reported that the age at onset of patients with AN with anti-NF155 antibodies was younger than that of serologically negative patients with CIDP, at 40 and 52, respectively ([9, 10](#)). Besides,

TABLE 1 Comparison of clinical characteristics of patients with AN with anti-NF155 antibodies with serologically negative patients with CIDP.

Characteristics	Anti-NF155 (n=9)	Negative (n=28)	p value
Demographics			
Female/Male	1/8	12/16	0.087
Age (years)	33.8 ± 16.5	51.3 ± 15.7	0.007
Onset age (years)	33.0 ± 16.3	49.8 ± 15.8	0.009
Onset characteristics, n/N(%)			
Triggering (infection/ vaccination)	3/9 (33.3%)	5/28 (17.9%)	0.292
Acute/subacute	3/9 (33.3%)	7/28 (25%)	0.463
Chronic	6/9 (66.7%)	21/28 (75%)	0.463
Course of disease, n/N(%)			
Remitting-relapsing	2/9 (22.2%)	14/28 (50%)	0.141
Progressing	7/9 (77.8%)	14/28 (50%)	0.141
Disease duration (month)	7.0 (5.0, 10.5)	7.5 (3.0, 21.0)	0.931
Clinical manifestations, n/N(%)			
Limb weakness	8/9 (88.9%)	22/28 (78.6%)	0.444
Limb numbness	6/9 (66.7%)	22/28 (78.6%)	0.377
Tremor	2/9 (22.2%)	1/28 (7.1%)	0.244
Sensory deficiency	8/9 (88.9%)	19/28 (67.9%)	0.216
Ataxia	8/9 (88.9%)	12/28 (42.9%)	0.019
Pain	0/9 (0%)	2/28 (7.1%)	0.568
Cranial nerve involvement	1/9 (11.1%)	5/28 (17.9%)	0.543
Electrophysiologic examination, n/N(%)			
Demyelinating predominant	8/9 (88.9%)	22/28 (78.6%)	0.444
Axon damage predominant	1/9 (11.1%)	6/28 (21.4%)	0.444
Axon damage	9/9 (100%)	17/28 (60.7%)	0.025
Prolonged DML	9/9 (100%)	24/28 (85.7%)	0.310
Prolonged F- wave latency	4/9 (44.4%)	11/28 (39.3%)	0.541
Reduced CV	9/9 (100%)	28/28 (100%)	NA
CB	1/9 (11.1%)	2/28 (7.1%)	0.578
TD	1/9 (11.1%)	2/28 (7.1%)	0.578
Reduced CMAP amplitude	9/9 (100%)	17/28 (60.7%)	0.025
CSF examination			
CSF protein (g/L)	3.37 (1.71,3.47)	0.79 (0.56,1.64)	0.001

(Continued)

TABLE 1 Continued

Characteristics	Anti-NF155 (n=9)	Negative (n=28)	p value
CSF examination			
Leucocyte (<5/μl)	4.0 (2.0, 7.0)	2.0 (2.0, 4.0)	0.566
Protein cell separation	8/9 (88.9%)	18/28 (64.3%)	0.163
Improvement by treatment, n/N(%)			
Corticosteroids	6/6 (100%)	18/20 (90%)	0.585
IVIG	0/1 (0%)	10/11 (90.9%)	0.167
PE	3/3 (100%)	1/1 (100%)	NA
RTX	1/1 (100%)	0 (0%)	NA
Cyclophosphamide	1/1 (100%)	1/1 (100%)	NA

DML, distal motor latency; CV, conduction velocity; CB, conduction block; TD, temporal dispersion; CMAP, compound muscle action potential; CSF, cerebrospinal fluid; IVIg, intravenous immunoglobulins; PE, plasma exchange; RTX, Rituximab; NA, not applicable. Bolded values represent statistically significant differences in clinical data between the two groups of patients with autoimmune nodopathy with anti-NF155 antibodies and serologically negative patients.

Delmont et al. prospectively tested sera from patients with suspected CIDP from France, Belgium, and Switzerland and found that the mean age of 15 patients with AN with NF155-IgG4 antibodies was younger than that of serologically negative patients with CIDP, with ages of onset of 54 and 66 years, respectively (11). This suggests that younger age and age at onset may be more representative of Asian populations, possibly due to ethnic differences; however, larger sample sizes are needed for further validation.

Ataxia is one of the clinical features that distinguishes patients with AN from serologically negative patients with CIDP, and our findings support this conclusion (6). Studies appear to be lacking on the specific mechanisms by which ataxia occurs in patients with CIDP with anti-NF155 antibodies. We hypothesize that ataxia may result from demyelination of sensory neurons or other unexplored mechanisms.

CSF albuminocytologic dissociation is a common phenomenon in CIDP, and the vast majority of patients with AN with anti-NF155 antibodies had markedly elevated CSF protein levels—up to more than 3.8 g/L—which were much higher than those in serologically negative patients with CIDP. At the meantime, magnetic resonance imaging (MRI) of peripheral nerves showed thickening of nerve roots in the cervical plexus or lumbosacral plexus, which is similar to what has been reported in previous studies (8, 12) (Figure 2). The reason for the elevated CSF protein levels remains unclear and may be related to thickening of the nerve root. Previous studies have found significant involvement of nerve roots of peripheral nerve in patients with AN compared to serologically negative patients with CIDP (6). Because of the absence of pathological manifestations of repeated demyelination and myelin regeneration in patients with AN as well as the lack of histological examination of proximal nerve roots, the mechanism of nerve roots thickening in patients with AN remains to be further explored. This antibodies may be involved in

TABLE 2 Clinical characteristics of nine patients with AN with anti-NF155 antibodies.

Characteristics	Anti-155 positive								
	1	2	3	4	5	6	7	8	9
Onset age/Sex	35/M	52/M	16/M	19/M	55/F	50/M	35/M	15/M	20/M
Onset	A	C	A	A	C	C	C	C	C
Disease duration(month)	8	24	5	5	6	7	9	3	12
Clinical features									
Limb weakness	+	+	+	+	+	+	+	+	–
Sensory dysfunction	+	+	+	+	+	+	+	+	–
Ataxia	+	+	+	+	+	+	+	+	–
Cranial nerve involvement	–	–	Facial nerve	–	–	–	–	–	–
Tremor	–	+	–	–	–	–	+	–	–
CSF protein level (g/L)	2.65	3.85	3.52	3.40	1.14	2.27	3.41	3.37	0.31
Leucocyte (<5/ μ l)	4	2	8	2	6	4	2	8	2
Peripheral nerve MRI	NA	NA	(+)	NA	NA	NA	NA	NA	NA
Electrophysiological study									
Demyelination	+	+	+	+	+	+	+	+	+
Axonal damage	+	+	+	+	+	+	+	+	+
Reduced CV	+	+	+	+	+	+	+	+	+
Prolonged DML	+	+	+	+	+	+	+	+	+
CB	–	–	–	–	+	–	–	–	–
TD	–	–	–	–	+	–	–	–	–
Reduced CMAP amplitude	+	+	+	+	+	+	+	+	+
Treatment response									
Corticosteroids	+	+	NA	+	+	+	+	NA	NA
IVIG	NA	NA	–	NA	NA	NA	NA	NA	NA
PE	NA	NA	+	+	NA	NA	NA	+	NA
RTX	NA	NA	+	NA	NA	NA	NA	NA	NA
Cyclophosphamide	NA	+	NA	NA	NA	NA	NA	NA	NA

M, male; F, female; A, acute; C, chronic; CSF, cerebrospinal fluid; MRI, Magnetic resonance imaging; CV, conduction velocity; DML, distal motor latency; CB, conduction block; TD, temporal dispersion; CMAP, compound muscle action potential; IVIg, intravenous immunoglobulins; PE, plasma exchange; RTX, Rituximab; NA, not applicable.
In clinical characteristics and electrophysiological studies, the symbol "–" indicates negative or absent, and the symbol "+" indicates positive or present; in response to treatment, the symbol "–" indicates no response, and the symbol "+" indicates a response.

the elevation of CSF protein levels and thickening of nerve roots by some complex mechanism.

Electrophysiological examination revealed frequent involvement of the motor and sensory nerves in patients with AN with anti-NF155 antibodies, and all patients had axonal damage, with demyelination predominating in the vast majority of the patients. This may correlate with the localization of NF155 in NR and its close relationship with axon, perhaps explained by the fact that nerve conduction depends on the structural and functional integrity of myelinated nerve fibers. NR consists of the node, paranode, and juxta-paranode, structurally and functionally specialized regions on the axon of myelinated nerve fibers that ensure the “jumping” conduction of nerve impulses on myelinated fibers (13). The structural basis is “septate-like” junctions at the

paranodes, consisting of NF155 located on myelin loops and CNTN1 and Caspr1 on the axolemma, which tightly bind the myelin sheath to the axon and act as electrical and molecular barriers limiting diffusion of ions and ion channels (14). Autoantibodies attacking on NF155 injure septate-like junctions leading to detachment of the myelin loops from the axon, thus affecting peripheral nerve conduction (15, 16). Mutant animal models have demonstrated impaired septal-like junction formation in the paranodal region and decreased peripheral nerve conduction velocity in mice lacking NF155, CNTN1, or Caspr1 (11, 17). All patients with AN in this study showed decreased or even inability to elicit CMAP amplitude, and the electromyography results of eight patients suggested neurogenic changes in the tested muscles, which

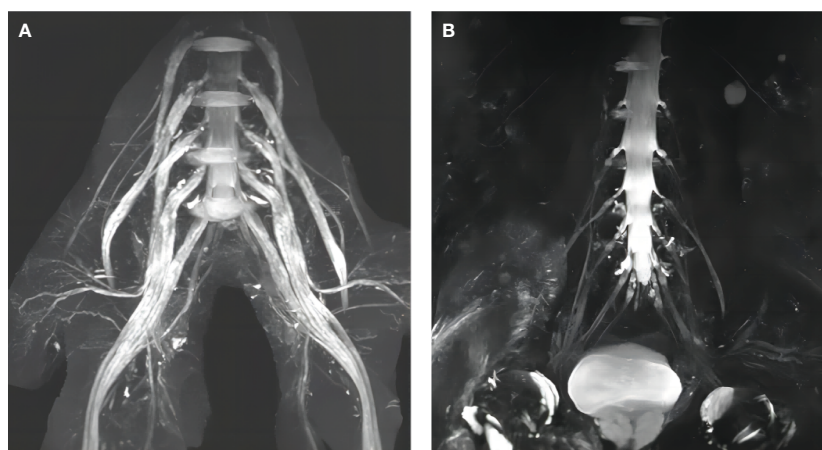


FIGURE 2

Lumbo-sacral plexus MRI findings. (A) Diffuse thickening of the lumbo-sacral nerve roots of a patient with AN with anti-NF155 antibodies; (B) Normal lumbo-sacral nerve roots of a serologically negative patient with CIDP.

suggests axonal damage to the peripheral nerves. Previous pathological findings did suggest that patients with AN with anti-NF155 antibodies have impaired formation of septate-like junctions, leading to secondary axonal damage (18). It can be hypothesized that NF155 may be a target antigen for autoantibody-mediated axonal damage in patients with AN with anti-NF155 antibodies.

Corticosteroids, IVIg, and plasma exchange therapy are first-line treatment options for CIDP, with most patients responding favorably. In this study, most patients with AN with anti-NF155 antibodies who received immunotherapy experienced varying degrees of improvement in their symptoms. However, the therapeutic efficacy of IVIg was unsatisfactory, which is in line with previous studies (19, 20). The poor response to IVIg may be explained by the fact that the predominant subtype of the anti-NF155 antibody is IgG4, which lacks complement-activating capability and has a low affinity for Fc-receptors, which constitute the mechanism of action of IVIg (6). Thus, antibody nature determines the lack of response to IVIg. Other patients with predominantly IgG1, IgG2, or IgG3 antibodies early in the course of the disease, responded favorably to IVIg; when the disease progressed to the middle and late stages, the antibody subtype shifted to IgG4, which may account for the different responses to IVIg at different stages of the disease (21, 22). Therefore, it is important to perform antibody typing and early treatment. Since there was only one patient with AN treated with IVIg in this study, further confirmation regarding the effectiveness of IVIg is still needed from studies with larger sample sizes.

Notably, we found no significant difference in the efficacy of corticosteroids between the two groups. We conjectured that the response to corticosteroids in the two groups may be independent of the serological status of the antibodies. Rituximab (RTX) is an option for patients with AN who do not respond well to first-line therapy (23). However, one patient with AN with anti-NF155 antibodies was treated with RTX and, despite symptomatic relief, the drug was discontinued because of oliguria and bilateral lower-extremity edema. Long-term observations are still needed to evaluate the effects of RTX treatment. Overall, the vast majority

of patients with AN patients in this study responded well to immunotherapy.

This study has some limitations. First, the number of patients with CIDP with anti-NF155 antibodies was small. Second, the antibody subtypes were unknown, preventing a more detailed characterization of the features corresponding to each subtype. Finally, complete and detailed follow-up information on efficacy could not be obtained because this was a retrospective study. Large-sample, multicenter prospective studies and long-term follow-up studies are needed.

In summary, the analysis and comparison revealed patients with AN with anti-NF155 antibodies have unique clinical characteristics that differ from serologically negative patients with CIDP, such as younger age and onset age, more frequent ataxia, significantly higher CSF protein levels, and more frequent electrophysiological manifestations of axon damage. Admittedly, these antibody-positive neuropathies should not be categorized as CIDP but rather as a new disease entity different from CIDP. If a patient presents with young onset, limb weakness, ataxia, markedly high CSF protein levels, and poor response to IVIg, detection of nodal or paranodal antibodies is recommended to help in the early diagnosis and guide treatment of AN.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the

participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

XH: Writing – review & editing, Writing – original draft, Data curation. LW: Writing – review & editing. JL: Writing – review & editing. SL: Writing – review & editing. YG: Writing – review & editing. JZ: Writing – review & editing, Supervision, Conceptualization. HL: Writing – review & editing. YJ: Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1345953/full#supplementary-material>

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Investigation of the causal association between Parkinson's disease and autoimmune disorders: a bidirectional Mendelian randomization study

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Background: To date, an increasing number of epidemiological evidence has pointed to potential relationships between Parkinson's disease (PD) and various autoimmune diseases (AIDs), however, no definitive conclusions has been drawn about whether PD is causally related to AIDs risk.

Methods: By employing summary statistics from the latest and most extensive genome-wide association studies (GWAS), we performed a bidirectional two-sample Mendelian randomization (MR) analysis to investigate the causal associations between PD and a variety of 17 AIDs, encompassing multiple sclerosis, neuromyelitis optica spectrum disorder, myasthenia gravis, asthma, inflammatory bowel disease, Crohn's disease, ulcerative colitis, irritable bowel syndrome, celiac disease, primary biliary cirrhosis, primary sclerosing cholangitis, type 1 diabetes, ankylosing spondylitis, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and vitiligo. Inverse-variance weighted (IVW) was adopted as the main statistical approach to obtain the causal estimates of PD on different AIDs, supplemented by a series of complementary analyses (weighted median, MR Egger regression, and MR-PRESSO) for further strengthening the robustness of results.

Results: Our MR findings suggested that genetically predicted higher liability to PD was causally associated with a decreased risk of irritable bowel syndrome (OR = 0.98; 95% CI: 0.96-0.99; $P = 0.032$). On the contrary, IVW analysis showed a potential positive correlation between genetically determined PD and the incidence of type 1 diabetes (OR = 1.10; 95%CI: 1.02-1.19; $P = 0.010$). Subsequent MR tests ended up in similar results, confirming our findings were reliable. Additionally, in the reverse MR analyses, we did not identify any evidence to support the causal relationship of genetic predisposition to AIDs with PD susceptibility.

Conclusion: In general, a bifunctional role that PD exerted on the risk of developing AIDs was detected in our studies, both protecting against irritable bowel syndrome occurrence and raising the incidence of type 1 diabetes. Future studies, including population-based observational studies and molecular experiments *in vitro* and *in vivo*, are warranted to validate the results of our MR analyses and refine the underlying pathological mechanisms involved in PD-AIDs associations.

KEYWORDS

Mendelian randomization, Parkinson's disease, autoimmune diseases, causal effect, irritable bowel syndrome, type 1 diabetes

1 Introduction

Parkinson's disease (PD) is widely perceived as the second most common neurodegenerative disease and the most prevalent movement disorder throughout the globe, characterized by a diverse array of clinical features including rigidity, bradykinesia, resting tremor, and postural instability (1). The number of patients suffering from PD worldwide has continued to rise substantially from 4.1 million in 2005 to 8.7 million by the year 2030 (2). Although the exact etiology remains elusive, a generally accepted hypothesis suggests that PD is a complicated multifactorial disease with interactions between toxic environmental factors and genetic determinants (3). Moreover, many recent studies indicated that in PD animal models as well as PD patients, a continuous inflammatory response was invariably observed at both the central nervous system (CNS) and peripheral levels (4–8). In the toxin-based PD rat models, CNS inherent immune cells, microglial cells, in the nigral tissue, could be over-activated to release substantial pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) (4). Besides, these centrally produced cytokines then flowed into the peripheral circulation through subarachnoid granules and/or lymphatic drainage, thereby regulating the inflammatory state of circulating myeloid cells (9). Reciprocally, a wide range of pro-inflammatory mediators generated by leukocytes in the peripheral blood were able to cross the blood-brain barrier (BBB) and activate microglia and astrocytes, which could alter the CNS neuroimmune environment (9). Thus, due to the central-peripheral immune crosstalk, it is particularly worth exploring whether PD is related to autoimmune diseases (AIDs) risk and vice versa.

Currently, an increasing number of observational studies reported the presence of comorbidities between PD and various autoimmune diseases. However, the epidemiological evidence on the possible relationships between PD and AIDs was seemingly inconsistent. A case-control study recruiting 3,276 participants from Mayo Clinic Biobank illustrated that individuals who self-reported a family history of Parkinson's disease among their first-degree relatives were 30% less likely to develop rheumatoid arthritis

(RA) (10). Other large-scale retrospective cohort research held a similar view that people with RA and systemic lupus erythematosus (SLE) exhibited a significantly reduced susceptibility to PD when compared to healthy individuals (11, 12). Conversely, a meta-analysis involving 833,004 patients and 10,175,890 controls demonstrated that ankylosing spondylitis (AS) and other AIDs were associated with a higher incidence of PD (13). Owing to uncontrolled confounding factors, reverse causality and limited sample size, these epidemiological investigations were prone to bias hindering the accurate inference of causal associations between PD and AIDs.

Mendelian randomization (MR) is a powerful technique that seeks to establish the causative link between exposure and outcome by utilizing genetic variations as instrumental variables (IVs) (14). In terms of overcoming the traditional bias present in observational studies, MR exhibited prominent advantages in eliminating reverse causation and avoiding the risk of confounding factors. Therefore, we conducted this bidirectional two-sample MR analysis to reveal the causal correlations between Parkinson's disease and a broad spectrum of 17 autoimmune disorders, including multiple sclerosis (MS), neuromyelitis optica spectrum disorder (NMOSD), myasthenia gravis (MG), asthma, inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), irritable bowel syndrome (IBS), celiac disease (CeD), primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), type 1 diabetes (T1D), AS, RA, SLE, psoriasis and vitiligo.

2 Methods

2.1 Study design, data sources and instrument selection

According to the core principles and main assumptions of MR, single-nucleotide polymorphisms (SNPs) used as the instrumental variables (IVs) need to satisfy three criteria: (1) relevance assumption: SNPs should be associated with the exposure of interest, (2) independence assumption: the genetic variants are

independent of confounders and (3) exclusion restriction assumption which proposes that SNPs should only affect outcome exclusively through path of the exposure (15). Considering that the data in our study were obtained from the publicly available genome-wide association studies (GWAS) database of PD and AIDs, no additional ethic approval and informed consent were required.

As shown in Table 1, we included the summary statistics for susceptibility to PD from the most comprehensive and updated genome-wide meta-analyses, which comprised 33,674 cases and 449,056 controls of European ancestry from International

Parkinson's Disease Genomics Consortium, Systems genomics of Parkinson's disease consortium and UK Biobank (16). With regard to the IVs associated with diverse AIDs, we extracted genetic variants from a total of 17 GWASs involving MS (47,429 cases and 68,374 controls) (17), NMOSD (215 cases and 1,244 controls) (18), MG (1,873 cases and 36,370 controls) (19), asthma (56,167 cases and 352,255 controls) (20), IBD (12,882 cases and 21,770 controls) (21), CD (5,956 cases and 14,927 controls) (21), UC (6,968 cases and 20,464 controls) (21), IBS (53,400 cases and 433,201 controls) (22), CeD (12,041 cases and 12,228 controls) (23), PBC

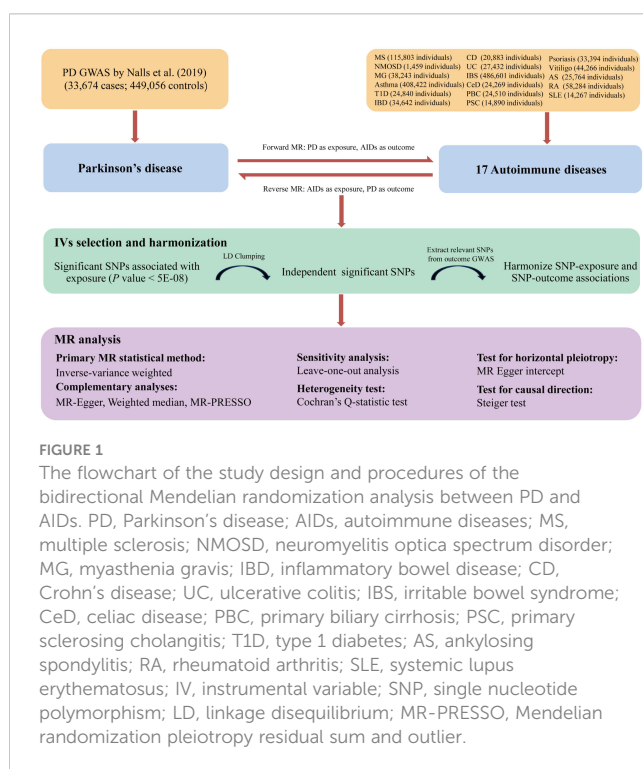
TABLE 1 Characteristics of the GWAS cohorts involving Parkinson's disease and 17 autoimmune diseases and results of the power analysis.

Traits	Data Source/Reference	Sample size (cases/controls)	Population	Forward min OR	Reverse min OR
Parkinson's disease (16)	IPDGC-NeuroX, UK Biobank, SGPD, IPDGC	482,730 (33,674/449,056)	European	–	–
Multiple sclerosis (17)	IMSGC	115,803 (47,429/68,374)	European	1.16	1.11
Neuromyelitis optica spectrum disorder (18)	GWAS by Estrada et al.	1,459 (215/1,244)	European	2.97	NA
Myasthenia gravis (19)	GWAS by Chia et al.	38,243 (1,873/36,370)	European	1.55	1.25
Asthma (20)	GWAS by Valette et al.	408,422 (56,167/352,255)	European	1.11	1.15
Inflammatory bowel disease (21)	GWAS by Liu et al.	34,652 (12,882/21,770)	European	1.30	1.05
Crohn's disease (21)	GWAS by Liu et al.	20,883 (5,956/14,927)	European	1.42	1.04
Ulcerative colitis (21)	GWAS by Liu et al.	27,432 (6,968/20,464)	European	1.37	1.07
Irritable bowel syndrome (22)	GWAS by Eijbsbouts et al.	486,601 (53,400/433,201)	European	1.11	1.51
Celiac disease (23)	GWAS by Trynka et al.	24,269 (12,041/12,228)	European	NA	1.04
Primary biliary cirrhosis (24)	GWAS by Cordell et al.	24,510 (8,021/16,489)	European	NA	1.05
Primary sclerosing cholangitis (25)	GWAS by Ji et al.	14,890 (2,871/12,019)	European	NA	1.09
Type 1 diabetes (26)	GWAS by Forgetta et al.	24,840 (9,266/15,574)	European	1.36	1.06
Ankylosing spondylitis (27)	IGAS	25,764 (10,619/15,145)	European	NA	1.07
Rheumatoid arthritis (28)	GWAS by Okada et al.	58,284 (14,361/43,923)	European	1.26	1.10
Systemic lupus erythematosus (29)	GWAS by Bentham et al.	14,267 (5,201/9,066)	European	1.52	1.03
Psoriasis (30)	GWAS by Tsoi et al.	33,394 (10,588/22,806)	European	NA	1.05
Vitiligo (31)	GWAS by Jin et al.	44,266 (4,680/39,586)	European	1.39	1.07

IPDGC, International Parkinson's Disease Genomics Consortium; SGPD, Systems genomics of Parkinson's disease consortium; IMSCG, International Multiple Sclerosis Genetics Consortium; IGAS, International Genetics of Ankylosing Spondylitis Consortium; GWAS, genome-wide association study; Forward min OR, minimum detectable odds ratios in forward MR with statistical power of 80%; Reverse min OR, minimum detectable odds ratios in reverse MR with statistical power of 80%; NA, not available.

(8,021 cases and 16,489 controls) (24), PSC (2,871 cases and 12,019 controls) (25), T1D (9,266 cases and 15,574 controls) (26), AS (10,619 cases and 15,145 controls) (27), RA (14,361 cases and 43,923 controls) (28), SLE (5,201 cases and 9,066 controls) (29), psoriasis (10,588 cases and 22,806 controls) (30) and vitiligo (4,680 cases and 39,586 controls) (31). Details of recruitment procedures and diagnostic standards were shown in the original publications.

The overall design of this bidirectional MR study is presented in Figure 1. First, we derived SNPs at genome-wide significance ($P < 5E-08$) from the summary-level GWAS statistics and the variants located in human leukocyte antigen (HLA) region (chr6:27,477,797-34,448,354) were removed (32). Then linkage disequilibrium (LD) tests were performed with a genetic window size of 10 Mb and a threshold of $r^2 = 0.001$ to ascertain independence between SNPs. In the next step, we extracted relevant SNPs from outcome GWAS, and then harmonized the summary effect estimates for each SNP in both exposure and outcome datasets. If exposure IVs were absent in the outcome datasets, proxy SNP in LD ($r^2 > 0.8$) would be searched on the basis of the European 1,000 Genomes Project reference panel. Furthermore, the F statistic was calculated from the formula $F = R^2(N-k-1)/[(1-R^2)k]$, for which a value above ten was regarded as an indicator of the strong instrument (N: sample size, k: number of IVs, R^2 : the variance of exposure explained by the selected IVs) (33). The same procedure was performed for the reverse MR studies, as such, we conducted a series of bidirectional MR analyses to infer the causality between PD and AIDs, where PD and AIDs were considered either as exposure or as the outcome. Additionally, MR analyses were performed in accordance with the STROBE-MR guidelines (Supplementary Table 1) (34, 35).



2.2 MR analysis

The inverse variance weighted (IVW) model, as principal analysis, was conducted to assess potential causal associations between genetically determined PD and the occurrence of AIDs. Besides, we applied Cochran's Q test in the IVW method for quantifying the potential impact of heterogeneity among causal effects of different SNPs (36). Subsequent leave-one-out analysis (LOO) was adopted to investigate whether any single genetic variant carried a disproportionate contribution to IVW estimates in each MR study.

Since the IVW method assumed that all SNPs incorporated in the analysis were valid instrumental variables, the other sensitivity analysis methods were used as the complement to IVW analysis when a violation of the standard MR assumptions existed (33). Specifically, the weighted median (WM) method generated consistent causal estimates even if half of the SNPs were invalid (37). Besides, the MR-Egger intercept, as part of the MR-Egger analysis, was used to identify the possible directional pleiotropic effects which were described as some IVs affecting outcome through alternative pathways other than via exposure. Similarly, Mendelian Randomization Pleiotropy Residual Sum and Outlier (MR-PRESSO) was an approach that allowed for the identification of horizontal pleiotropic effects and removal of outlier SNPs in multi-instrument summary-level MR testing (38). We further carried out the Steiger test to validate direction of causal relationship of exposure with outcome. Using the mRnd power calculation online tool, we had sufficient statistical power greater than 80% to detect the minimum odds ratio (OR).

2.3 Genetic correlation

Although SNPs that are directly related to outcomes was eliminated during the IV selection process, SNPs with no relevance could also influence the occurrence of outcome. MR analysis might bias the estimation of causal effects if we found genetic correlations between the exposure and outcome (39). Therefore, after excluding SNPs within the HLA region due to biased LD structure, linkage disequilibrium score (LDSC) regression was performed to estimate latent genetic overlap between two traits by the regression slope utilizing GWAS summary data. When the $P > 0.05$, it indicated that the associations observed in our MR study were truly causal and were not affected by residual pleiotropy. Otherwise, the MR findings might be doubtful.

2.4 Statistical analysis

In particular, to adjust for multiple comparisons in assessing the association between PD and AIDs, we utilized Bonferroni correction method. A statistically significant association was identified when the P -value was below the Bonferroni-corrected threshold of 0.004 (0.05/13) for AIDs as outcome, and 0.003 (0.05/

17) for AIDs as exposure. Moreover, if the *P*-value was lesser than 0.05 but the Bonferroni-corrected *P*-value exceeded 0.05, it was considered a suggestive indication of a causal association. All statistical analyses were performed with LDSC Version 1.0.1, “TwoSampleMR”, “MendelianRandomization” and “MR-PRESSO” packages (R version 4.2.2).

3 Results

3.1 Causal effect of PD on AIDs

The minimum detectable causal effect sizes of genetic liability to PD on AIDs ranged from 1.11 to 2.97 at a statistical power of 0.8 (Table 1). Overall, the number of genetic variants in the PD dataset that ultimately applied as IVs fluctuated from 75 for SLE to 87 for asthma (76 for RA, 82 for IBD, 83 for CD, 84 for MS, UC, vitiligo, 85 for NMOSD, IBS and 86 for MG, T1D). Regarding these various AIDs, the variance explained by IVs ranged from 1.2 to 1.5%, respectively. Additionally, the *F*-statistics of IVs were consistently above 74, which was greater than the standard cutoff of 10 (Table 2).

According to the IVW estimator, we observed that a higher genetic predisposition to PD was associated with decreased risk of IBS (OR = 0.98; 95% CI: 0.96-0.99; *P* = 0.032), which was corroborated by the weighted median approach (OR = 0.95; 95% CI: 0.93-0.98; *P* = 0.003) (Table 2) (Figures 2, 3). Besides, the estimates of the MR-Egger regression analysis showed a directionally consistent but insignificant result (OR = 0.97; 95% CI: 0.92-1.01; *P* = 0.181). Although moderate heterogeneity among chosen SNPs was identified by Cochran’s *Q* statistics (*Q* = 123.66; *P* = 0.003), the results of LOO analysis reported that the causal association between PD and IBS was not driven by any individual IV. No horizontal pleiotropic effect could be found due to the MR Egger intercept that was significantly close to zero (*P* = 0.690). Additionally, MR-PRESSO analyses did not detect any outliers, and the Steiger test revealed the causal directions were true, indicating that our MR results were reliable and robust (Supplementary Table 2). Furthermore, the results of LDSC analysis provided weak evidence of genetic relevance between PD and IBS (*r*_g = 0.072; *se* = 0.045; *P* = 0.110), implying the shared genetic components could not confound the causal estimates.

Concerning the causal association between PD and T1D, our study suggested a positive relationship of genetically predicted PD on

TABLE 2 Mendelian randomization estimates for causal effects of Parkinson’s disease on 12 autoimmune disorders.

Outcome	No. of SNPs	R ²	F-statistics	Inverse variance weighted			MR Egger			Weighted median		
				OR	CI	P-value	OR	CI	P-value	OR	CI	P-value
MS	84	0.013	75	1.02	0.97-1.08	0.448	0.94	0.83-1.06	0.287	0.99	0.92-1.06	0.724
NMOSD	85	0.013	76	1.15	0.87-1.51	0.334	1.40	0.78-2.50	0.259	1.08	0.69-1.68	0.735
MG	86	0.015	84	0.97	0.87-1.07	0.536	0.85	0.69-1.06	0.149	0.95	0.82-1.11	0.531
Asthma	87	0.015	83	0.98	0.96-1.00	0.100	1.00	0.96-1.04	0.824	0.97	0.95-1.00	0.059
IBD	82	0.013	76	1.04	0.98-1.11	0.182	1.06	0.93-1.21	0.380	1.05	0.97-1.13	0.214
CD	83	0.013	76	1.06	0.97-1.15	0.177	1.03	0.87-1.24	0.710	1.00	0.91-1.11	0.954
UC	84	0.013	76	1.04	0.98-1.12	0.211	1.08	0.94-1.25	0.280	1.07	0.97-1.17	0.178
IBS	85	0.013	76	0.98	0.96-0.99	0.032	0.97	0.92-1.01	0.181	0.95	0.93-0.98	0.003
T1D	86	0.013	76	1.10	1.02-1.19	0.010	1.15	0.98-1.35	0.084	1.10	0.99-1.22	0.050
RA	76	0.012	75	0.96	0.91-1.02	0.203	1.04	0.92-1.17	0.568	1.00	0.93-1.08	1.000
SLE	75	0.012	77	1.02	0.92-1.13	0.675	1.12	0.90-1.40	0.321	1.03	0.89-1.19	0.645
Vitiligo	84	0.013	76	0.94	0.85-1.03	0.177	0.83	0.68-1.01	0.059	1.00	0.89-1.13	1.000

MR, Mendelian Randomization; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; MG, myasthenia gravis; IBD, inflammatory bowel disease; CD, Crohn’s disease; UC, ulcerative colitis; IBS, irritable bowel syndrome; T1D, type 1 diabetes; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

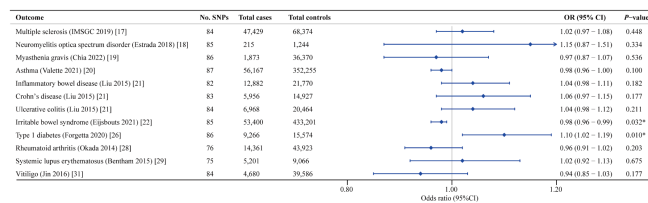


FIGURE 2
Forest plot of the causal associations between genetically predicted PD and the risk of 12 AIDs.

the incidence of T1D (OR = 1.10; 95% CI: 1.02-1.19; $P = 0.010$). Albeit of lower precision, the risk estimates given by the weighted median (OR = 1.10; 95% CI: 0.99-1.22; $P = 0.050$) and MR-Egger method (OR = 1.15; 95% CI: 0.98-1.35; $P = 0.084$) demonstrated a similar causal trend with IVW finding (Table 2) (Figures 2, 4). A degree of heterogeneity among SNPs for T1D was detected in the Cochran's Q test ($Q = 129.84$; $P = 0.001$), however, by utilizing the leave-one-out approach, we discovered that the causal effect estimates were not influenced by a sole outlier SNP. MR-Egger intercept showed no evidence suggesting significant deviation from zero ($P = 0.549$), which implied the absence of horizontal pleiotropy. Besides, after correction for one outlier variant, the causal effect of PD on T1D remained significant with an OR of 1.09 in the MR-PRESSO test (95% CI: 1.02-1.17; $P = 0.015$). Moreover, the results of the Steiger test showed that the causal pathway was in the direction from PD to T1D (Supplementary Table 2). In addition, the genetic correlation results indicated an absence of polygenic pleiotropy between PD and T1D ($r_g = 0.041$; $se = 0.073$; $P = 0.578$), supporting the issue that pleiotropic IVs unlikely exist in our MR analysis.

As for the remaining AIDs, we found that genetic susceptibility to PD was not relevant to MS, NMOSD, MG, asthma, IBD, CD, UC, RA, SLE, and vitiligo in the IVW as well as subsequent sensitivity analyses (Table 2) (Supplementary Figures 1, 2). Moreover, MR

analyses of PD on several autoimmune disorders showed low to moderate heterogeneity of used instruments, while MR-Egger regression revealed a lack of horizontal pleiotropic effect (all intercept P values were more than 0.05). Although 1, 1, 2, 1, 1, 2, 1 and 1 outliers were depicted by the MR-PRESSO method in the casual estimate of PD on MS, asthma, IBD, CD, UC, RA, SLE and vitiligo, the results of our MR studies did not reflect any significant changes. The MR Steiger test pointed out that all causal impacts except NMOSD were in the intended direction (Supplementary Table 2).

3.2 Causal effect of AIDs on PD

Our study had 80% power to detect the causal estimates of genetic predisposition to AIDs on PD, whose ORs ranged from 1.03 to 1.51 (Table 1). In addition, a total of 4, 6, 12, 20, 30, 32, 32, 33, 34, 37, 38, 48, 57, 60, 62 and 70 SNPs for detecting causal relationship of MG, IBS, PSC, AS, CeD, UC, T1D, RA, vitiligo, SLE, PBC, CD, IBD, MS, psoriasis as well as asthma on PD risk were enrolled in our reverse-direction MR analyses, respectively. Genetic instruments from the GWAS of autoimmune disorders collectively accounted for 0.1 to 22.3% of the variance. Besides, instrumental variables for

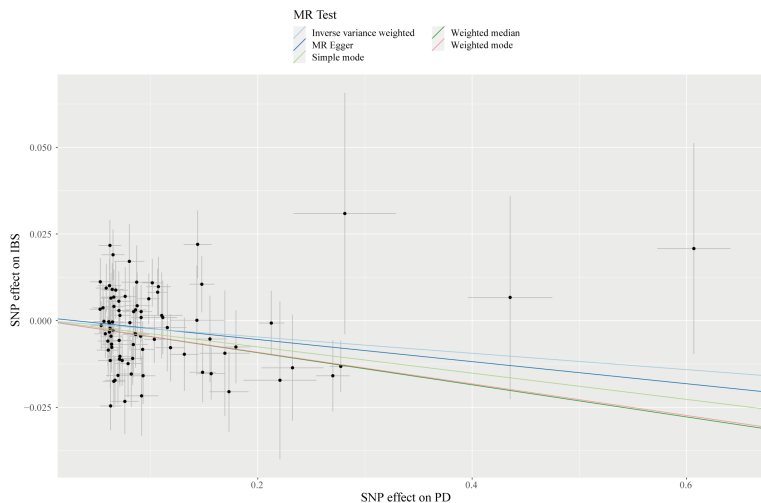


FIGURE 3
Scatterplot of genetic association with PD against the genetic association with IBS risk. Each black dot indicates an SNP, plotted by the estimate of SNP on PD and the estimate of SNP on IBS risk with standard error bars. The slope of the line represents the causal relationship, and each method has a different line.

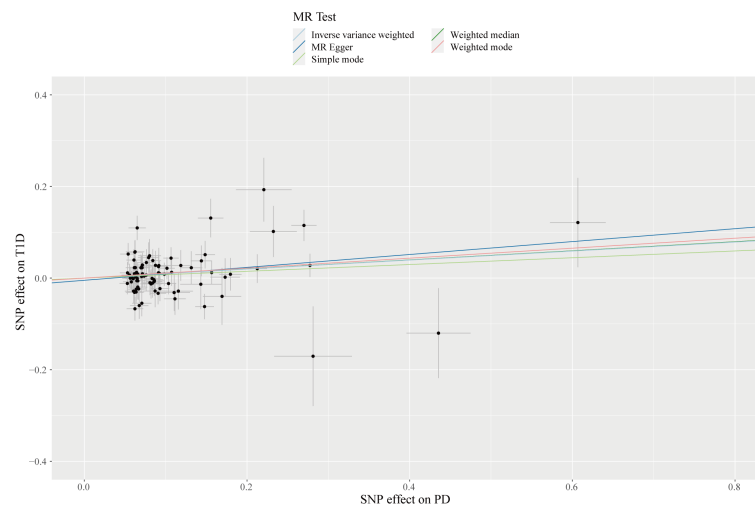


FIGURE 4

Scatterplot of genetic association with PD against the genetic association with T1D risk. Each black dot indicates an SNP, plotted by the estimate of SNP on PD and the estimate of SNP on IBS risk with standard error bars. The slope of the line represents the causal relationship, and each method has a different line.

each AID had F-statistics from 32 to 123, eliminating potential weak instrument bias.

When tested with IVW analyses, no causal association was observed between genetically determined MS (OR = 1.00; 95% CI: 0.96-1.05; $P = 0.846$), MG (OR = 1.05; 95% CI: 0.97-1.14; $P = 0.261$), asthma (OR = 0.99; 95% CI: 0.91-1.08; $P = 0.817$), IBD (OR = 0.98; 95% CI: 0.94-1.03; $P = 0.530$), CD (OR = 1.00; 95% CI: 0.96-1.03; $P = 0.843$), UC (OR = 0.99; 95% CI: 0.94-1.04; $P = 0.599$), IBS (OR = 0.76; 95% CI: 0.48-1.22; $P = 0.254$), CeD (OR = 0.99; 95% CI: 0.95-1.03; $P = 0.601$), PBC (OR = 0.98; 95% CI: 0.95-1.02; $P = 0.274$), PSC (OR = 1.03; 95% CI: 0.98-1.09; $P = 0.223$), T1D (OR = 0.97; 95% CI: 0.93-1.00; $P = 0.068$), AS (OR = 0.84; 95% CI: 0.66-1.07; $P = 0.149$), RA (OR = 0.97; 95% CI: 0.90-1.05; $P = 0.501$), SLE (OR = 1.00; 95% CI: 0.97-1.03; $P = 0.853$), psoriasis (OR = 1.00; 95% CI: 0.99-1.01; $P = 0.769$) and vitiligo (OR = 1.03; 95% CI: 0.99-1.06; $P = 0.190$) and the risk of PD (Table 3) (Figure 5; Supplementary Figures 3, 4). For the supplementary analyses we performed, the MR-Egger and weighed median estimator yielded similar results despite some with reduced statistical power. We detected slight heterogeneity in the principal analyses of causal relationships of IBD, CD, UC, PBC, RA, SLE and vitiligo on PD. As the MR-Egger intercept test corresponded to the statistics with a P -value > 0.05 , it meant that our MR findings, except for IBD as exposure, were not affected by genetic pleiotropy. Additionally, the MR-PRESSO indicated that the causal estimates of IBD, CD, PBC, RA and vitiligo with PD were retained after pruning outliers. Lastly, our Steiger test provided evidence of significant inferred causal direction between 16 AIDs and PD (Supplementary Table 3).

4 Discussion

Nowadays, Parkinson's disease and autoimmune disorders have imposed a huge health and economic burden on individuals and

society around the world. However, the profound understanding of the potential genetic mechanism and pathophysiological link remains limited, which leads to an absence of effective prevention and treatment strategies. Although a large body of epidemiological researches emerged to investigate the correlation between PD and AIDs, these observational studies were vulnerable to reverse causation and residual confounding bias. Thus, in the present analysis, we conducted a bidirectional two-sample MR study for comprehensively exploring the mutual causal associations between genetically determined PD and AIDs by leveraging publicly available summary statistics from large GWAS datasets. Based on the MR results after correction for multiple sensitivity tests, our study provided evidence that PD had a protective influence on IBS risk, whereas a possible detrimental link was reported between genetic predisposition to PD and T1D susceptibility. Additionally, in the reverse MR analyses, we found no indication to support the causal effect of AIDs on PD occurrence.

Our MR results supported that individuals with high genetic risk for PD suggestively have a lower susceptibility to developing IBS. However, a previous register-based observational study from the Swedish Patient Register showed that IBS diagnosis was related to an increased risk of PD (40). Nonetheless, as an observational case-control study, it was inevitably vulnerable to confounding factors and encountered challenges in accurately determining the diagnostic sequence of PD and IBS. Unlike findings from the observational investigations, our MR analysis improved the reliability of causal inference with the use of genetic variants as IVs, avoiding systemic bias derived from modifiable confounders and reverse causation. Furthermore, the following biological mechanisms could account for the protective effect of PD against IBS risk. The altered gastrointestinal microbiota in PD patients might be a major factor in the pathophysiology of IBS. On the one hand, through the application of high-throughput 16S rRNA sequencing, gut microbiome dysbiosis was commonly detected in PD patients, revealing a higher relative

TABLE 3 Mendelian randomization estimates for causal effects of 16 autoimmune disorders on Parkinson’s disease.

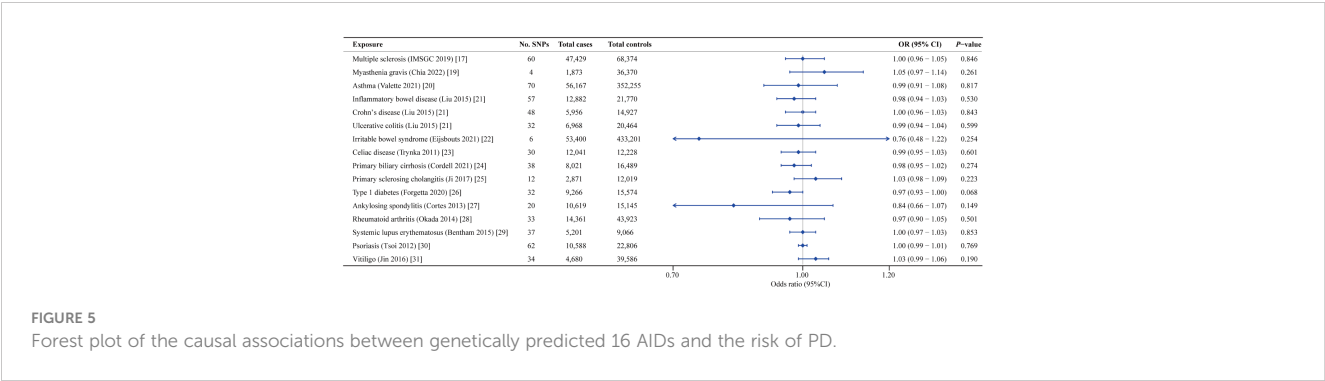
Exposure	No. of SNPs	R ²	F-statistics	Inverse vari- ance weighted			MR Egger			Weighted median		
				OR	CI	P-value	OR	CI	P-value	OR	CI	P-value
MS	60	0.023	46	1.00	0.96-1.05	0.846	1.04	0.90-1.20	0.573	0.99	0.93-1.05	0.761
MG	4	0.004	39	1.05	0.97-1.14	0.261	0.87	0.49-1.54	0.674	1.05	0.95-1.16	0.328
Asthma	70	0.012	70	0.99	0.91-1.08	0.817	1.02	0.80-1.28	0.897	0.96	0.85-1.09	0.550
IBD	57	0.094	63	0.98	0.94-1.03	0.530	1.23	1.01-1.51	0.047	1.01	0.95-1.06	0.917
CD	48	0.134	67	1.00	0.96-1.03	0.843	1.11	0.99-1.26	0.087	1.01	0.97-1.05	0.709
UC	32	0.057	52	0.99	0.94-1.04	0.599	1.05	0.83-1.34	0.690	1.00	0.94-1.06	0.945
IBS	6	0.001	32	0.76	0.48-1.22	0.254	0.03	0.00-16.10	0.339	0.76	0.45-1.31	0.313
CeD	30	0.132	123	0.99	0.95-1.03	0.601	1.02	0.95-1.09	0.639	1.03	0.97-1.09	0.300
PBC	38	0.122	91	0.98	0.95-1.02	0.274	0.97	0.88-1.07	0.527	0.99	0.95-1.03	0.618
PSC	12	0.032	41	1.03	0.98-1.09	0.223	0.98	0.79-1.22	0.879	1.01	0.94-1.07	0.871
T1D	32	0.072	60	0.97	0.93-1.00	0.068	0.92	0.85-0.99	0.026	0.93	0.88-0.99	0.012
AS	20	0.048	63	0.84	0.66-1.07	0.149	1.12	0.53-2.37	0.775	0.85	0.61-1.19	0.346
RA	33	0.028	50	0.97	0.90-1.05	0.501	1.10	0.80-1.52	0.548	0.98	0.90-1.07	0.716
SLE	37	0.223	111	1.00	0.97-1.03	0.853	0.97	0.91-1.04	0.467	1.00	0.96-1.04	0.944
Psoriasis	62	0.100	60	1.00	0.99-1.01	0.769	1.00	0.99-1.01	0.844	1.00	0.99-1.01	0.599
Vitiligo	34	0.047	64	1.03	0.99-1.06	0.190	1.06	0.93-1.20	0.398	1.03	0.98-1.07	0.235

MR, Mendelian Randomization; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; MS, multiple sclerosis; MG, myasthenia gravis; IBD, inflammatory bowel disease; CD, Crohn’s disease; UC, ulcerative colitis; IBS, irritable bowel syndrome; CeD, celiac disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; T1D, type 1 diabetes; AS, ankylosing spondylitis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

abundance of *Lactobacillus* and *Bifidobacterium* (41). Furthermore, O’Mahony et al. observed that *Lactobacillus* and *Bifidobacterium* could participate in peripheral immunity through up-regulating IL-10 and down-regulating IL-12 levels in peripheral blood mononuclear cells (42). On the other hand, a prior meta-analysis composed of 22 studies by Romano et al. demonstrated the microbiological pattern of reduced *Lachnospiraceae* genus content in individuals with PD (43). Moreover, flagellin from *Lachnospiraceae* was reported to activate innate and adaptive immunity, eliciting anxiety-like behavior, abnormal gastrointestinal transit, and intestinal barrier dysfunction in a humanized mouse model of IBS (44, 45). In addition to gut microbiome dysbiosis, another possible mechanism linking PD to IBS was dysregulation of neurotransmitters and neurotrophic factors.

It is well-known that deficits in dopamine and 5-HT signaling are implicated in the initiation and progression of PD (46, 47), but as for IBS, reduced levels of these neurotransmitters could mitigate pathological symptoms like diarrhea, abdominal pain and discomfort and stabilize intestinal permeability (48–51). Additionally, although decreased netrin-1 concentrations in the brain and gut of PD patients resulted in significant loss of motor function, it provided an obvious alleviation effect on visceral hypersensitivity, a core characteristic in approximately half of patients with IBS (52, 53). Thus, future large-scale prospective cohort studies are urgently required to verify this causal relationship.

Regarding the causal effect of PD on T1D occurrence, we found that those who were genetically predisposed to PD exhibited a 10%



increased incidence of experiencing T1D. This causal association could be explained by perturbation of the immune system and inflammatory response. It was reported that PD patients were afflicted with persistent neuroinflammation, which disrupted BBB integrity and resulted in BBB leakage (54, 55). Following the changes in BBB permeability, more pro-inflammatory cytokines (TNF- α , IL-1 β , etc.) yielded by overactivated neurotoxic microglia had the opportunity to enter the systemic circulation (9, 56). Meanwhile, in a PD rat model induced by injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), nitrated alpha-synuclein (N- α -synuclein) was detected to break immunological tolerance and activate T cells into Th1 and Th17 subsets of T helper cells (56–58). Moreover, increased activation of natural killer cells, B lymphocytes and monocytes was also found in the peripheral blood samples of PD patients compared to healthy controls using flow cytometric analyses (59–61). Thus, accompanied by elevated concentrations of pro-inflammatory mediators, including IFN- γ , TNF- α , IL-6, IL-17 and activated peripheral immune cells, an enhanced immune response might mistakenly lead to attack and destroy the insulin-producing beta cells in pancreatic islets (62). Additionally, Li et al. identified that in A53 transgenic (A53T) PD mice, the expression levels of cytotoxic-T-lymphocyte-antigen-4 (CTLA-4) and inducible costimulator (ICOS) were markedly reduced in T regulatory lymphocytes (Tregs), a critical player in maintaining immune homeostasis (63). Similarly, Tregs were also found to exhibit functional defects in PD patients, implying an abolished suppressive effect on autoimmune response and a contributory factor for the emergence of T1D (64). Meanwhile, in the reverse direction MR analysis, we failed to observe a causal effect of T1D on the susceptibility to PD. However, a previous MR analysis observed that T1D might play a protective role against PD with an OR of 0.97 (95% CI: 0.94–0.99; $P = 0.039$) (65). The primary factors contributing to the inconsistency could stem from the utilization of disparate GWAS datasets and selection strategy for instrumental variables. Specifically, the T1D GWAS adopted by Senkevich et al. only encompassed 714,850 SNPs, half of which lacked summary statistics (beta, SE, p -value), whereas the GWAS data utilized in our MR analysis included over 1.2 million SNPs with complete data. The evident increase in the number of available SNPs guaranteed more comprehensive genome coverage, thereby minimizing the possibility of falsely detecting genetic variants associated with T1D and PD. Additionally, we applied stringent criteria to choose instrumental variables. The SNPs related to more

than one autoimmune disease were excluded to mitigate pleiotropic effects. Interestingly, after adding this rigorous screening procedure, we re-analyzed the GWAS data used in previous MR study by Senkevich et al. and the results demonstrated that the protective effect of T1D on PD occurrence disappeared (data not shown).

Concerning the relationships between AIDs and PD, a comprehensive meta-analysis by Li et al. showed that aging individuals diagnosed with IBD, CD and UC had a 1.24, 1.30 and 1.31-fold increased likelihood of developing PD than the healthy controls (66). Likewise, Yeh et al. found that in a population-based cohort study of 6,440 AS patients and 25,760 matched controls, the incidence of PD was significantly higher in the AS cohort than in reference individuals (67). However, compared with previous findings in observational studies, our reverse MR analyses showed that genetically determined AIDs were not causally associated with PD risk. This paradox might be ascribed to the reasons that observational studies were prone to bias owing to the limited sample size, inevitable confounding factors and reverse causation. Therefore, we carried out these two-sample MR analyses and obtained reliable and robust results demonstrating no causality of 16 AIDs on PD susceptibility.

Our study benefits from several noteworthy strengths. First, to the best of our knowledge, our MR study is the first to systematically elucidate the bidirectional causative links between PD and AIDs, which yields instructive insight for the management of autoimmune disorders in PD patients as well as the prevention strategies for PD problems in individuals with AIDs. An additional advantage of this MR analysis is that the largest and latest GWAS datasets for AIDs and PD were implemented, providing strong statistical power and precise effect estimates to explore the causal associations. Additionally, except for IVW analysis, we also employed exhaustive complementary analyses containing MR Egger regression, weighted median and MR-PRESSO. The directionally consistent results observed from multiple MR techniques ensured the robustness of our MR findings. Lastly, since genetic variants within the HLA region (chr6:27,477,797–34,448,354) were in strong LD and had high pleiotropic effects on a variety of autoimmune disorders, our study excluded these variants to help us avoid possible bias in MR instruments and more accurately identify causality. However, some potential limitations should also be acknowledged in our MR analyses. First, although we selected the largest GWAS datasets to date for various AIDs, the screened IVs for AIDs such as NMOSD remained somewhat underrepresented,

making it difficult to obtain sufficient genetic instruments for bidirectional MR studies. As such, larger-scale GWAS should be updated in the future to boost the statistical power for investigating the causal associations between PD and AIDs. Second, it should be noted that the majority of GWAS participants were of European ancestry. Thus, our viewpoint on causal inferences between PD and AIDs should be applied with caution to other racial and ethnic populations. Last but not least, although our MR studies support the evidence for causal relationships of genetically determined PD with IBS and T1D occurrence, these findings and underlying pathophysiological mechanisms need to be further confirmed by cross-sectional, longitudinal cohort studies and molecular biology experiments *in vivo* and *in vitro*.

5 Conclusion

In conclusion, we performed this bidirectional 2-sample MR analysis for the first time to explore the causal associations between PD and AIDs. Our forward MR studies identified that genetically predicted PD played a dual role in AIDs, not only protecting against IBS but also exerting a detrimental effect on T1D. Simultaneously, in the reverse direction, we found a lack of evidence for the causal effect of AIDs on PD risk. We hope our study could provide new insights into the causal relationships between PD and AIDs, thereby contributing to the development of clinical strategies for autoimmune disorders in PD individuals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

Author contributions

JY: Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft. WL: Data curation, Formal analysis, Writing – original draft. YM: Writing – original draft, Visualization. HS: Writing – original draft. CM: Writing – original draft, Data curation. QW: Writing – original draft. CH: Writing – original draft. JZ: Conceptualization, Data curation, Writing – review & editing. XL: Conceptualization, Funding acquisition, Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1370831/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Scatter plots of MR tests assessing the effect of PD on AIDs. (A) PD on MS; (B) PD on NMOSD; (C) PD on MG; (D) PD on Asthma; (E) PD on IBD; (F) PD on CD; (G) PD on UC; (H) PD on RA; (I) PD on SLE; (J) PD on Vitiligo. PD, Parkinson's disease; AIDs, autoimmune diseases; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; MG, myasthenia gravis; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

SUPPLEMENTARY FIGURE 2

Leave-one-out plots of MR tests assessing the effect of PD on AIDs. (A) PD on MS; (B) PD on NMOSD; (C) PD on MG; (D) PD on Asthma; (E) PD on IBD; (F) PD on CD; (G) PD on UC; (H) PD on IBS; (I) PD on T1D; (J) PD on RA; (K) PD on SLE; (L) PD on Vitiligo. PD, Parkinson's disease; AIDs, autoimmune diseases; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; MG, myasthenia gravis; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBS, irritable bowel syndrome; T1D, type 1 diabetes; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

SUPPLEMENTARY FIGURE 3

Scatter plots of MR tests assessing the effect of AIDs on PD. (A) MS on PD; (B) MG on PD; (C) Asthma on PD; (D) IBD on PD; (E) CD on PD; (F) UC on PD; (G) IBS on PD; (H) CeD on PD; (I) PBC on PD; (J) PSC on PD; (K) T1D on PD; (L) AS on PD; (M) RA on PD; (N) SLE on PD; (O) Psoriasis on PD; (P) Vitiligo on PD. AIDs, autoimmune diseases; PD, Parkinson's disease; MS, multiple sclerosis; MG, myasthenia gravis; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBS, irritable bowel syndrome; CeD, celiac disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; T1D, type 1 diabetes; AS, ankylosing spondylitis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

SUPPLEMENTARY FIGURE 4

Leave-one-out plots of MR tests assessing the effect of AIDs on PD. (A) MS on PD; (B) MG on PD; (C) Asthma on PD; (D) IBD on PD; (E) CD on PD; (F) UC on PD; (G) IBS on PD; (H) CeD on PD; (I) PBC on PD; (J) PSC on PD; (K) T1D on PD; (L) AS on PD; (M) RA on PD; (N) SLE on PD; (O) Psoriasis on PD; (P) Vitiligo on PD. AIDs, autoimmune diseases; PD, Parkinson's disease; MS, multiple sclerosis; MG, myasthenia gravis; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBS, irritable bowel syndrome; CeD, celiac disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; T1D, type 1 diabetes; AS, ankylosing spondylitis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

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Discovery of biomarkers in the psoriasis through machine learning and dynamic immune infiltration in three types of skin lesions

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Introduction: Psoriasis is a chronic skin disease characterized by unique scaling plaques. However, during the acute phase, psoriatic lesions exhibit eczematous changes, making them difficult to distinguish from atopic dermatitis, which poses challenges for the selection of biological agents. This study aimed to identify potential diagnostic genes in psoriatic lesions and investigate their clinical significance.

Methods: GSE182740 datasets from the GEO database were analyzed for differential analysis; machine learning algorithms (SVM-RFE and LASSO regression models) are used to screen for diagnostic markers; CIBERSORTx is used to determine the dynamic changes of 22 different immune cell components in normal skin lesions, psoriatic non-lesional skin, and psoriatic lesional skin, as well as the expression of the diagnostic genes in 10 major immune cells, and real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry are used to validate results.

Results: We obtained 580 differentially expressed genes (DEGs) in the skin lesion and non-lesion of psoriasis patients, 813 DEGs in mixed patients between non-lesions and lesions, and 96 DEGs in the skin lesion and non-lesion of atopic dermatitis, respectively. Then 144 specific DEGs in psoriasis via a Venn diagram were identified. Ultimately, UGGT1, CCNE1, MMP9 and ARHGEF28 are identified for potential diagnostic genes from these 144 specific DEGs. The value of the selected diagnostic genes was verified by receiver operating characteristic (ROC) curves with expanded samples. The area under the ROC curve (AUC) exceeded 0.7 for the four diagnosis genes. RT-qPCR results showed that compared to normal human epidermis, the expression of UGGT1, CCNE1, and MMP9 was significantly increased in patients with psoriasis, while ARHGEF28 expression was significantly decreased. Notably, the results of CIBERSORTx showed that CCNE1 was highly expressed in CD4⁺ T cells and neutrophils, ARHGEF28 was also expressed in mast cells. Additionally, CCNE1 was strongly correlated with IL-17/CXCL8/9/10 and CCL20.

Immunohistochemical results showed increased nuclear expression of CCNE1 in psoriatic epidermal cells relative to normal.

Conclusion: Based on the performance of the four genes in ROC curves and their expression in immune cells from patients with psoriasis, we suggest that CCNE1 possess higher diagnostic value.

KEYWORDS

psoriasis, atopic dermatitis, diagnosis genes, immune infiltration, machine learning algorithm

1 Introduction

Psoriasis (PsO) and atopic dermatitis (AD) are common chronic inflammatory skin diseases (1). Typical psoriasis is characterized by the appearance of erythema, scaling, itching and pain on the surface of the skin, and its etiology is not yet fully understood but is thought to be related to genetics, the immune system and environmental factors (2, 3). AD, on the other hand, also has similar lesion characteristics to those of psoriatic lesions, and AD in particular can present as psoriasiform during the resistance phase (4). In addition to this, PsO manifests as eczema-like in the acute phase, there are also patients with psoriasis-like AD, and interestingly, there are even patients in whom PsO and AD can coexist (5, 6). Therefore, in view of the more complex manifestations of PsO and AD patients, there are really diagnostic difficulties in diagnosis and stereotyping (7). The correct diagnosis of these two diseases involves the correct selection and use of biologics, and the correct and effective use of biologics can realize its true treatment meaning (8, 9). Thus, for mixed patients or PsO patients whose lesions are difficult to distinguish from AD patients, more accurate diagnostic markers are needed to carry out differential diagnosis (10).

In this study, we carried out the GSE182740 datasets from the Gene Expression Omnibus (GEO) database for differential analysis, which included 20 AD (10 lesional and 10 non-lesional), 33 overlap phenotype of AD and PsO (17 lesional and 16 non-lesional), 16 PsO (9 lesional and 7 nonlesional), and 6 normal skin. By GEO2R analysis, we obtained 580 differentially expressed genes (DEGs) in the skin lesion and non-lesion of PsO patients, 813 DEGs in mixed patients between non-lesions and lesions, and 96 DEGs in the skin lesion and non-lesion of AD, respectively. The advantage of this kind of differential analysis between the RNA-seq of lesional and non-lesional skin in the same patient is that circumvent the problem of mismatch between healthy individuals and patients, thereby improving the accuracy of DEGs.

In recent years, machine learning algorithms have been increasingly used in the medical field to construct optimal classification models to filter out characterized variables (11). During our research, two machine algorithms, “least absolute shrinkage and selection operator” (LASSO) and “support vector

machine - recursive feature elimination” (SVM-RFE), were utilized for diagnostic gene screening of differential genes (12, 13), and at the same time, CIBERSORT, as a widely used machine algorithm for classifying populations of immune cells (14), was applied to identify three types skin lesion (normal skin, psoriasis non-lesional skin and psoriasis lesional skin) and potential diagnosis genes of PsO associated with immune infiltration, and were verified by real-time quantitative polymerase chain reaction (RT-qPCR) experiments.

2 Materials and methods

2.1 Datasets information

The GSE182740 datasets obtained from <http://www.ncbi.nlm.nih.gov/geo/was> used in the present study. Specifically, the microarray data included 20 cases of AD (10 lesional and 10 non-lesional), 33 cases of AD and PsO with overlapping phenotypes (17 lesional and 16 non-lesional), 16 cases of PsO (9 lesional and 7 non-lesional), and 6 cases of normal skin (from GSE78097 datasets). Next, GEO2R was performed to obtain the DEGs between the non-lesional and lesional of AD, the non-lesional and lesional of PsO and the non-lesional and lesional of overlapping phenotypes in an attempt to reduce individual variation and improve the specificity of the DEGs. GEO2R is an interactive R-based online analysis tool that utilizes limma R packages to analyze the original data with the moderated t-statistic for significance analysis, and Benjamini & Hochberg false discovery rate method for applying *P-value adjustment* for multiple testing correction. In addition, normal skins and psoriatic lesions and psoriatic paraneoplastic lesions data were used for immune infiltration analysis with the aim of observing the immunological dynamics of three types of lesions ranging from mild to severe. $P < 0.05$ and $|\log_2 \text{fold change (FC)}| > 1$ were used as threshold points for screening DEGs.

2.2 Functional pathway analyses

Sangerbox which is a comprehensive bioinformatics analysis platform (15) was performed on the 144 unique DEGs between

psoriasis lesional (PsO-LS) and psoriasis non-lesional (PsO-NL) skin to uncover the possible gene functional annotation and pathway enrichment. *P value less than 0.05* and *adjusted P value less than 0.05* were selected as cutoff criteria.

2.3 Candidate gene biomarker identification

We employed two machine learning algorithms, namely the least absolute shrinkage and selection operator (LASSO) (16) and the support vector machine (SVM), to identify specific diagnostic gene biomarkers for psoriasis. As a regression analysis algorithm, LASSO is characterized by variable selection and regularization, which can avoid overfitting and enhance prediction accuracy. SVM is a supervised machine learning technique for classification and regression. The recursive feature elimination (RFE) algorithm can obtain the optimal variable combination that maximizes model performance. Combining these two techniques, we used the SVM-RFE algorithm to identify biomarkers with excellent discriminative abilities (17). The overlapping genes obtained from the above two algorithms were considered as candidate gene biomarkers. Specifically, LASSO was performed in conjunction with the glmnet R package to obtain the feature selections, important parameters are set as follows: standardize=Ture, alpha=1, family=gaussian, nfolds=3). In addition, we used mlbench and caret R packages to perform SVM-RFE, and all data were normalized prior to training the model. The 144 special DEGs between PsO-NL and PsO-LS, the results of LASSO and SVM-RFE, and the veen data of the two algorithms were provided respectively in the [Supplementary Tables 1-3](#).

2.4 RNA extraction and real-time PCR

Skin lesion samples and peripheral blood were collected (samples were from the Second Xiangya Hospital of Central South University), and skin lesions were soaked in the dispase enzyme for 24h, isolation of epidermis for RNA extraction, peripheral blood was used to fractionate CD4+T cells. RNA was extracted using the TRIzol method. A reverse transcription kit produced by Takara in Japan was used to produce one nanogram of first-strand complementary DNA (cDNA). An ABI 7900 Real-Time PCR System was utilized for the execution of the real-time PCR. The relative expressions of were calculated using with a $2^{-\Delta\Delta Ct}$ method and normalized using GAPDH as an internal control. The primers used in this study were shown below [Table 1](#).

TABLE 1 Real-time PCR primer sequences.

Gene	Forward primer	Reverse primer
UGGT1(Human)	TTCACCGCCAGCTTATATCAAAA	CCCCTGAACCTCATCAATAGGA
MMP9(Human)	GGGACGCAGACATCGTCATC	CGTCATCGTCGAAATGGGC
CCNE1(Human)	GCCAGCCTTGGGACAATAATG	CTTGACCGTTGAGTTTGGGT
ARHGEF28(Human)	AGGTGATGAAGTCTACGCTAACT	AGTGGCAGTGATTCCCTCTAT
RPLPO(Human)	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCA

2.5 Diagnostic effectiveness examination

To further examine the validity of the prediction of diagnostic genes in PsO, we plotted receiver operating characteristic (ROC) curves on the mRNA expression data of normal samples and PsO patients in GSE182740, while calculating the area under the ROC curve (AUC) to determine the diagnostic validity of the diagnosis for PsO.

2.6 Immune infiltration analysis

To examine the expression of infiltrating immune cells in normal skin, psoriatic paralesional skin, and psoriatic lesions, we used a bioinformatics algorithm that identifies cell types by relative subsets of RNA transcripts(CIBERSORT, <https://cibersortx.stanford.edu/>). Putative immune cell abundance was calculated using a reference set of 22 immune cell species (LM22) and visualized using sangerbox. In addition, the expression of four diagnostic genes in immune cells was specifically analyzed. Correlation analysis was used to analyze the correlation between CCNE1 and inflammatory factors.

2.7 Immunohistochemistry

Skin lesions from patients with psoriasis vulgaris confirmed by histopathological examination, as well as skin tissues from normal individuals (all from the outpatient operating room of the Department of Dermatology, the Second Xiangya Hospital of Central South University) were collected after signing an informed consent form. Paraffin sections of the corresponding tissues were placed in an oven at 70°C for 2 hours, deparaffinized with turpentine for 30mins, then to 95%, 75%, 50%, deionized water for gradient rehydration, and autoclaved with antigenic restorative solution at pH 9.0 for 7mins, then cooled at room temperature. The slides were removed from the antigen repair solution and rinsed twice with TBST. Then 1 drop of 3% H₂O₂ was added to each section and incubated for 15 min at room temperature to block endogenous peroxidase activity. The sections were rinsed twice with TBST and 10ul cyclin E1 polyclonal antibody (proteintech, Cat No. 11554-1-AP) (dilution 1:400) was added to each section and incubated for 1 hour at room temperature. Rinse twice with TBST, add horseradish peroxidase (HRP)-labeled antibody to each section, and incubate for 30 min at room temperature. And then rinsed twice with TBST and observed under ordinary slice panoramic scanning (Hamamatsu Company, Japan). In multicolor

immunofluorescence, add 10ul cyclin E1 polyclonal antibody (dilution 1:100), CD4 rabbit monoclonal antibody (RMA-0620, Fuzhou Maixin Biotechnology Company, China), Rb mAb to CD11b (Abcam, ab13357, 1:1000) incubated for 1 hour at room temperature to each tissue circle. After washing, use secondary antibody (Abcam, Anti-rabbit IgG H&L-HRP ab205722) (dilution 1:1000), apply and incubate for 20 minutes. After cleaning, add 10ul Opal 520, Opal 570 or Opal 650 respectively, to each tissue circle and incubate for 20 minutes, then 10ul DAPI (Servicebio Cat: G1012) for each tissue. After incubation for 20 minutes, Mantra pathological analysis system was used for analysis and photography (Akoya Company, USA).

2.8 Statistical analysis

Statistical analyzes used in this study used R (Version 4.3.1) and GraphPad Prism 8. Student's unpaired t test was performed for normally distributed variables, while Mann-Whitney U test was performed for abnormally distributed variables. Differences were considered to be significant when the *P value* was <0.05.

3 Results

3.1 DEGs identification

In the present study, we conducted the GSE182740 for differential analysis on 9 PsO-LS and 7 PsO-NL skin biopsy samples, yielding 580 DEGs. Additionally, we obtained 96 DEGs from 10 atopic dermatitis non-lesional (AD-NL) and 10 atopic dermatitis lesional (AD-LS), as well as 813 DEGs from 16 mixed non-lesional (Mixed-NL) and 17 mixed lesional (Mixed-LS). As illustrated, we will primarily focus on the 580 DEGs between PsO-LS and PsO-NL, as well as the specific 144 DEGs that exclude those

overlapping with the mixed DEGs and AD DEGs between non-lesional and lesional (Figure 1).

3.2 Functional enrichment analyses

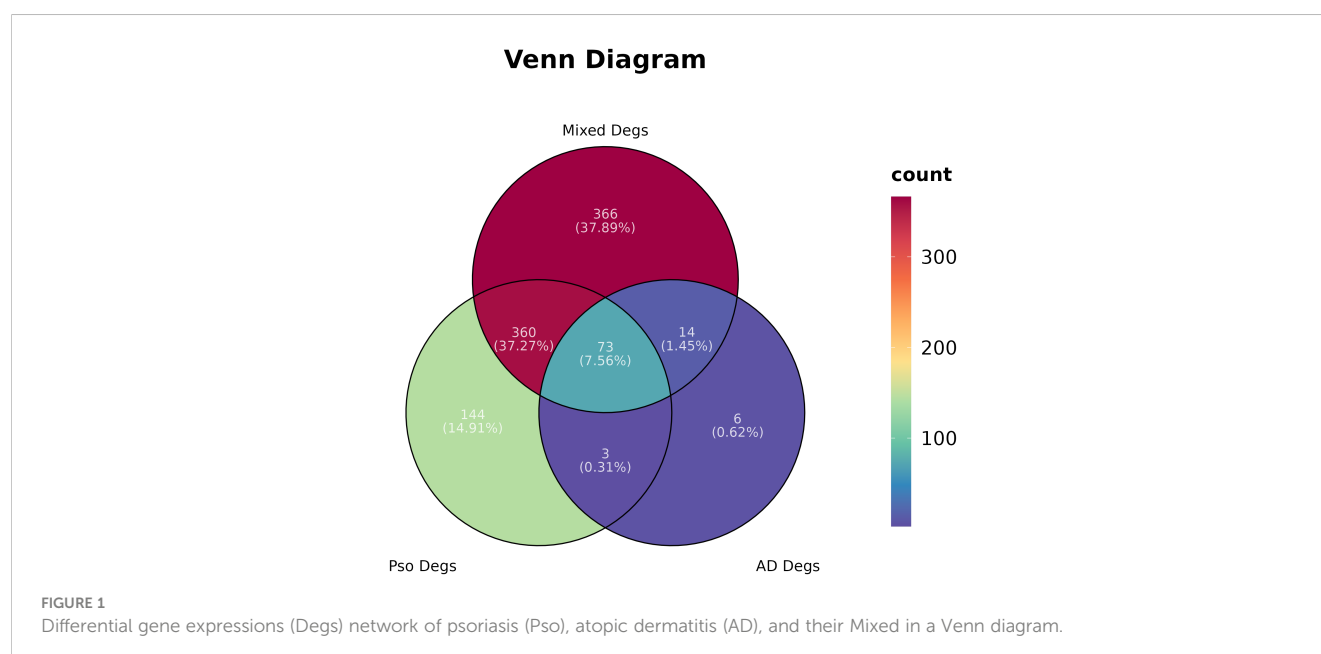
Moreover, we conducted functional enrichment analysis on the 144 unique DEGs between PsO-NL and PsO-LS. The top 20 pathways that were enriched for specific differential genes between PsO-NL and PsO-LS are shown in Figure 2. Proteoglycans in cancer, cytokine-cytokine receptor interaction, micro RNAs in cancer and JAK-STAT signaling pathway are the enrichment results for higher generatio.

3.3 Four DEGs were identified as specific diagnostic genes for psoriasis

We employed LASSO and SVM-RFE algorithms to screen potential diagnostic genes from the above-mentioned 144 distinctive genes between PsO-NL and PsO-LS. Firstly, we utilized LASSO logistic regression and performed a penalty parameter tuning process with three-fold cross-validation, resulting in the identification of 8 genes as potential PsO biomarkers. Subsequently, the SVM-RFE algorithm identified 15 feature variables for diagnosing PsO. Ultimately, both algorithms selected 4 genes (UGGT1\MMP9\CCNE1\ARHGGEF28) as the optimal candidate genes for characteristic gene expression (Figure 3).

3.4 The expressing patterns of diagnostic genes in psoriasis

To further confirm the expression of the optimal candidate genes in patients with PsO, we validated the training and validation cohorts.



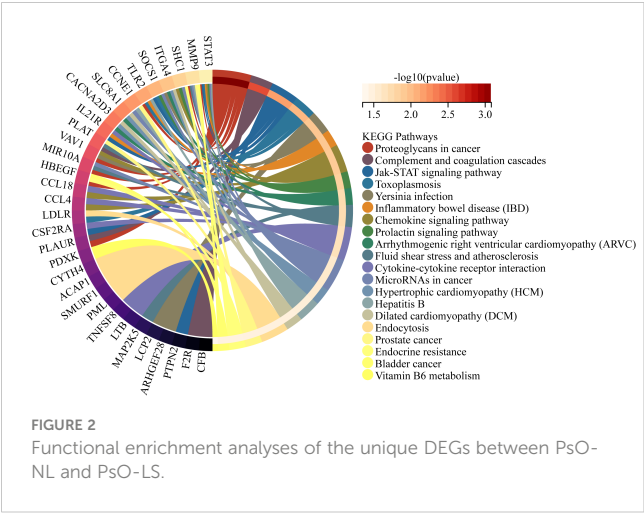


FIGURE 2
Functional enrichment analyses of the unique DEGs between PsO-NL and PsO-LS.

The results revealed that the expression of UGGT1, MMP9, and CCNE1 was significantly increased in PsO-LS compared with the PsO-NL, while ARHGEF28 exhibited a decreased status compared with the PsO-NL (Figure 4).

3.5 Diagnostic effectiveness examination via RT-qPCR & ROC

The RT-qPCR results revealed compared with the normal epidermis, a significant upregulation of CCNE1, MMP9, and UGGT1

expression in psoriatic epidermis, in the contrast, a notable downregulation of ARHGEF28 in psoriatic epidermis. These findings were consistent with those observed in the cohort study. Moreover, ROC curves were generated for these four diagnosis genes to assess their discriminative power in distinguishing normal from psoriatic patient samples. The AUC of the ROC curve for the four potential diagnostic genes was 1.0 for ARHGEF28, 0.96 for UGGT1, 0.8854 for CCNE1, and 0.77 for MMP9 (Figure 5).

3.6 Association of three types lesions with the proportion of infiltrating immune cells

We employed CIBERSORTx to analyze the composition of 22 immune cell types in 6 normal skins, 7 PsO-NL, and 8 PsO-LS within this dataset. First, in normal and PsO-LS as well as in PsO-NL and PsO-LS, significant differences were observed in CD4⁺ T cells memory activated, T cells follicular helper, NK cells activated, and mast cell resting populations. Additionally, meaningful statistical differences were exhibited by B cell and Tregs cell only in the normal skin and PsO-LS (Figure 6).

3.7 Four diagnostic genes in immune cell types and its correlation with chemokines

Further, we analyzed the distribution of four diagnosis genes identified by LASSO and SVM-RFE in 10 major immune cell types.

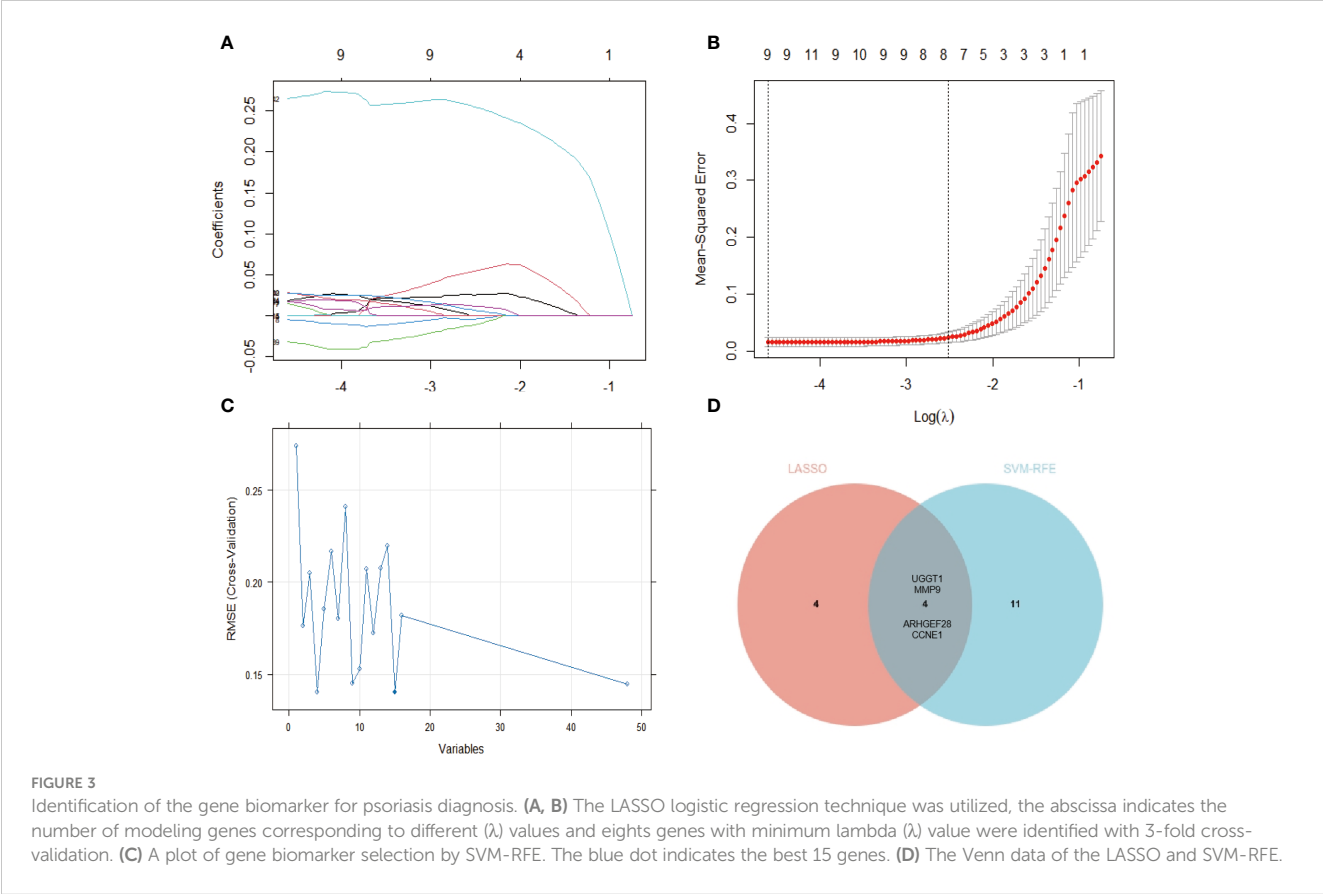


FIGURE 3
Identification of the gene biomarker for psoriasis diagnosis. (A, B) The LASSO logistic regression technique was utilized, the abscissa indicates the number of modeling genes corresponding to different (λ) values and eight genes with minimum lambda (λ) value were identified with 3-fold cross-validation. (C) A plot of gene biomarker selection by SVM-RFE. The blue dot indicates the best 15 genes. (D) The Venn data of the LASSO and SVM-RFE.

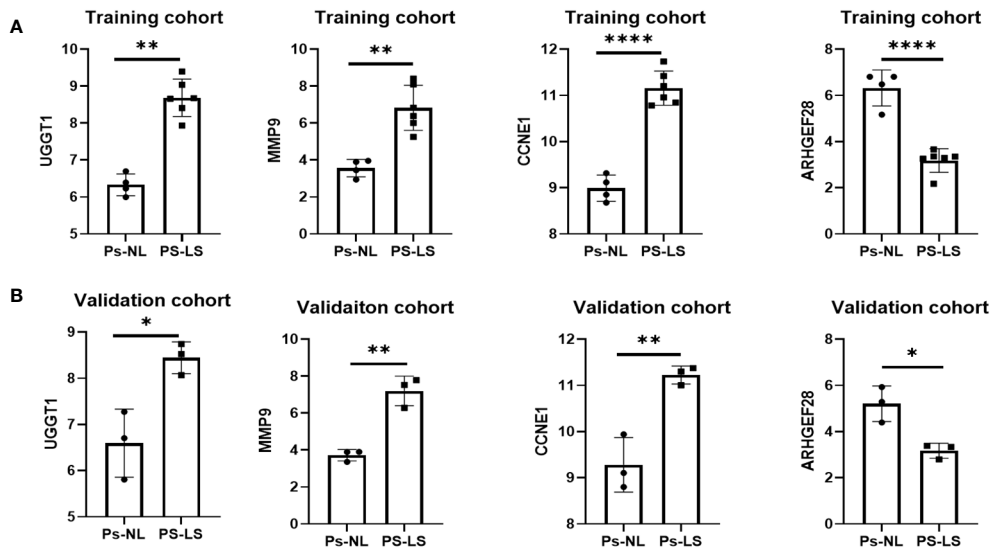


FIGURE 4 The expression level of UGGT1, MMP9, CCNE1, ARHGEF28 in training (A) and validation (B) cohorts. Ps-NL, psoriasis non-lesion; Ps-LS, psoriasis lesion. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

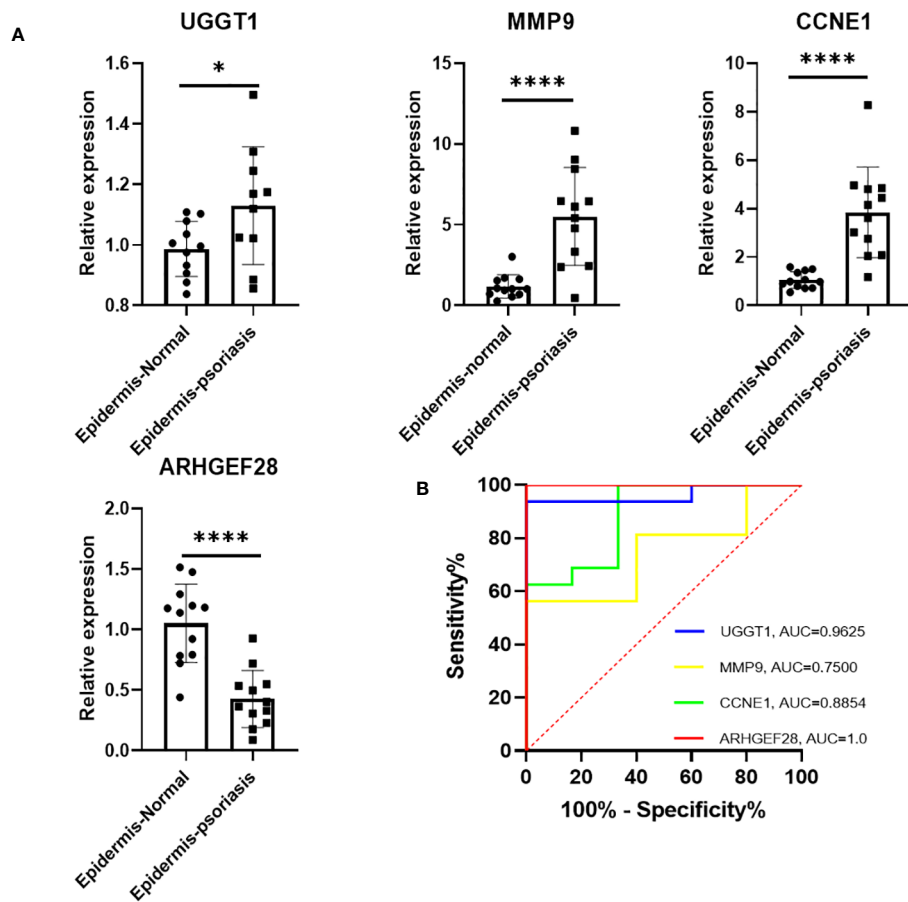


FIGURE 5 (A) Expression of four diagnostic genes in epidermis of PsO and normal human as revealed by RT-qPCR. (B) The determination of its diagnostic effectiveness between normal and psoriatic samples via ROC curves for UGGT1/MMP9/CCNE1/ARHGEF28. ROC, receiver operating characteristic. * $P < 0.05$, **** $P < 0.0001$.

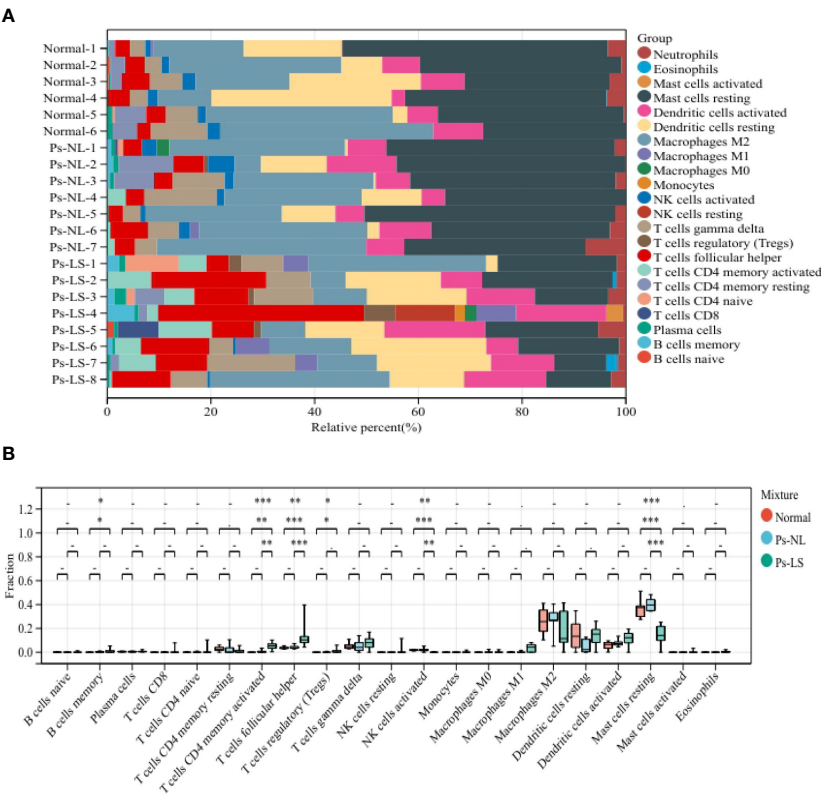


FIGURE 6 (A) A bar plot comparing the percentage of 22 different types of immune cells found infiltrating in three types lesions (B) three types lesions were compared using an histogram plot, which displayed the ratio differentiation of 22 distinct types of immune cells. Ps-NL, psoriasis non-lesion; Ps-LS, psoriasis lesion. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

As shown in Table 2, we found that CCNE1 is highly expressed in CD4⁺ T cells and neutrophils in psoriatic lesions, at the same time, validation of the expression of CCNE1 in CD4⁺ T cells was performed by RT-qPCR (Figure 7A), and ARHGEF28 is highly expressed in mast cells. In addition, CCNE1 is closely related to the chemokines IL-17A, CXCL8/9/10 and CCL20 (Figure 7B).

3.8 The elevation of CCNE1 in the psoriasis epidermis

Given the importance of CD4⁺T in psoriasis and the results of the four genes in immune cells, finally, CCNE1 was chosen for

further validation. As shown, CCNE1 expression was significantly increased especially in the nucleus in psoriatic epidermis relative to normal controls (Figure 8).

3.9 The up-regulation of CCNE1 in immune cells in psoriatic skin lesions

Further, we used multi-color immunofluorescence technology to detect the expression of CCNE1 in CD4⁺ cells and neutrophils in PsO and normal control lesions. As shown in Figure 9, CCNE1 is expressed in both CD4⁺ cells and CD11b-labeled cells, and the expression in patients with PsO is significantly higher than that in normal people.

TABLE 2 The expression of four diagnosis genes in 10 major immune cell types.

GeneSymbol	B cells	Plasma cells	T cells CD8	T cells CD4	NK cells	Monocytes	Dendritic cells	Mast cells	Eosinophils	Neutrophils
MMP9	-	-	-	-	0	-	-	0	-	-
UGGT1	-	-	-	-	-	-	-	-	-	-
CCNE1	-	-	-	12.26	0	-	-	0	-	12.38
ARHGEF28	-	-	-	-	-	-	-	10.88	0	-

Red highlight means that the gene is highly expressed in certain cell. Blue highlight means that the expression of certain gene is not obvious.

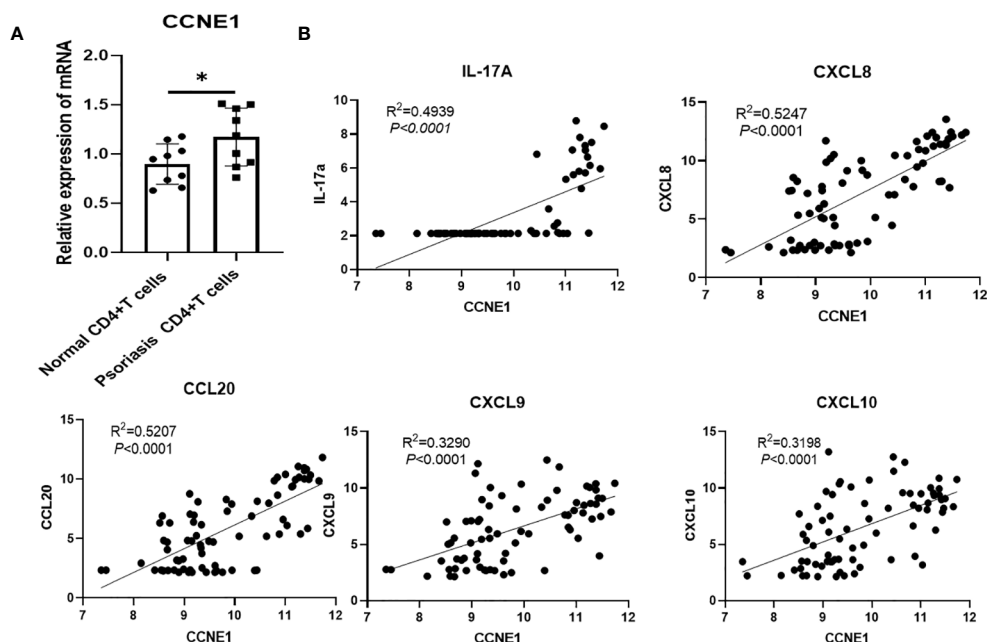


FIGURE 7
(A) Validation of CCNE1 in CD4+ T cells from controls and psoriasis patients (B) Correlation between CCNE1 and IL-17a, CXCL8/9/10 and CCL20.
* $P < 0.05$.

4 Discussion

Psoriasis vulgaris is a chronic inflammatory skin disease, affects approximately 125 million individuals globally, including 6.5 million in China (18, 19). Psoriasis with the character of recurrent episodes seriously affects the psychological and physical health of patients and aggravates the socio-economic burden and public health resources (20). Currently, biological agents show promising therapeutic effects for patients with typical psoriasis, however, patients with psoriasis-like atopic dermatitis often present

challenges in differentiation from psoriasis (21), thus affecting the selection of biological agents and disease treatment progress.

Furthermore, we utilized the Venn diagram to exclude DEGs shared between the lesion and no-lesion of atopic dermatitis and mixed patients, obtaining 144 specific differential genes in psoriasis non-lesions and lesions. By employing the LASSO algorithm, we identified eight specific diagnostic genes, including TRIM13, CCNE1, UGGT1, MMP9, ARHGEF28, NAPG, CCNYL1, and NFASC. The SVM-RFE model selected 15 specific diagnostic genes. Taking the intersection of these two algorithms, we finally

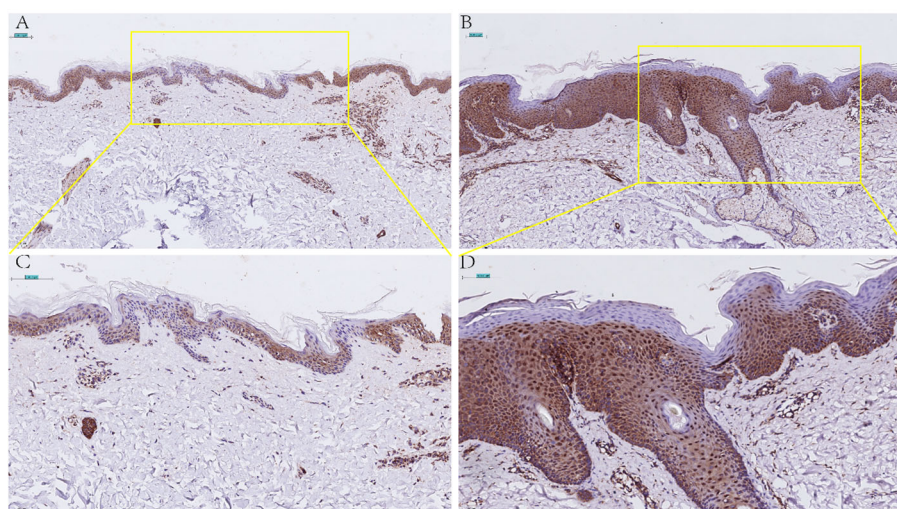


FIGURE 8
Immunohistochemical detection of CCNE1 expression in normal skin lesions (A 20X, C 40X) and psoriatic lesions (B 20X, D 40X).

obtained four specific gene biomarkers for psoriasis diagnosis (UGGT1\MMP9\CCNE1\ARHGEF28).

To further explore the expression patterns of the four diagnostic genes in psoriasis patients, we analyzed the training and validation cohorts in the models. We observed a significant increase in UGGT1, MMP9, CCNE1 expression in psoriatic lesional skin compared with the psoriatic non-lesion, and a noteworthy decrease in ARHGEF28 expression in psoriatic lesional skin.

Next, we collected the epidermis of healthy volunteers and psoriasis, extracted RNA, and performed RT-qPCR for further validation. The results demonstrated a significant upregulation of UGGT1, MMP9, and CCNE1 expression in psoriatic patients, accompanied by a significant downregulation of ARHGEF28 expression. These findings were consistent with the algorithm-analysis outcomes. Additionally, we expanded the sample size and compared the diagnostic effectiveness with psoriatic and normal individuals. The ROC curve analysis revealed that the AUC of four diagnostic genes were greater than 0.7, with ARHGEF28 reaching 1.0, UGGT1 0.96, CCNE1 0.8854, and MMP9 0.77, indicating effective discriminative significance.

Additionally, we performed enrichment analysis on the 144 unique DEGs in psoriatic lesional and non-lesional skin. As shown in [Figure 2](#), JAK-STAT signaling pathway, yersinia infection, inflammatory bowel diseases (IBD), cytokine-cytokine receptor interaction were performed in the psoriasis-specific enrichment results. As we all know, JAK-STAT signaling pathway is a key mediator underlying the immunopathogenesis of psoriasis, and its presence in our psoriasis-specific differential gene enrichment results provides a new piece of evidence that drugs targeting the JAK-STAT pathway for the treatment of psoriasis although they are still in the early stages of development ([22, 23](#)). Intriguingly, the results of the psoriasis-specific differential gene enrichment showed an association with IBD, which coincides with the results of the study in JAMA Dermatology ([24](#)), a cohort study that showed that patients with psoriasis had a 2.53-fold increased risk of Crohn's disease and a 1.71-fold increased risk of ulcerative colitis. Furthermore, our results show that the genes mediating the development of IBD in psoriasis may be associated with the following genes CSF2RA/SOCS1/PTPN2/STAT3/IL21R (in the [Supplementary Table 4](#)), which has implications for the exploration of co-morbid targets in PsO combined with IBD. In addition, vitamin efficacy has been shown to be important in the treatment of psoriasis ([25](#)). We found that vitamin B6 metabolism occupies a place in the gene enrichment analysis specific to psoriasis, suggesting that vitamin B6 supplementation is an important part of vitamin therapy for psoriasis. It is worth mentioning that four diagnostic genes screened by our machine learning were shown to be associated with toxoplasmosis, yersinia infection, arrhythmogenic right ventricular cardiomyopathy (ARVC), and hepatitis B, dilated cardiomyopathy (DCM) disease in the enrichment results (in the [Supplementary Table 4](#)), which needs to draw attention to the importance of screening for cardiovascular and intestinal diseases in psoriasis patients as well as the development of therapeutic targets for patients with co-morbidities.

As we all know, psoriasis is an autoimmune inflammatory disease closely associated with the immune system. It is characterized by chronic inflammation and epidermal hyperplasia, with a substantial

number of inflammatory and immune cells functioning simultaneously ([26](#)). CIBERSORTx is also a type of machine learning algorithm, also known as digital cytometry, which implements an inverse convolution approach through linear support vector regression (SVR) techniques that can analyze the gene expression patterns of cells in complex tissues to determine the cellular composition of the tissue ([27, 28](#)). In our research, we performed CIBERSORTx to analyze the infiltration of 22 immune cell types in the RNA-seq of normal skin lesions and the non-lesional and lesional skin regions of psoriatic patients. This three types skin biopsy ranged from mild to severe allowed us to dynamically observe the immune cell dynamics in the three distinct stages of inflammation lesions. As shown in [Figure 6](#), in adaptive immunization, we first observed a positive correlation between T cells CD4⁺ memory activated and T cells CD4⁺ follicular helper in normal skin lesions, non-lesional areas in psoriatic patients, and lesional areas in psoriatic patients, therefore, we speculate that T cells CD4⁺ memory activated and T cells CD4⁺ follicular helper might play an indispensable regulatory role throughout the whole process of change from mild to severe process of psoriasis-like skin lesions. Numerous studies on the immune pathogenesis of psoriasis have focused on T lymphocytes, and their importance in this disease has been widely acknowledged ([29, 30](#)). Additionally, we found activated dendritic cells (DCs) showed an upward trend in normal individuals, psoriatic non-lesional areas, and psoriatic lesions, which is consistent with the conclusion that DCs are increased in psoriatic lesions and are thought to contribute to the formation of a T-cell response ([31](#)). Interestingly, B cells memory and Tregs cells exhibited significant differences only in normal skins and psoriatic lesional areas, suggesting that B cells memory or Tregs might carry a more prominent role in the later stages of lesion progression.

On the other hand, in the innate immune response, the results of immune infiltration showed a significant decrease in activated NK cells in psoriatic lesional skin compared to normal skin and non-lesional skin in psoriatic patients. This phenomenon is consistent with the reduced number of NK cells in the peripheral blood of psoriatic patients, but there are few reports on the changes in the proportion of NK cells in lesions, based on the results of this study, we prefer that NK cells activation can maintain skin immune homeostasis ([32, 33](#)). Also interestingly, the results of CIBERSORTx add a new piece of evidence for the role of mast cells in the progression of psoriatic skin lesions. And our three skin lesion immuno dynamic infiltration results showed that resting state mast cells showed a gradual decrease in skin lesions in psoriasis patients compared with non-lesional skin in psoriasis patients, suggesting that resting mast cells may play an important role in the maintenance of skin homeostasis. In addition, mast cells may activate and degranulate during the progression of psoriasis, ultimately leading to chronic inflammation ([34](#)). In brief, we suggest that the reduction of resting mast cells may cause disturbances in skin homeostasis and thus exacerbate the changes in the lesions. Taken together with the reduction of NK cells and mast cells in psoriatic lesions, we hypothesize that innate immunity plays an important role in skin homeostasis, and that the progression of lesions toward psoriasis-like conditions may be associated with a reduction in innate immune cell function.

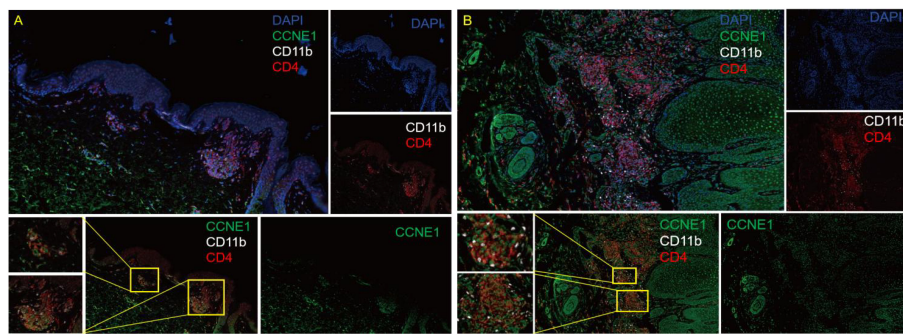


FIGURE 9

Multicolor immunofluorescence technology to detect the expression of CCNE1 in the CD4⁺ cells and neutrophils in normal human skin lesions (A), 10X eyepiece, 10X objective) and PsO lesions (B), 10X ocular lenses, 10X objective). Blue represents DAPI, green represents CCNE1, white represents CD11b positive, and red represents CD4⁺ positive.

Further we analyzed the expression of the four diagnostic genes screened by the two machine algorithms in 10 major immune cells and found that CCNE1 was elevated in CD4⁺ T cells as well as neutrophils in psoriasis lesions, and ARHGEF28 was elevated in mast cells. UGGT1 and MMP9 were not significantly enriched in 10 major immune cells. It is worth mentioning that we verified the expression of CCNE1 in CD4⁺ cells and neutrophils which located in the lesion through multicolor immunofluorescence technology. Actually, psoriasis is a T-cell mediated autoimmune skin disease, IFN- γ , TNF- α , IL-17A, CXCLs, CCLs, and antimicrobial peptide secreted by immune cells or keratinocytes undergo important functions in the crosstalk between keratinocyte and immune cells in the pathogenesis of psoriasis (35–38), thus, in view of the results that we validated in CD4⁺ T cell with CCNE1, we pay more attention to the correlation analysis between CCNE1 and inflammatory mediators mentioned above, and the results showed that CCNE1 was significantly correlated with IL-17A/CXCL8/CXCL9/CXCL10/CCL20 ($P < 0.0001$, $R^2 > 0.3$). Indeed, CCNE1 is a positive regulator of the cell cycle, which can activate cyclin-dependent kinases 2 (CDK2) to regulate the G1-S transition of the cell division cycle, and plays an important role in the regulation of cell cycle progression and cell proliferation (39). To further investigate the role of CCNE1 in psoriasis, we performed immunohistochemical experiments, which showed that CCNE1, as a nuclear protein, was increased in the nuclei of cells in the epidermis of psoriasis relative to normal human skin. It has been demonstrated that IL-17A stimulates keratinocytes to upregulate the cell cycle-related genes, such as CCNE1, which made contribution to epidermal KC proliferation (40), therefore we hypothesize that enhanced IL-17 may contribute to the elevated CCNE1 in epidermal cells of psoriasis patients. And studies have shown that CXCL8 could recruit neutrophils and CCL20 chemotactically attracts IL-17A-producing immune cells, further creating an IL-17A-rich environment and accelerating the pathological progression of psoriasis (41). In addition, elevated expression of CXCL9 and CXCL10 in epidermal keratinocytes may induce type 1 T cells to migrate from the dermis to the epidermis and trigger inflammation in the skin (42–44). Nevertheless, whether CCNE1 is able to regulate the expression of

CXCL8/9/10, CCL20, and thus mediate the above mentioned progression of psoriasis pathological mechanisms is not enough to rely only on correlation analysis, more experiments should be performed. In addition, CCNE1 was also highly expressed in CD4⁺ T cells as well as neutrophils in our immune infiltration results, although the mechanism of CCNE1 elevation in CD4⁺ as well as neutrophils has indeed been rarely studied, Neutrophil traps and TH17 release of IL-17 in psoriatic lesions is common (45), and based on the fact that IL-17 induces epidermal cells to produce CCNE1 expression, we speculate whether there is a similar stimulatory effect in CD4⁺ T cells as well as neutrophils, but more studies are needed to confirm this. Based on existing research, and we found that CCNE1 may not only play a role in skin lesions through the cell cycle, but may also intervene in the progression of psoriasis lesions by modulating immune cells as well as secretion of inflammatory factors.

Overall, we circumvented the problem of mismatch between healthy individuals and patients by differential gene analysis of lesions and non-lesional areas in psoriasis, atopic dermatitis, and overlapping patients and obtained 144 psoriasis-specific differential genes, then four diagnostic genes were then screened by two machine algorithms, and finally the dynamics of the immune cells in the three degrees of lesions were analyzed by using a reverse convolutional algorithm. The manifestations of the four diagnostic genes in immune cells were also specifically analyzed, and we also performed RT-qPCR and immunohistochemistry for experimental validation, and the results confirmed to be consistent with those obtained by the machine analysis algorithms, which suggests that the application of machine algorithms in the medical field is reliable and valuable. Finally, we obtained four diagnostic genes with research value, and we have a greater interest in CCNE1, in view of the AUC of ROC and their expression in immune cells. However, in the future, we should pay more effort into the validation and investigation of the specific regulatory mechanisms of CCNE1 with CXCL8/9/10 and CCL20, and whether IL-17 can stimulate CCNE1 expression in keratinocytes, CD4⁺ T cells and neutrophils in the pathogenesis of psoriasis, in addition to this, expanded samples are also needed to further improve the sensitivity of the diagnostic genes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

Ethics statement

The studies involving humans were approved by Institutional Review Boards of Hanyang University, Seoul, Korea, and Rockefeller University, New York, NY, USA. Written informed consent was obtained from all patients. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

XZ: Conceptualization, Methodology, Supervision, Visualization, Data curation, Writing – original draft, Writing – review & editing. HZ: Conceptualization, Methodology, Supervision, Visualization, Data curation, Formal analysis, Writing – review & editing. XL: Conceptualization, Validation, Visualization, Writing – review & editing. R-FW: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1388690/full#supplementary-material>

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CD8 T cells are dispensable for experimental autoimmune prostatitis induction and chronic pelvic pain development

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Introduction: Chronic Pelvic Pain Syndrome or Chronic Prostatitis (CPPS/CP) is the most prevalent urologic affliction among young adult men. It is a challenging condition to treat, which significantly decreases patient quality of life, mostly because of its still uncertain aetiology. In that regard, an autoimmune origin is a prominent supported theory. Indeed, studies in patients and in rodent models of Experimental Autoimmune Prostatitis (EAP) have provided compelling evidence suggesting a key role of CD4 Th1 cells in disease pathogenesis. However, the implication of other prominent effectors of the immune system, such as CD8 T cells, has yet to be studied.

Methods: We herein analyzed the induction of prostatitis and the development of chronic pelvic pain in EAP using CD8 T cell-deficient animals.

Results: We found similarly elevated PA-specific immune responses, with high frequencies of specific IFN γ +CD4+ and IL17+CD4+ T cells in prostate draining lymph nodes from PA-immunized either CD8 KO or wild type animals with respect to controls. Moreover, these peripheral immune responses were paralleled by the development of significant chronic pelvic pain, and accompanied by prostate histological lesions, characterized by hemorrhage, epithelial cell desquamation, marked periglandular leukocyte infiltration, and increased collagen deposition in both, PA-immunized CD8 KO and wild type animals. As expected, control animals did not develop prostate histological lesions.

Discussion: Our results indicate that CD8 T cells do not play a major role in EAP pathogenesis and chronic pelvic pain development. Moreover, our results corroborate the previous notion that a CD4 Th1 associated immune response drives the induction of prostate tissue inflammation and the development of chronic pelvic pain.

KEYWORDS

autoimmunity, prostatitis, inflammation, CD8 T cells, chronic pelvic pain, animal model, pathogenesis

1 Introduction

Chronic pelvic pain syndrome or chronic prostatitis (CPPS/CP) stands as the most prevalent urologic condition among males under 50 years of age and the third most common in older males, accounting for up to 25% of all outpatient visits and urology consults (1–3). It has a considerable negative impact on quality of life of patients, comparable to other major long-term health conditions such as Crohn's disease, diabetes mellitus, angina, or myocardial infarction (4, 5). CPPS/CP manifests with a wide array of pain and inflammatory symptoms, varying in type and intensity, affecting regions such as the pelvis, rectum, perineum, penis, testes, and lower back, persisting for at least 3 of the preceding 6 months. Although in most patients pain is accompanied by semen and/or prostate inflammation, no invading infectious agents are detected (5–8). Since its aetiology and pathogenesis remain unclear, most therapies are empiric and ineffective (9, 10). In fact, the therapeutic options currently available for patients are inadequate and often fail to meet the expectations of both physicians and patients alike (9). In consequence, CPPS/CP is usually misdiagnosed and poorly managed, resulting in chronic signs and symptoms, substantial morbidity, and overall patient dissatisfaction despite being common in clinical practice (11). CPPS/CP seems to involve similar clinical phenotypes resultant from several heterogeneous conditions, and dysregulated inflammation as a consequence of autoimmunity against the prostate has been proposed to be involved in its onset and/or progression (1, 10, 12–14). Cumulative data obtained from the study of both, the human disease and rodent models of Experimental Autoimmune Prostatitis (EAP), have provided compelling evidence regarding the immune mechanisms that underlie the induction and development of the disease and its associated consequences (10, 15–17). Indeed, self-reactive T cell and antibody responses specific to prostate antigens (PA) in CPPS/CP patients have been reported (18–22). Interestingly, increased IFN γ -secreting Th1 cell responses specific to PA, concomitant with urogenital inflammation as well as reduced semen quality, have been detected in a substantial fraction of men suffering from CPPS/CP, thus indicating that PA-specific Th1 cell-mediated immune responses may contribute to the development of male reproductive tract inflammation and chronic pelvic pain (22–24). Besides, EAP animal models serve as valuable tools in investigating the pathogenesis of CPPS/CP, as they closely mimic key features of the human disease, demonstrating their reliability and utility (10, 12, 17). They have shown that, upon PA immunization, animals develop specific Th1 cell responses that are accompanied by important lesions and infiltration of the prostate (17, 25, 26). Cumulative evidence indicated a key role of CD4 Th1 cells in the induction of inflammation in the prostate gland and the development of chronic pelvic pain, the characteristic symptom observed in patients with CPPS/CP (27–32). Although they have provided important data on the pathogenesis of the disease, useful for the development of new and more rational therapies, the possible pathogenic role of other prominent effectors of the immune system like CD8 T cells has not been studied yet.

CD8 T lymphocytes are crucial for the elimination of tumor cells and intracellular pathogens, demonstrating functional plasticity and complexity (33, 34). However, it is currently known that CD8 T cells play a plethora of functions, encompassing direct and indirect cytotoxic and non-cytotoxic actions (35). Upon activation in secondary lymphoid organs following antigen presentation, naïve CD8 T cells undergo rapid clonal expansion, resulting in large numbers of antigen-specific acute effector and memory CD8 T cells. This process is intricately regulated by various cell types, including CD4 T cells, which contribute to establishing optimal niches for the development and maintenance of effective CD8 T cell immunity (36). Effector CD8 T cells migrate to primary sites of infection or tumors, where they secrete cytokines such as IFN γ and effector molecules like perforin and granzyme, aimed at specifically eliminating the target cells *in situ* (37). Most of these acute effector CD8 T cells are eventually eliminated following antigen clearance, leading to the formation of a residual pool of diverse memory cells (38). Memory CD8 T lymphocyte subsets are thought to be structured in a developmental hierarchy, where tissue stem cell memory CD8 T cells (T_{SCM}) self-renew and give rise to long-lasting central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and tissue resident T cells (T_{RM}) (39). Most CD8 T cells found in peripheral tissues are genuine T_{RM}, which establish particular niches in nearly every organ (40). Regarding that, it is interesting to highlight that CD8 T cells are commonly found infiltrating inflamed tissues in several autoimmune diseases (35, 38). Interestingly, increased levels of CD8 T cells have been reported in semen from CPPS/CP patients (24). Moreover, several researchers have reported significant CD8 T cell infiltration in the prostate from animals with EAP (29, 31, 41, 42), suggesting they could play a major role in disease induction and progression, and the consequent pelvic pain. However, to the best of our knowledge, there is currently no documented evidence regarding the assessment of these cells involvement in the pathogenesis of chronic prostatitis. Considering that, with the aim of expanding the current knowledge of the pathogenesis of CPPS/CP, we herein analyzed the role of CD8 T cells in the induction of prostate inflammation and the development of chronic pelvic pain using an animal model of EAP.

2 Materials and methods

2.1 Ethics statement

Animal studies were conducted under protocols EXP-UNC: 0002734/2016 and EXP-UNC: 60699/2018 approved by the Institutional Laboratory Animal Care and Use Committee (CICUAL) at Facultad de Ciencias Químicas, Universidad Nacional de Córdoba (FCQ-UNC). CICUAL conducts its reviews strictly adhering to the guidelines of the Canadian Council on Animal Care (CCAC), the Directive of the European Union on the protection of animals used for scientific purposes (2010/63/EU), and the recommendations outlined in the Guide for the Care and Use of Laboratory Animals by the NIH (NIH publication 86–23).

2.2 Mice and antigens

Male CD8 α knock-out (B6.129S2-Cd8atm1Mak/J) (43) and C57BL/6 wild type mice were procured from Jackson Laboratory (Bar Harbor, ME, USA). The mice were kept and cared for under specific pathogen-free (SPF) conditions at the Animal Care Facility of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, and were utilized at the age of 6–8 weeks. The animal care facilities and programs of CIBICI meet the requirements of the national laws and institutional guidelines that are based on NIH regulations. Our facility has been certified by OLAW-NIH, Animal Welfare Assurance Number F16-00193.

The preparation of PA and the purification of Prostataein, also known as Prostate Steroid-Binding Protein (PSBP), the main target autoantigen in EAP, were conducted following established protocols as previously described (29). Briefly, PA extracts were prepared from Wistar rat prostate glands. Pooled glands were homogenized in protease inhibitors-supplemented PBS in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 100,000 $\times$ g for 30 min, and the supernatant was used as PA mixture. Protein concentration was determined using the Folin phenol reagent. Aliquots were kept frozen at -20°C until use. PSBP was purified following the procedure described by Chen et al. (44). Ventral prostates were homogenized in 20 mM Tris-HCl using an Ultra-Turrax homogenizer, and the cytosolic fraction was obtained after 60 min of centrifugation at 100,000 $\times$ g and 4°C. The resultant supernatant was applied onto a Mono-Q FPLC column. Proteins were eluted with a linear 0–60% NaCl gradient in 20 mM Tris-HCl. Protein concentration of each fraction was evaluated by measuring its OD280, which were run under non-denaturing conditions in 15% polyacrylamide gels (SDS-PAGE). Fractions containing two bands of 18 and 20 kDa (corresponding to both subunits of PSBP) were also run under denaturing conditions to verify their identity. The purity of the PSBP preparation was >95% evaluated by Western blot using a mouse mAb recognizing the C3 polypeptide (kindly given by Dr. P. Bjork, Pharmacia and Upjohn, Uppsala, Sweden); and was LPS free tested by Gel clot 0.03 endotoxin units/ml sensitivity (Charles River, Laboratories International, Wilmington, NY, USA).

2.3 Antibodies

The antibodies utilized in various experiments, along with their respective manufacturers, were as follows: anti-CD3 (145–2C11), anti-CD4 (RM4–5), anti-Gr1 (RB6–8C5), anti-CD11b (M1/70), anti-anti-IL-10 (JES5–16E3), IFN γ (XMG1.2), and IgG1 isotype controls were procured from BD Biosciences (San Diego, CA, USA). The following antibodies were obtained from eBioscience (San Diego, CA, USA): anti-CD45 (30-F11) and anti-IL-17A (eBIO17B7). Antibodies including anti-CD45 (30-F11), anti-CD19 (6D5), anti-CD11b (M1/70), anti-NK-1.1 (S17016D), anti-CXCR3 (CXCR3–173), and anti-CCR5 (HM-CCR5) were

purchased from BioLegend (San Diego, CA, USA). The antibodies were conjugated with APC, FITC, PE, PerCP-Cy5.5, Alexa Fluor 647, or PE-Cy7 and appropriately paired.

2.4 EAP induction and histopathological score

Male CD8-KO and C57BL/6 wild-type mice, aged six to eight weeks, were subcutaneously immunized in the hind footpad and at the base of the tail. The immunization comprised PA (300 μ g/mouse, PA-CD8 KO and PA-WT groups, respectively) or saline solution (control group, C-WT) emulsified in complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA) at a total volume of 150 μ l/mouse, following established protocols (25, 26, 29). Mice were immunized on days 0 and 15 and subsequently euthanized on day 24 according to the experimental timeline. A total of 58 mice were analyzed; n=4–6 mice per experimental group, being experiments repeated at least thrice. EAP severity was evaluated through histological scoring, conducted in a double-blind manner as described previously (27, 29). The inflammation level was graded on a scale of 0–3: 0 for no inflammation, 1 for mild perivascular cuffing with mononuclear cells, 2 for moderate perivascular cuffing with mononuclear cells, and 3 for marked perivascular cuffing, hemorrhage, and abundant mononuclear cells in the parenchyma. Evaluation was performed on 5 μ m thick prostate tissue sections from each organ per animal, processed using conventional hematoxylin and eosin staining. Slides were examined under a Nikon TE 2000U microscope (Nikon, Osaka, Japan), and images were analyzed using Adobe Photoshop CS6 version 13 image analysis software (Adobe Systems Inc., San Jose, CA, USA).

2.5 Collagen quantification by picrosirius red staining

Prostate tissue samples were fixed in 4% paraformaldehyde, dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin. Subsequently, 5 μ m sections were stained with PSR according to a previously established protocol (45). Fluorescent imaging was conducted utilizing a BZ-X710 digital microscope (Keyence, Itasca, IL) equipped with a 20X dry objective (PlanFluor, NA 0.45) and illuminated by full spectrum light filtered with Texas red and FICZ filters. Tiled images were captured using Keyence image acquisition software (Keyence, Itasca, IL) and stitched to create a comprehensive image of the entire prostate section. Specific collagen staining was isolated by subtracting tissue autofluorescence from the acquired images. The total tissue area was determined by outlining the perimeter of the prostate lobe using a freehand selection tool and measuring the pixel area of the selected region. Collagen density was assessed by quantifying the area of PSR staining relative to the total tissue area using CT-FIRE software (LOCI, Madison, WI).

2.6 Cell culture

Mononuclear cell suspensions were individually prepared from prostate draining lymph nodes of mice in HBSS (Sigma-Aldrich) using Ficoll-Paque PREMIUM 1.084 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) centrifugation gradients. Live cells were counted via Trypan blue exclusion, then resuspended in RPMI 1640-GlutaMAX medium (Life Technologies, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin (Life Technologies), 50 mM 2-ME (Life Technologies), and 10% FCS (Life Technologies). They were cultured in round-bottomed plates either with PSBP (20 µg/ml) or medium alone. Plates were incubated at 37°C in a water-saturated 5% CO₂ atmosphere. Subsequently, cells were subjected to surface and/or intracellular cytokine staining and analyzed using FACS.

For lymphoproliferation assays, cells were seeded at a density of 3×10^5 cells per well in a volume of 0.2 ml in flat-bottomed 96-well plates. Cells were cultured in the presence of PSBP (20 µg/ml) or medium alone. All cell combinations were replicated in quadruplicate. Plates were then incubated for 4 days at 37°C in an atmosphere saturated with 7.5% CO₂ and pulsed during the final 18 hours with 1 µCi [methyl-³H] thymidine per well. The labelled material was automatically harvested and counted using a β-plate scanner. The response was quantified as a proliferation index, computed from counts per minute (cpm) incorporated in antigen-pulsed cultures divided by cpm incorporated in cultures with medium alone, as described previously (29).

2.7 Quantification of PSBP-specific antibodies in serum

PSBP-specific serum levels of total IgG, IgG1, and IgG2a were evaluated using conventional ELISA methods as detailed previously (29). In detail, multiwell plates (Maxisorp) were coated with 50 µl/well of PSBP (20 µg/ml) in 0.05 M carbonate buffer pH 9.6 and left to incubate overnight at 4°C. Following this, microwells were washed twice and subsequently blocked with 3% BSA (Sigma-Aldrich) in PBS for 2 hours at 37°C. After rinsing with PBS-Tween 20 at 0.05%, 100 µl of serum (obtained post-cardiac puncture) serial dilutions (starting at 1/50) were added to the wells and allowed to incubate for 1 hour at 37°C. To identify specific total IgG, IgG1, and IgG2a, the plates underwent another washing step and were then incubated with a solution containing HRP-conjugated rat anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2a (BD Biosciences) for 1 hour at 37°C. Following thorough washing, the reaction was developed with BD OptEIA TMB Substrate Reagent Set (BD Biosciences). Serum reactivity was quantified as OD measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.8 Flow cytometry

Prostate draining lymph node cells were stimulated with PSBP *in vitro*, followed by staining for cell surface markers and intracellular cytokines, according to established protocols (27, 29).

In brief, cells were stimulated *in vitro* with PSBP (20 µg/ml) for 48 hours and, during the final 4 hours, incubated with 50 nM PMA and 0.5 µg/ml ionomycin (Sigma-Aldrich) along with GolgiStop and GolgiPlug (BD Biosciences). Following this, cell-surface staining for various molecules and chemokine receptors was conducted, followed by intracellular staining for different cytokines using the BD CytoFix/CytoPerm and Perm/Wash kit (BD Biosciences) according to the manufacturer's instructions. APC-labelled antibodies to IL-17A (eBiosciences), IL-4 (eBiosciences), or CCR5 (BioLegend), as well as PE-labelled antibodies to IFNγ (BD Biosciences), IL-10 (BD Biosciences), or CXCR3 (BioLegend), were employed. Cells were analyzed using a FACSCanto II flow cytometer (BD Bioscience). The data were analyzed using FlowJo software (Tree Star).

2.9 Analysis of prostate infiltrating leukocytes

Analysis of prostate infiltrating leukocytes was conducted following previously established protocols (27, 29). In brief, the prostates were mechanically disrupted and enzymatically digested in RPMI 1640 medium containing 1 mg/ml collagenase D (Roche, Basilea, Switzerland) and 50 U/ml DNase I (Sigma-Aldrich) for 45 minutes at 37°C. Following digestion, the suspensions were filtered through 75- and 40-µm cell strainers (BD Biosciences), and single-cell suspensions were washed twice in RPMI 1640 medium supplemented with 10% FBS, 2 mM EDTA, and 50 mM 2-ME. Cells were then stained with various antibodies for flow cytometry analysis and acquired using a FACSCanto II. The data were analyzed using FlowJo software (Tree Star).

2.10 Assessment of chronic pelvic pain

The development of chronic pelvic pain was analyzed by behavioral testing as described previously (29, 46, 47). Mice were analyzed before immunization (baseline, day 0), and at 7, 14, and 23 days after the initial immunization. Tests were conducted in individual Plexiglas chambers featuring a stainless-steel wire grid floor, following an acclimation period. Referred hyperalgesia and tactile allodynia were evaluated using von Frey filaments with forces ranging from 0.04 to 4 g (Bioseb, Chaville, France). Each filament was applied for 1–2 s with an inter-stimulus interval of 5 s, totaling 10 times, and the filaments were applied in ascending order of force. Stimulation was confined to the lower abdominal area proximal to the prostate, and efforts were made to stimulate various areas within this region to prevent desensitization or “wind-up” effects. An investigator blinded to the treatment groups graded animal responses. Positive responses to filament stimulation were categorized as: 1) sharp retraction of the abdomen; 2) immediate licking or scratching of the stimulated area; or 3) jumping. Response frequency was calculated as the percentage of positive responses, and data were presented as the mean percentage of response frequency ± SEM.

2.11 Statistical analyses

Statistical analyses were conducted using one-way ANOVA with Bonferroni *post hoc* test analysis. Tactile allodynia testing results were analyzed using two-way ANOVA with Šidák's multiple comparisons test *post hoc* analysis. Mean values along with their standard error of the mean (SEM) are depicted in the graphs. Statistical computations were carried out using GraphPad Prism 5.0 software. A significance level of $*p < 0.05$ was applied to all analyses.

3 Results

3.1 CD8 T cell deficiency does not impair the induction of mixed Th1/Th17 immune responses upon PA-immunization in EAP

With the aim of assessing whether the absence of CD8 T cells had an effect on the specific Th1/Th17 immune response underlying EAP pathogenesis and chronic pelvic pain development, we analyzed the induced immune response upon PA immunization in CD8 α -deficient (CD8 KO) mice and in wild type (WT) mice. Animals were immunized and, 24 days after, the cellular and humoral specific immune responses specific to Prostatein or PSBP, the major target autoantigen in EAP (28, 41), were analyzed. As shown in Figure 1, comparable positive lymphoproliferative responses to PSBP were detected from prostate-draining lymph node cells from both groups of PA-immunized animals, either wild type (PA-WT) or CD8 KO (PA-CD8 KO) mice, when compared with control animals (C-WT, Figure 1A). Besides, these cellular immune responses were accompanied by comparable high serum levels of PBSP-specific total IgG, IgG1 and IgG2a in either PA-WT or PA-CD8-KO mice respect to control animals (C-WT, Figure 1B). To confirm whether the typical mixed prostate-specific Th1/Th17 immune responses underlying EAP induction were observed in our setting, intracellular cytokine staining was conducted on mononuclear cells from prostate-draining lymph nodes after 48 hours of *in vitro* stimulation with PSBP. Interestingly, the induction of similarly higher frequencies of IFN γ + or IL-17A+ CD4 T cells was observed in both groups of PA-immunized mice (PA-WT or PA-CD8 KO) compared with control animals (C-WT, Figure 1C). Conversely, there were no significant differences in the frequencies of IL-10+ and/or IL-4+ CD4 T cells among experimental groups (C-WT, PA-WT and PA-CD8 KO, Figure 1C). Interestingly, significantly higher frequencies of autoreactive CD4 T cells expressing the chemokine receptors CXCR3 and CCR5 were detected in either PA-WT or PA-CD8 KO mice than in control animals (C-WT, Figure 1C).

These findings demonstrate that mice lacking CD8 T cells can generate comparable prostate-specific Th1 and Th17 immune responses to those observed in their wild-type counterparts during EAP induction.

3.2 The development of prostate chronic inflammation in EAP does not depend on CD8 T cells

Then, the prostates from animal groups under study underwent histopathological analysis, while collagen deposition was measured as an indicator of chronic inflammation. As shown in Figure 2A, both groups of PA-immunized mice, PA-WT and PA-CD8 KO, developed similarly severe tissue damage and inflammation revealed by marked multifocal perivascular and stromal infiltration of mononuclear cells, oedema, hemorrhage, epithelial cell desquamation, and severe tissue disorganization were noted, resulting in EAP histopathological scores of 2.33 ± 0.65 and 2.58 ± 0.67 , respectively (Figures 2A, B). As expected, no significant tissue alterations were observed in prostate tissue sections from control mice, yielding an EAP histopathological score of 0.08 ± 0.29 (Figures 2A, B). Moreover, staining with PSR, a collagen stain capable of binding to both fibrillar and non-fibrillar collagen subtypes (45), revealed significantly increased collagen deposition in prostate tissue sections from both groups of PA-immunized mice, PA-WT and PA-CD8 KO with respect to control animals (C-WT, Figures 2C, D). The latter suggested the presence of fibrosis, a fundamental element of chronic inflammation. In parallel, prostate infiltrating leukocytes were quantified and characterized by flow cytometry (Figure 3). In agreement with these findings, significantly higher amounts of total leukocytes (CD45+ cells) were observed in prostate tissue samples from both groups of PA-immunized mice, PA-WT and PA-CD8 KO, compared with control animals (approximately 3–4 fold increase), which exhibited minimal to no prostate leukocyte infiltration (C-WT, Figures 3A–C). The leukocyte infiltrates predominantly consisted of CD4+ T lymphocytes, Gr1+ granulocytes, and CD11c+ cells, while CD19+ B lymphocytes and NK cells were present in either lower proportions (Figures 3B, C). As expected, no prostate infiltrating CD8 T cells were detected in PA-CD8 KO mice, whereas significantly higher counts were observed in PA-WT mice than in control animals (C-WT, Figures 3B, C). These findings show that both, CD8 KO mice and wild type (C57BL/6) mice, exhibit similar types and extents of prostate tissue lesions and chronic inflammation upon EAP induction, indicating that CD8 T cells do not significantly contribute to EAP pathogenesis.

3.3 CD8 T cells are dispensable for chronic pelvic pain development

To evaluate whether the onset of prostate inflammation and leukocyte infiltration was accompanied by pelvic pain development, PA-CD8 KO and PA-WT mice along with control (C-WT) mice underwent suprapubic allodynia assessments at baseline and following PA immunization. Pelvic area mechanical stimulation in PA-immunized mice resulted in positive response frequencies correlating with the applied force (48), with similar response profiles observed in PA-CD8 KO and PA-WT mice (Figure 4). Significant increases in tactile allodynia were noted at both early (7

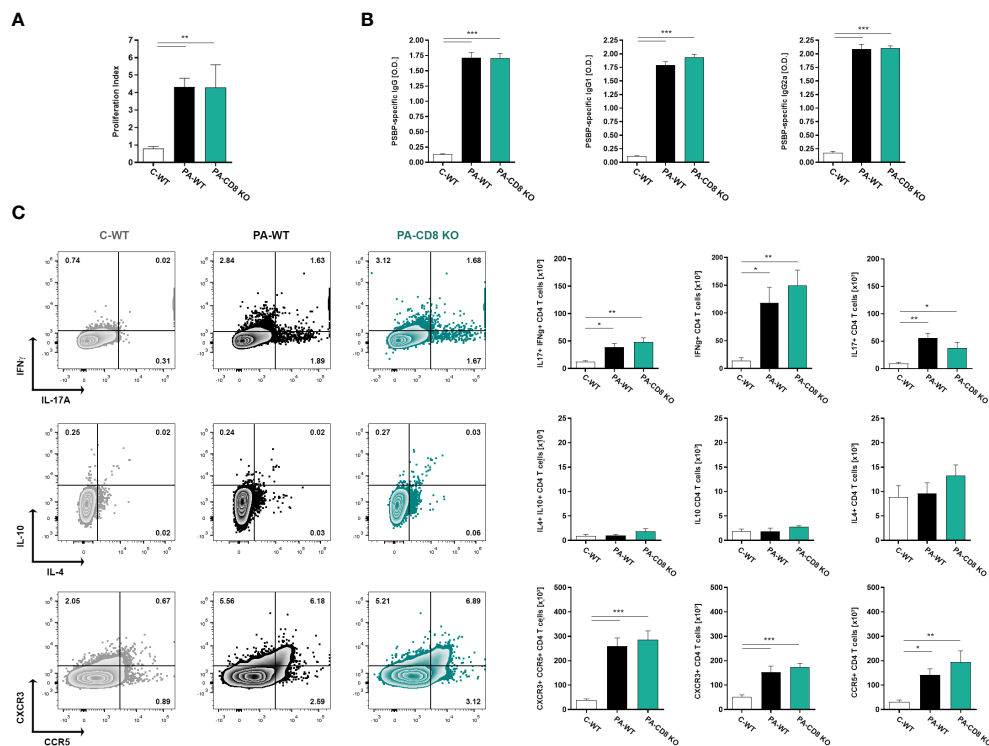


FIGURE 1

Mixed prostate-specific Th1/Th17 immune underlying EAP development are similarly induced upon PA-immunization in either CD8 KO or wild type mice. CD8 KO or C57BL/6 mice were immunized with PA emulsified in CFA (PA-CD8 KO and PA-WT groups) or with saline-CFA (control animals, C-WT). Mice were euthanized at day 24 after immunization, and sera and prostate draining lymph nodes were obtained. Prostate draining lymph node cells were cultured for 48 or 72 h in the presence of PSBP or medium. (A) PSBP specific lymphoproliferative responses from prostate-draining lymph node cells were expressed as proliferation index. (B) Serum levels of PSBP-specific total IgG, IgG1, and IgG2a, assessed by indirect ELISA (serum specimens diluted 1/200). (C) Representative flow cytometry dot plots of intracellular staining for IFN γ , IL-17A, IL-10 and IL-4, and of surface staining for CXCR3 versus CCR5, performed on prostate draining lymph node cells stimulated with PSBP for 48 h. Numbers indicate percentage among gated CD4+ T cells. Bar graphs depict absolute numbers of the indicated cytokine- or chemokine receptor-expressing CD4+ T cells. Data are shown as mean $\pm$ SEM, $n=4-6$ per group, and are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test analysis. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

dpi) and late (14 and 24 dpi) time points in either PA-CD8 KO or PA-WT mice, indicating comparable chronic pelvic pain development kinetics (Figure 4). Altogether, these findings indicate that CD8 T cells do not play a pivotal role in the induction and progression of prostate inflammation and the development of chronic pelvic pain in EAP.

4 Discussion

CPPS/CP is an urological condition that poses a significant healthcare challenge due to its considerable incidence among young and middle-aged men and its significant deleterious impact in the quality of life of patients (2–6, 11, 49–51). Furthermore, its aetiology remains largely unknown, leading to empirically prescribed therapies that often yield suboptimal clinical outcomes, exacerbating patient discomfort and dissatisfaction (1, 5, 9–11, 50, 52, 53). Accumulating evidence suggests that the syndrome is likely a result of dysregulated inflammation manifested through autoimmunity targeting more than one prostate autoantigen, a typical feature of autoimmune diseases. Indeed, several studies have reported evidence indicating an autoimmune aetiology for

CPPS/CP, as a significant proportion of patients display self-reactive T-cell responses, notably Th1 and Th17, targeting various prostate antigens coupled with Treg dysfunction. These responses correlate with inflammation in semen and the prostate, in the absence of detectable infections (8, 10, 12, 19–24, 54–59). Furthermore, treatment with immunosuppressants significantly improves CPPS/CP symptoms (53, 60). Moreover, rodent models of EAP have provided compelling evidence supporting the autoimmune basis of CPPS/CP (17, 55, 61, 62). EAP models have been extensively used to investigate the pathogenesis of CPPS/CP, demonstrating reliability and validity as they closely mirror key findings observed in patients, having provided fundamental evidence about the immune mechanisms underlying disease and chronic pelvic pain development and the associated pathological consequences (10, 14, 17, 61, 62). After immunization with a mixture of PA, purified prostate proteins such as PSBP, or peptides, animals develop mixed PA-specific T cell and antibody responses that associate with florid histologic prostatitis, and the development of chronic pelvic pain (17, 61, 62). As in patients, mice strains susceptible to EAP typically develop mixed PA-specific Th1 and Th17 immune responses, semen and/or prostate inflammation, and chronic pelvic pain (25, 26, 41, 42, 46, 63). Interestingly, a

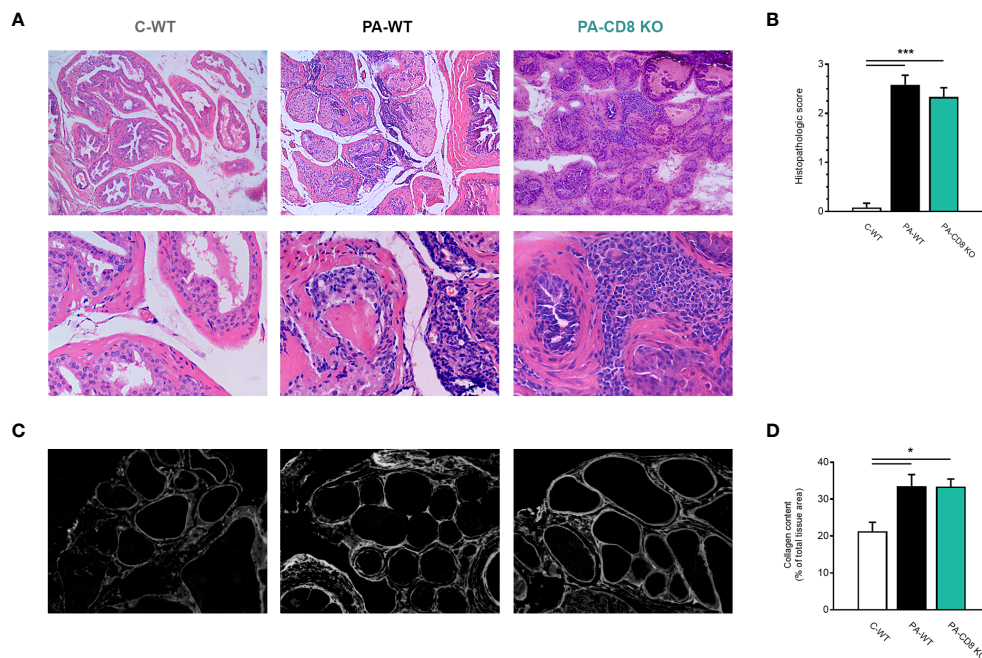


FIGURE 2

Mice deficient in CD8 T cells develop prostate chronic inflammation after EAP induction. CD8 KO or C57BL/6 mice were immunized with PA emulsified in CFA (PA-CD8 KO and PA-WT groups) or saline-CFA (control animals, C-WT). Mice were euthanized at day 24 after immunization and the prostate glands were excised and processed for histopathology analysis. **(A)** Representative hematoxylin and eosin stained histological assays in prostate tissue sections (original magnifications 100X and 400X), and **(B)** Prostate histopathological scores and from mice under study. **(C)** Representative picrosirius red (PSR) stained histological assays in prostate tissue sections, conducted to assess prostate collagen content (original magnification 400X). **(D)** Bar graph depicting collagen content quantification in prostate tissue from mice from the different experimental groups. Data are shown as mean $\pm$ SEM, $n=4-6$ per group, and are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni *post hoc* test analysis. * $p < 0.05$, and *** $p < 0.001$.

fundamental role for Th1 cells in driving disease pathogenesis and chronic pelvic pain development was already shown (27, 29, 64). In fact, the adoptive transfer of sorted PA-specific CD4 Th1 lymphocytes expressing the associated chemokine receptors CXCR3 and CCR5 was sufficient to induce histological prostatitis, with the local expression of several cytokines and chemokines, which subsequently recruited more leukocytes worsening prostate inflammation and inducing chronic pelvic pain (27). Prostate tissue inflammation in EAP is characterized by significant infiltration of macrophages, helper and cytotoxic T cells, granulocytes, and mast cells, and the expression of inflammatory cytokines and chemokines such as IFN γ , IL-17, IL-12, TNF, IL-1 β , CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, and CCL5 (26, 27, 41, 42, 64). Interestingly, mast cells, their chemoattractants CCL2 and CCL3, and the tryptase-PAR2 axis have shown to be essential for chronic pelvic pain development and maintenance (42, 65–67). Once again, these findings mirror the human disease, since elevated levels of CCL2 and CCL3, the Th1-associated chemokine CXCL10, mast cell-derived tryptase and other mediators, and significantly increased effector Th1/Th17 cell proportions have been found elevated in expressed prostatic secretions or peripheral blood from CPPS/CP patients, and correlating with pelvic pain symptoms (32, 66–71). Remarkably, recently reported data indicates that a mast cell-directed therapy improved the quality of life of CPPS/CP patients by alleviating pain and urinary symptoms in association with a downregulation of immune-related pathways including Th1 and

Th17 T cell differentiation (72). From all these evidence, IFN γ -secreting Th1 lymphocytes emerge as central drivers in the pathogenesis of the disease and the development of chronic pelvic pain, the characteristic symptom observed in CPPS/CP patients. Nonetheless, the potential contribution of other fundamental components of the adaptive immune response, such as CD8 T cells, to disease development and pelvic pain induction and/or amplification remains largely unexplored. In that regard, Ye et al. reported significantly decreased levels of CD8 T cells in peripheral blood from CPPS/CP patients than in healthy control individuals (73). On the contrary, an elevation of CD8 T cells in semen from CPPS/CP patients has been described (24). Although the significance of these findings remains uncertain, one possible explanation is that CD8 T cells may be recruited to the prostate during an ongoing local inflammatory process, subsequently leading to an increase in their presence in semen. Interestingly, recently reported data by Zhang et al. suggest that while there were no significant differences in the proportions of peripheral blood naïve, central memory, effector memory, or terminally differentiated effector cells (TEMRA) CD8 T cell subsets between CPPS/CP patients and healthy controls, the CD8 TEMRA cell subset from CPPS/CP patients exhibited significantly elevated expression of IFN γ and TNF and an enriched inflammatory pathway signature compared to controls (74). Although very interesting, these results remain to be confirmed by further research since only 2 CPPS/CP patients and 2 healthy controls

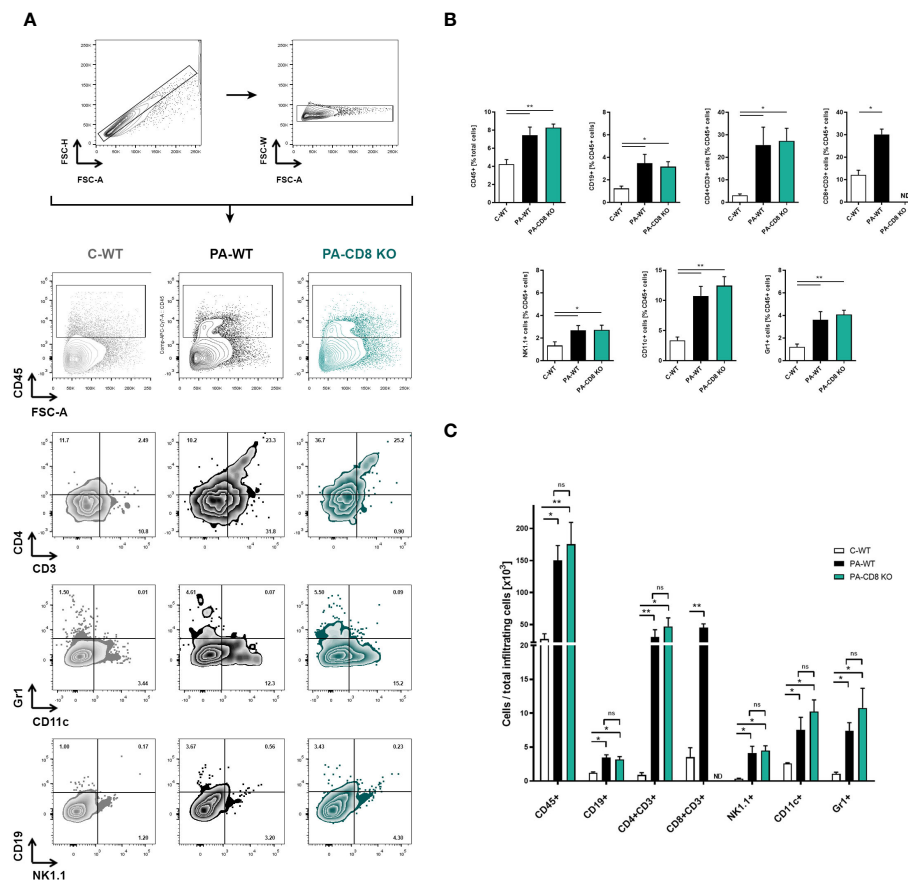


FIGURE 3

Prostate leukocyte infiltration is comparably induced in either CD8 KO or wild type after EAP induction. CD8 KO or C57BL/6 mice were immunized with PA emulsified in CFA (PA-CD8 KO and PA-WT groups) or saline-CFA (control animals, C-WT), and mice were euthanized at day 24 after immunization. The prostates were excised and processed for flow cytometry analysis. Live cells were counted and flow cytometry was performed to evaluate leukocyte infiltration in the prostate glands from animals under study. (A) Gating strategy and representative flow cytometry density plots of the analysis of different leukocyte subpopulations: total leukocytes were gated on plots showing CD45⁺ cells versus forward scatter, and the different leukocyte cell subsets were analyzed in plots showing CD4⁺ versus CD3⁺ cells, Gr1⁺ versus CD11c⁺ cells, and CD19⁺ versus NK1.1⁺ cells. Bar graph depicts relative (B) or absolute numbers (C) of CD45⁺, CD19⁺, CD4⁺CD3⁺, CD8⁺CD3⁺, NK1.1⁺, CD11c⁺, and Gr1⁺ cells in the prostate gland from mice under study. Numbers indicate the percentage of cells in each quadrant. Data are shown as mean $\pm$ SEM, $n=4-5$ per group, and are representative of three independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni *post hoc* test analysis. * $p < 0.05$, and ** $p < 0.01$; ns, non-significant; ND, not detected.

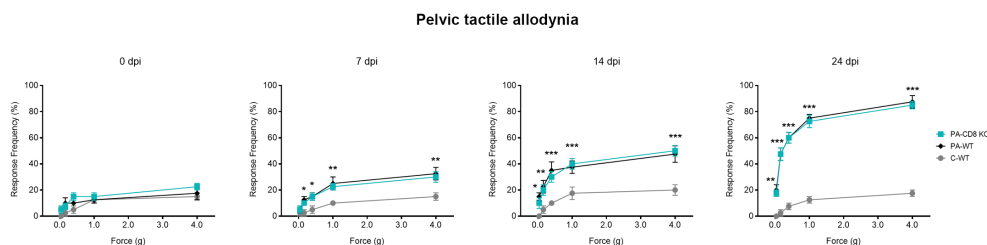


FIGURE 4

Chronic pelvic pain is developed by animals able to mount prostate-specific Th1 immune responses, even in the absence of CD8 T cells. CD8 KO or C57BL/6 mice were immunized with PA emulsified in CFA (PA-CD8 KO and PA-WT groups) or CFA alone (control animals, C-WT), and referred visceral hyperalgesia was measured over time of EAP induction as responses to mechanical stimulation of the pelvic region and hind paw using von Frey filaments of 5 calibrated forces. Data are shown as the mean percentage of response frequency $\pm$ SEM (e.g., 5 responses of 10 = 50%) before (baseline, Day 0) or at 7, 14, and 24 days after PA immunization. Tactile allodynia responses to pelvic stimulation of every experimental group under study over different time points of EAP induction. Shown data ($n=4-5$ per group) are representative of three independent experiments. Statistical analysis was performed using two-way ANOVA with Šidák's multiple comparisons *post hoc* test analysis. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

were analyzed (74). Therefore, the role of CD8 T cells in the pathogenesis of CPPS/CP remains uncertain and warrants further investigation.

An increasing body of evidence indicates that CD8 T cells play pivotal roles in the initiation, progression, pathogenesis, and protection for autoimmune diseases (75, 76). In that regard, it is noteworthy to highlight that CD8 T cells can exert not only direct cytotoxic effects but also a myriad of actions, including indirect cytotoxicity, as well as direct and indirect non-cytotoxic functions via crosstalk with other immune and non-immune cells (35). These actions include the recruitment of other immune cells to inflammation sites, the activation of NK cells and dendritic cells to ensure antigen presentation and the induction of effector innate and adaptive immunity (mainly in an IFN γ dependent manner), the help to B cells to support antibody production, immunoregulation, and the induction of tissue healing and regeneration (35, 76, 77). Interestingly, memory CD8 T cells were shown to protect dendritic cells from the killing by effector cytotoxic T cells via the upregulation of an endogenous inhibitor of granzymes, thus enhancing antigen presentation and thereby augmenting the consequent induction of Th1 immune responses (78). Remarkably, effector memory CD8 T cells express T-bet and secrete Th1-associated cytokines such as IFN γ , and chemokines including CCL3, CCL4 and CCL5, and express the chemokine receptor CXCR3 (75, 79). Given the significant implications of these mediators in CPPS/CP and chronic pelvic pain, and the documented elevation of CD8 T cells in semen from CPPS/CP patients and in the prostates of animals with EAP (24, 29, 31, 41, 42), different possibilities arise. On the one hand, CD8 T cells may play a central role in EAP pathogenesis and chronic pelvic pain development. Alternatively, rather than pathogenic, their recruitment to and infiltration of the prostate (and consequently their increase in semen) could represent an amplification of a Th1-driven ongoing inflammatory process in the prostate, where elevated levels of cytokines and chemokines that bind to their receptors (e.g. CXCR3) are secreted. Finally, it is also possible that both scenarios are occurring simultaneously. To assess these possibilities, in our study we analyzed the induction of EAP and the development of chronic pelvic pain in CD8 KO and wild type mice. As already reported (26, 27, 29), our findings revealed that, following PA immunization, wild-type mice elicited prostate-specific Th1 and Th17 immune responses, leading to chronic inflammation of the prostate, characterized by hemorrhage, epithelial cell desquamation, marked periglandular leukocyte infiltration, and increased collagen deposition. Furthermore, these animals exhibited markedly heightened tactile allodynia responses as the disease advanced, indicating that the development of chronic pelvic pain was a result of inflammation of the prostate (26, 27, 29). Interestingly, comparable results were obtained when analyzing immunized CD8 KO mice. In fact, similarly elevated PA-specific Th1 and Th17 immune responses were detected in PA-immunized CD8 KO animals. Moreover, even in the absence of CD8 T cells, immunized animals developed significant chronic inflammation of the prostate and pelvic pain. Therefore, these results indicate that CD8 T cells do not play a major role in EAP pathogenesis and chronic pelvic pain development. Our findings are in line with the

limited reported data regarding the involvement of CD8 T cells in prostate inflammation and the development of chronic pelvic pain. Our data support previously reported findings indicating that NOD mice lacking expression of beta 2-microglobulin are as susceptible to EAP as their wild type counterparts, indirectly indicating that CD8 T cells are dispensable for EAP pathogenesis (28). Moreover, our results corroborate previous data showing that, after PA immunization, a CD4 Th1 associated immune response develops and drives the induction of prostate tissue inflammation and chronic pelvic pain (10, 12, 29, 70). Collectively, these findings support the notion that CD8 T cells are not pivotal for EAP and chronic pelvic pain development. Instead of being inherently pathogenic, the observed increase in CD8 T cells in the semen of CPPS/CP patients and in the prostates of animals with EAP might reflect an amplification of the established Th1-driven inflammatory process in the prostate during EAP. Given that effector memory CD8 T cells express the chemokine receptor CXCR3 (75, 79), and their cognate ligands are elevated in the prostate of mice with EAP and in semen from patients with CPPS/CP (24, 29, 31, 41, 42), CD8 T cells would be recruited as a consequence of the ongoing inflammatory process in the prostate rather than being pathogenic. Remarkably, there are no reported studies assessing the direct role of CD8 T cells in the pathogenesis of EAP and the development of chronic pelvic pain. Thus, we consider the present work provides novel evidence that add to the current knowledge in the field. However, future research employing other complementary experimental approaches beyond the use of CD8 α KO and wild-type C57BL/6 mice, and assessing their putative link with cells involved in pelvic pain development such as mast cells, may be necessary to confirm our findings.

In summary, we provided compelling data showing that CD8 T cells do not play a major role in EAP pathogenesis and chronic pelvic pain development. Moreover, our results corroborate the previous notion that, after PA immunization, a CD4 Th1 associated immune response develops and drives the induction of prostate tissue inflammation and chronic pelvic pain. These findings constitute a significant contribution to the field and improve the current knowledge about the immune mechanisms underlying EAP, a reliable experimental model for the study of CPPS/CP, a prevalent clinical condition in which the precise aetiology and pathogenesis remain to be unveiled.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Institutional Laboratory Animal Care and Use Committee (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio - CICUAL) at Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba,

Argentina. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FS: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. MM: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Writing – review & editing. DP: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. YC: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. CO: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. GG: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. EA-R: Funding acquisition, Resources, Writing – review & editing. VR: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. RM: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Genetically predicted N-methylhydroxyproline levels mediate the association between naive CD8+ T cells and allergic rhinitis: a mediation Mendelian randomization study

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Background: Allergic rhinitis (AR), a prevalent chronic inflammatory condition triggered by immunoglobulin E (IgE), involves pivotal roles of immune and metabolic factors in its onset and progression. However, the intricacies and uncertainties in clinical research render current investigations into their interplay somewhat inadequate.

Objective: To elucidate the causal relationships between immune cells, metabolites, and AR, we conducted a mediation Mendelian randomization (MR) analysis.

Methods: Leveraging comprehensive publicly accessible summary-level data from genome-wide association studies (GWAS), this study employed the two-sample MR research method to investigate causal relationships among 731 immune cell phenotypes, 1400 metabolite levels, and AR. Additionally, employing the mediation MR approach, the study analyzed potential mediated effect of metabolites in the relationships between immune cells and AR. Various sensitivity analysis methods were systematically employed to ensure the robustness of the results.

Results: Following false discovery rate (FDR) correction, we identified three immune cell phenotypes as protective factors for AR: Naive CD8br %CD8br (odds ratio (OR): 0.978, 95% CI = 0.966–0.990, $P = 4.5 \times 10^{-4}$), CD3 on CD39+ activated Treg (OR: 0.947, 95% CI = 0.923–0.972, $P = 3 \times 10^{-5}$), HVEM on CD45RA-CD4+ (OR: 0.967, 95% CI = 0.948–0.986, $P = 4 \times 10^{-5}$). Additionally, three metabolite levels were identified as risk factors for AR: N-methylhydroxyproline levels (OR: 1.219, 95% CI = 1.104–1.346, $P = 9 \times 10^{-5}$), N-acetylneuraminate levels (OR: 1.133, 95% CI = 1.061–1.211, $P = 1.7 \times 10^{-4}$), 1-stearoyl-2-arachidonoyl-gpc (18:0/20:4) levels (OR: 1.058, 95% CI = 1.029–1.087, $P = 5 \times 10^{-5}$). Mediation MR analysis indicated a causal relationship between Naive CD8br %CD8br and N-methylhydroxyproline levels, acting as a protective factor (OR: 0.971, 95% CI = 0.950–0.992, $P = 8.31 \times 10^{-3}$). The mediated effect was -0.00574, accounting for

26.1% of the total effect, with a direct effect of -0.01626 . Naive CD8+ T cells exert a protective effect on AR by reducing N-methylhydroxyproline levels.

Conclusion: Our study, delving into genetic information, has substantiated the intricate connection between immune cell phenotypes and metabolite levels with AR. This reveals a potential pathway to prevent the onset of AR, providing guiding directions for future clinical investigations.

KEYWORDS

allergic rhinitis, immunity, metabolites, causal inference, Mendelian randomization, mediation

1 Introduction

Allergic rhinitis (AR), a prevalent chronic inflammatory condition triggered by immunoglobulin E (IgE) in response to inhaled allergens (1), manifests widely across age groups, with estimated prevalence rates in European populations ranging from 17% to 28.5% (2, 3). Moreover, its incidence has steadily increased in recent years (4–6), imposing substantial health and psychological burdens due to its seasonal and recurrent nature.

Long-term exposure to allergens may compromise the airway epithelial cells' barrier function, inducing inflammatory responses in the airways and triggering the onset of AR (7). Elevated levels of group 2 innate lymphoid cells (ILC2s) have been observed in AR patients (8), while myeloid dendritic cells (mDCs) are capable of secreting IL-33 to activate ILC2s via the IL-33/ST2 pathway (9). Additionally, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) can individually or collectively stimulate ILC2s to produce IL-4, IL-5, and IL-13, initiating immune responses that may manifest as inflammation and allergic symptoms in nasal mucosal tissues, such as congestion and rhinorrhea (10). Moreover, memory-type pathogenic Th2 cells, detected in the peripheral blood of symptomatic AR patients, exhibit heightened IL-5 and IL-9 levels compared to conventional Th2 cells (11), potentially correlating with clinical symptoms (12). Furthermore, a circulating memory B cell subset expressing high levels of CD23 correlates with allergen-specific IgE levels and symptom severity in AR patients (13). Dendritic cells, as primary antigen-presenting cells, uptake allergens and present them to CD4+ T cells, provoking allergen-induced inflammation (14, 15). Thus, allergic rhinitis, characterized as a chronic hypersensitivity disorder, involves diverse immune cells and cytokine-mediated responses in its progression (16). Elucidating these possible pathways is crucial for advancing AR treatment.

With the continuous advancement of high-throughput sequencing technology, there has been a more profound exploration of immune cell functions. Immune cells undergo intricate genetic regulatory processes, and a deeper understanding of these mechanisms holds the potential to unveil the mysteries of

the immune system. Identifying and targeting specific points can enhance drug design and development, offering novel perspectives for the treatment and prevention of AR (17).

Allergen Immunotherapy (AIT) induces allergen tolerance by disrupting immune pathogenic mechanisms of allergic reactions and is currently considered an effective treatment for AR (18). Retrospective cohort studies have demonstrated that AIT can reduce the incidence of asthma attacks and pneumonia in AR patients (19). Over the years, various treatment forms have been developed for different patients, including Subcutaneous Immunotherapy (SCIT), Sublingual Immunotherapy (SLIT), and Intralymphatic Immunotherapy (ILIT) (20). However, owing to the complexity and uncertainty of clinical studies, our understanding of the interaction between the immune system and AR remains somewhat limited. Convincing evidence regarding the regulatory effects of AIT on immune cells in the human body is lacking due to variations in race, region, and individual factors. This may be attributed to insufficient sample sizes in real-world studies, divergent study designs, and challenges in eliminating confounding factors. Given that AIT is an individualized treatment approach based on the clinical and immunological characteristics of patients, identifying and validating biomarkers as treatment targets for AR has become a crucial direction in current AIT research (21).

With the disclosure of phenotype-related genetic information, a novel scientific research method has emerged—Mendelian randomization (MR). In recent years, MR has gained popularity as a research approach that employs genetic information loci strongly correlated with exposure factors as instrumental variables (IVs) to infer causal relationships between exposure factors and study outcomes. The method is grounded in Mendel's laws of inheritance, where alleles are randomly allocated to offspring during meiosis, largely mitigating the influence of confounding factors and reverse causation (22). Immune cells play a pivotal role in the onset and progression of AR. However, current research has only addressed a fraction of them. Due to the intricate biological mechanisms of the immune system, the precise roles of many immune cells remain incompletely understood. Hence, we

conducted a comprehensive causal association analysis between immune cell phenotypes and AR through MR, leveraging extensive genome-wide association studies (GWAS) summary-level data of immune cell phenotypes. The goal is to reveal the intricate relationship between immune cell phenotypes and AR.

Employing a two-sample MR approach, we conducted a study investigating the causal relationships involving 731 immune cell phenotypes and AR. Additionally, recognizing the involvement of metabolite levels in the onset and progression of AR, we further evaluated the causal associations between 1400 blood metabolite levels and AR. As part of the MR research, mediation MR can assess the effects of intermediate factors between exposure and outcome, offering insights into whether exposure factors exert their effects through these intermediaries (23). Consequently, we also scrutinized the mediated effect of blood metabolite levels in the relationship between immune cell phenotypes and AR. It is imperative to emphasize that for immune cell phenotypes with a causal relationship to AR, there should be no reverse causation. Accordingly, for positive results, we conducted an MR analysis of AR and immune cell phenotypes to eliminate reverse causation.

2 Materials and methods

2.1 MR assumptions

It is essential to clarify that MR relies on three fundamental assumptions: (1) The assumption of association, suggesting a robust correlation between the selected genetic variations serving as IVs and the exposure factors. (2) The assumption of independence, stating that genetic variations are unrelated to confounding factors. (3) The assumption of exclusivity, proposing that genetic variations can solely influence the outcome through exposure (24). The study adheres to the STROBE-MR statement (25). Furthermore, the GWAS summary-level data used in this research are publicly accessible, and the ethics committees of each institutional review board granted written informed consent from all participants in individual studies. Since this study involves a secondary analysis of previously published data, ethical approval is considered not required. Significantly, there was no observed overlap, as samples of AR, immune cells, and metabolites originated from distinct consortia.

2.2 GWAS data sources for AR

Summary-level data regarding AR were extracted from a comprehensive European population-wide GWAS conducted within the FinnGen project in Finland (26). We specifically selected the most recent R10 version summary data on AR, which encompasses 12,240 cases and 392,069 controls. The diagnosis of AR relied on the International Classification of Diseases codes, specifically ICD-10 (J30.1-J30.4) and ICD-9 (477). Additional details can be accessed at (https://www.finnngen.fi/en/access_results).

2.3 Immune cells GWAS data sources

A genetic variation analysis was conducted on 731 immune cell phenotypes within a cohort of 3,757 Sardinian individuals. This analysis covered 118 absolute cell counts (AC), 389 median fluorescence intensities (MFI) representing surface antigen levels, 32 morphological features (MP), and 192 relative cell counts (RC) (17). The GWAS data for these phenotypes can be accessed on the IEU Open GWAS project (<https://gwas.mrcieu.ac.uk>) through identifiers (ebi-a-GCST0001391 to ebi-a-GCST90002121).

2.4 Metabolites GWAS data sources

In another investigation with 8,299 participants from the Canadian Longitudinal Study on Aging (CLSA) cohort, researchers performed a GWAS on 1,091 blood metabolites and 309 metabolite ratios (27). This study yields vital insights into elucidating the genetic architecture of metabolites. The summary data for 1,400 metabolite levels are available in the GWAS Catalog (<https://www.ebi.ac.uk/gwas>) under identifiers (GCST90199621 to GCST90201020).

2.5 Selection of IVs

In MR analysis, we employed single nucleotide polymorphisms (SNPs) strongly correlated with the exposure as IVs. Recent studies revealed a limited number of SNPs strongly associated with immune cell phenotypes and blood metabolite levels using a genome-wide significance threshold of $P < 5 \times 10^{-8}$. Therefore, drawing on prior research, we adopted a less stringent threshold ($P < 1 \times 10^{-5}$) for SNPs selection (28, 29). Linkage disequilibrium was eliminated within a genetic distance of kb = 10,000 (correlation coefficient $r^2 < 0.001$) to ensure SNP independence. In reverse MR analysis, SNPs in AR summary data were chosen with a threshold of $P < 5 \times 10^{-8}$, and the same method (kb = 10,000, $r^2 < 0.001$) was applied to eliminate linkage disequilibrium (30). Palindrome SNPs, having identical alleles on the forward and reverse strands, were excluded due to the ambiguity in determining the effect allele strand. Finally, for each SNP meeting the specified criteria, R^2 and F-statistic were calculated, excluding SNPs with F-statistic < 10 as weak IVs, indicating a weak correlation between genetic variation and exposure (31).

2.6 Statistical analysis

Data analyses were conducted using MRPRESSO (version 1.0) and TwoSampleMR (version 0.5.6) packages in R software (version 4.3.1). Employing five MR analysis methods, with the Inverse Variance Weighted (IVW) method as the primary one, we assessed the causal relationship between exposure and outcome. To ensure robustness, we conducted sensitivity analyses using methods with different assumptions about horizontal pleiotropy, such as MR-Egger and Mendelian Randomization Pleiotropy

Residual Sum and Outlier (MR-PRESSO). MR-Egger analysis evaluated instrumental variable pleiotropy, with a non-zero intercept indicating bias in IVW estimates (32). MR-PRESSO identified horizontal pleiotropy through a global test and, if necessary, corrected potential pleiotropy by removing outliers (33). Additionally, we implemented the “leave-one-out” method to exclude abnormal SNPs, preventing undue influence of individual SNPs on the causal relationship between exposure and outcome. Furthermore, heterogeneity assessment used the Cochran Q test, and a P-value below 0.05 indicated present heterogeneity. In such cases, we employed the IVW random-effects model to estimate causal effects. In the absence of heterogeneity, the IVW fixed-effects model was considered the result of the IVW method. Moreover, considering multiple MR analyses between different exposures and a single outcome, we controlled for potential false-positive results due to multiple hypothesis testing using the false discovery rate (FDR) (34). Finally, we visually represented the results through forest plots, funnel plots, scatter plots, and “leave-one-out” plots.

3 Results

3.1 Exploration of the causal effect of immune cell phenotypes on AR

For the three GWAS summary-level datasets mentioned above, we selected IVs based on established significance threshold levels and included them in this study. Their F-statistics were all above 10, indicating that weak instrument bias is unlikely to be significant (Supplementary Tables 1–3, Sheet 1). Employing the IVW method as the primary MR analysis, we conducted a two-sample MR analysis investigating the relationship between immune cell phenotypes and AR, accompanied by tests for heterogeneity and pleiotropy. Post multiple testing correction using the FDR method, we identified two immune cell phenotypes demonstrating a causal relationship with AR at a significance level of 0.05, both exerting protective effects: CD3 on CD39+ activated Treg (Treg panel) and HVEM on CD45RA- CD4+ (Maturation stages of T cell). The odds ratio (OR) of CD3 on CD39+

activated Treg against AR, calculated by the IVW method, was 0.947 (95%CI = 0.923–0.972, $P = 3 \times 10^{-5}$, $P_{FDR} = 0.02278$), and the OR of HVEM on CD45RA- CD4+ against AR, calculated by IVW, was 0.967 (95%CI = 0.948–0.986, $P = 4 \times 10^{-5}$, $P_{FDR} = 0.01463$). Additionally, following insights from previous research, a $P_{FDR} < 0.2$ was considered suggestive correlation (35). At a significance level of 0.2, we identified a third immune cell phenotype with a causal relationship with AR, also displaying a protective effect: Naive CD8br %CD8br (Maturation stages of T cell panel). The OR of Naive CD8br %CD8br against AR, calculated by IVW, was 0.978 (95%CI = 0.966–0.990, $P = 4.5 \times 10^{-4}$, $P_{FDR} = 0.10862$). Results from the other four MR analysis methods for these three immune cell phenotypes and AR mirrored those of the IVW method, with OR values less than 1. MR-Egger and MR-PRESSO indicated no horizontal pleiotropy (P-values > 0.05, Supplementary Table 1, Sheet 1), confirming the reliability of the analysis results. Forest plots representing three immune cell phenotypes as exposure and AR as an outcome are illustrated in Figure 1. Scatter plots in Figures 2A–C, and Supplementary Figures 1–3 A, B, C offer comprehensive insights into funnel plots, ‘leave-one-out’ plots, and individual forest plots. Additionally, the results of five MR analyses for the above three immune cell phenotypes and AR are provided in the Supplementary Table 1, Sheet 2.

3.2 Exploration of the causal effect of AR on immune cell phenotypes

Additionally, to meet the requirements of mediation MR studies, we further treated AR as the exposure and the three aforementioned immune cell phenotypes as outcomes, conducting a reverse MR analysis with IVW as the primary method. We did not observe any reverse causal relationships between the three immune cell phenotypes and AR (P-values all greater than 0.05). Details of the heterogeneity and pleiotropy tests are provided in the Supplementary Table 2, Sheet 1, the results of five MR analyses for AR and three immune cell phenotypes are provided in the Supplementary Table 2, Sheet 2, and the forest plots are illustrated in Figure 3.

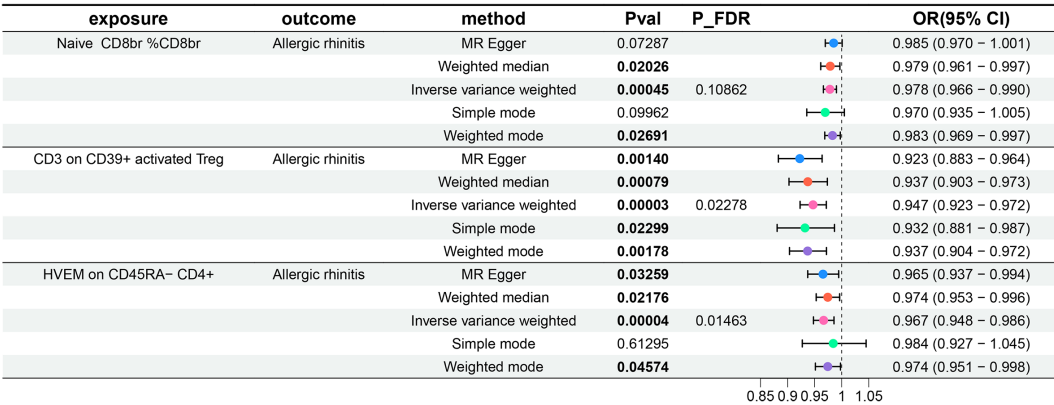
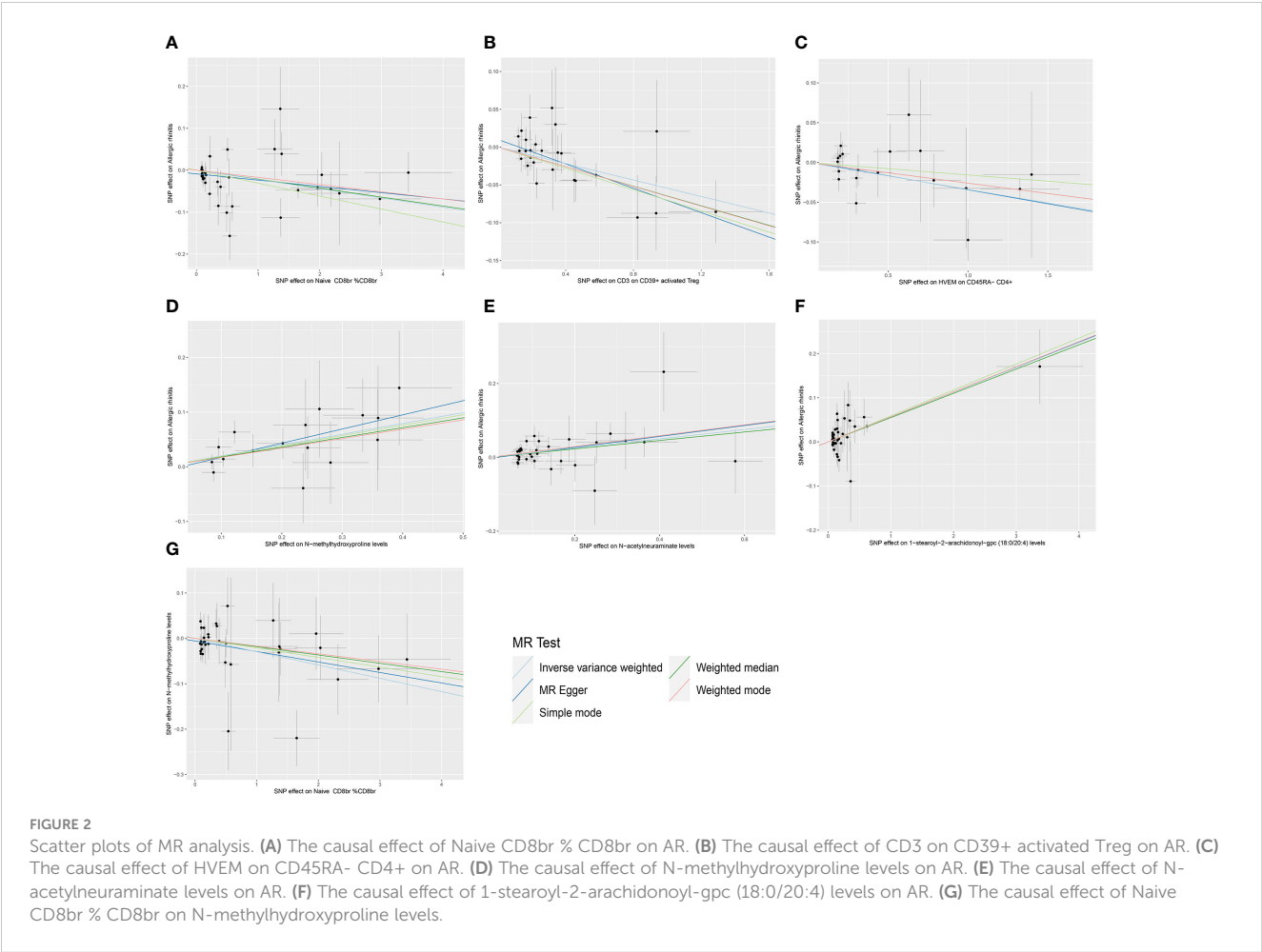


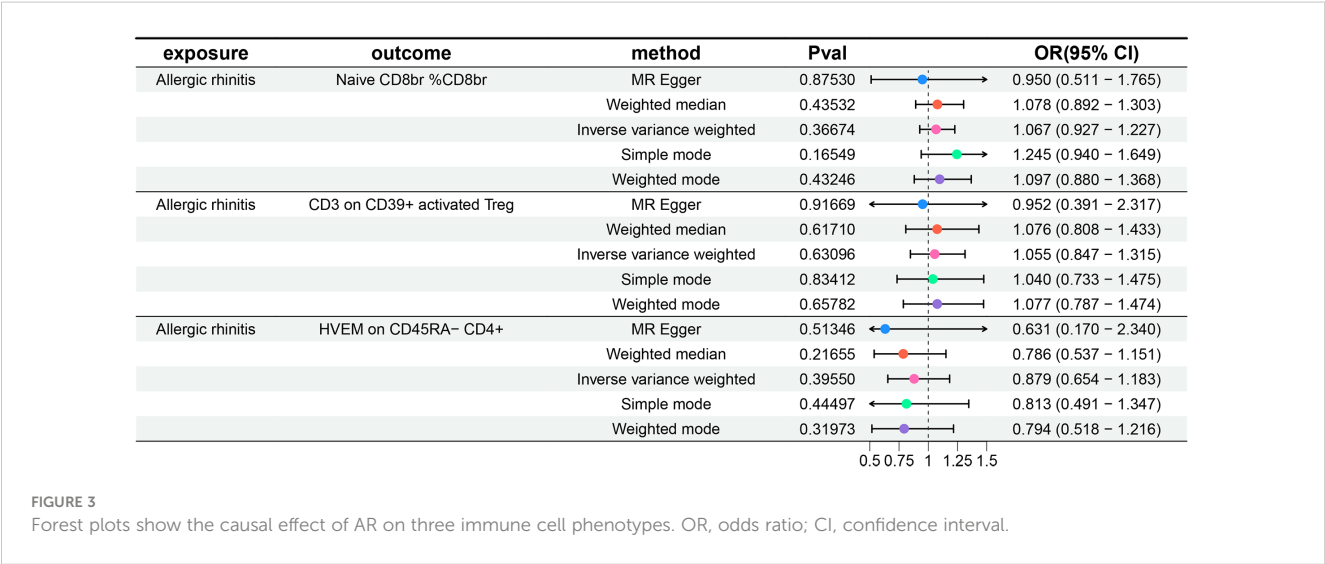
FIGURE 1 Forest plots show the causal effect of three immune cell phenotypes on AR. OR, odds ratio; CI, confidence interval.



3.3 Exploration of the causal effect of metabolite levels on AR

Subsequently, a two-sample MR analysis was conducted with metabolite levels as the exposure and AR as the outcome, utilizing IVW as the primary analytical method. Despite no significant

associations at the 0.05 level after FDR correction for multiple testing, we identified three metabolite levels with causal relationships with AR at the 0.2 level, all acting as risk factors: N-methylhydroxyproline levels, N-acetylneuraminate levels, and 1-stearoyl-2-arachidonoyl-gpc (18:0/20:4) levels. The OR of N-methylhydroxyproline levels against AR, calculated by the IVW



method, was 1.219 (95% CI = 1.104–1.346, $P = 9 \times 10^{-5}$, $P_{FDR} = 0.06427$). The OR of N-acetylneuraminate levels against AR, calculated by the IVW method, was 1.133 (95% CI = 1.061–1.211, $P = 1.7 \times 10^{-4}$, $P_{FDR} = 0.08076$). The OR of 1-stearoyl-2-arachidonoyl-gpc (18:0/20:4) levels against AR, calculated by the IVW method, was 1.058 (95% CI = 1.029–1.087, $P = 5 \times 10^{-5}$, $P_{FDR} = 0.07172$). Results from the other four methods in the MR analysis between these three metabolite levels and AR were consistent with the IVW method, with OR values all greater than 1. MR-Egger and MR-PRESSO indicated no horizontal pleiotropy (P -values all greater than 0.05, [Supplementary Table 3, Sheet 1](#)), confirming the reliability of the analysis results. Forest plots for these three metabolite levels as exposure and AR as the outcome are presented in [Figure 4](#), scatter plots in [Figures 2D–F](#), and [Supplementary Figures 1–3D–F](#) offer comprehensive insights into funnel plots, ‘leave-one-out’ plots, and individual forest plots. Furthermore, the detailed results for the five MR analyses between above three metabolite levels and AR can be found in the [Supplementary Table 3, Sheet 2](#).

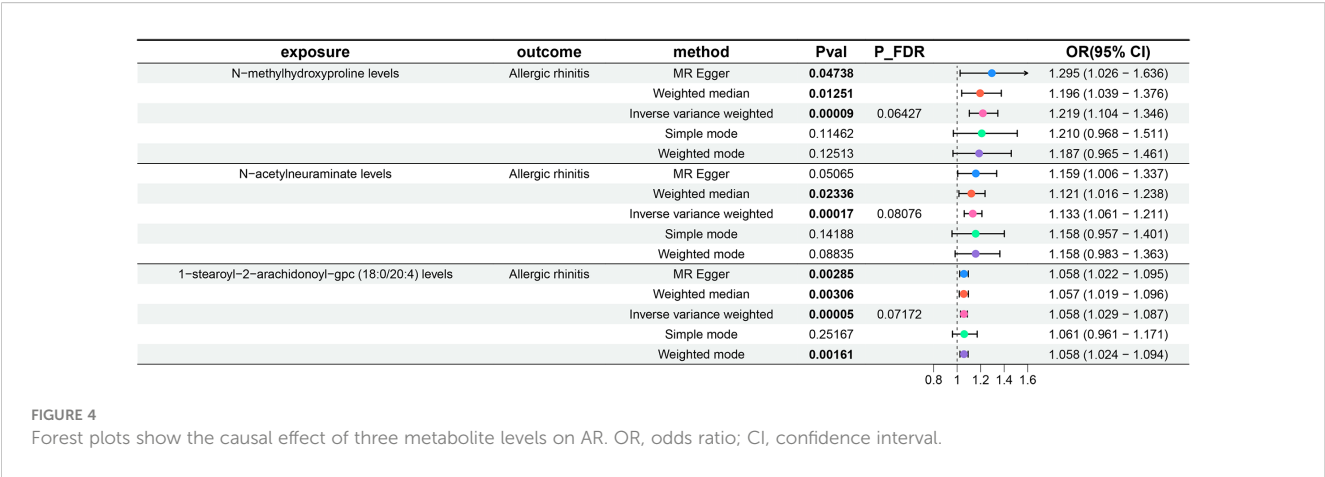
3.4 Exploration of the causal effect of immune cell phenotypes on metabolite levels

Following the identification of immune cell phenotypes and metabolite levels causally associated with AR, we conducted two-sample MR analyses between them. Since no reverse causal relationships were found in the second step of the MR analysis, the three immune cell phenotypes from the initial step met the criteria for mediation MR analysis. Due to the relatively small number of tests (only three tests between multiple exposures and a single outcome), we refrained from performing FDR correction in this segment of the study, considering a P -value less than 0.05 as statistically significant. Utilizing IVW as the primary analytical method, we established a causal relationship between the Naive CD8br %CD8br phenotype and N-methylhydroxyproline levels, indicating a protective effect. The OR of Naive CD8br %CD8br

method, was 0.971 (95% CI = 0.950–0.992, $P = 8.31 \times 10^{-3}$). OR values from all five MR analysis methods were consistently greater than 1, and both MR-Egger and MR-PRESSO indicated no horizontal pleiotropy (P -values all greater than 0.05, [Supplementary Table 4, Sheet 1](#)), affirming the robustness of the results. For the remaining eight MR analyses, given that the IVW method is the primary MR analysis method, we refrain from considering causal relationships between them, as their IVW method P -values were all greater than 0.05. The forest plots for these three immune cell phenotypes as exposure and three metabolite levels as the outcome are displayed in [Figure 5](#), while [Figure 2G](#) illustrates the scatter plot for Naive CD8br %CD8br and N-methylhydroxyproline levels. [Supplementary Figures 1–3G](#) offer comprehensive insights into funnel plots, ‘leave-one-out’ plots, and individual forest plots. Moreover, the detailed results for the five MR analyses between three metabolite levels and three metabolite levels can be found in the [Supplementary Table 4, Sheet 2](#).

3.5 Genetically predicted N-methylhydroxyproline levels mediate the association between Naive CD8+ T cells and AR

Finally, the findings of our mediation MR analysis were summarized, and an intermediary factor was identified. [Figure 6](#) presents the summarized forest plot, while [Figure 7](#) depicts the schematic diagram of the mediation MR analysis. Notably, Naive CD8br %CD8br demonstrated a protective role in relation to N-methylhydroxyproline levels ($\beta = -0.029$, $P = 8.31 \times 10^{-3}$), with N-methylhydroxyproline levels identified as a risk factor for AR ($\beta = 0.198$, $P = 9 \times 10^{-5}$). Furthermore, Naive CD8+ %CD8+ emerged as a protective factor against AR ($\beta = -0.022$, $P = 4.5 \times 10^{-4}$). The mediated effect was -0.00574, accounting for 26.1% of the total effect, while the direct effect was -0.01626. Consequently, Naive CD8+ T cells contribute to the protection against AR by mitigating N-methylhydroxyproline levels.



against N-methylhydroxyproline levels, calculated by the IVW

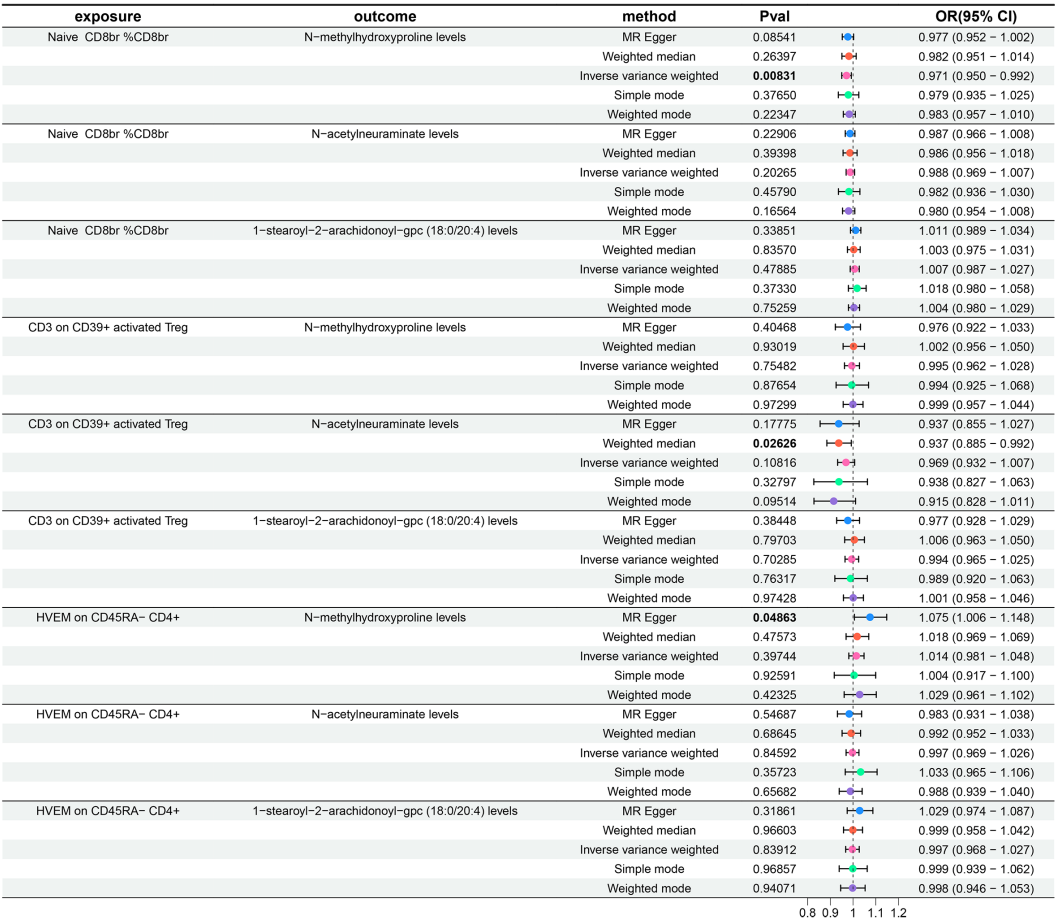


FIGURE 5 Forest plots show the causal effect of three immune cell phenotypes on three metabolite levels. OR, odds ratio; CI, confidence interval.

4 Discussion

Utilizing publicly available GWAS summary-level data, we systematically explored the causal relationships between 731 immune cell phenotypes and AR. Furthermore, acknowledging

the potential mediating influence of metabolites, we expanded our investigation to encompass the causal associations between 1400 metabolite levels and AR.

Previous studies have conducted MR analyses to investigate the causal relationship between eight immune-related diseases and AR.

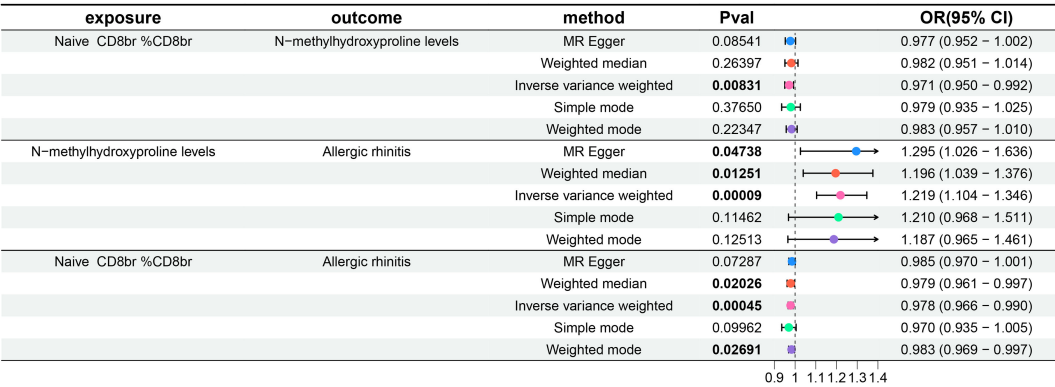


FIGURE 6 Forest plot of mediation MR analysis. OR, odds ratio; CI, confidence interval.

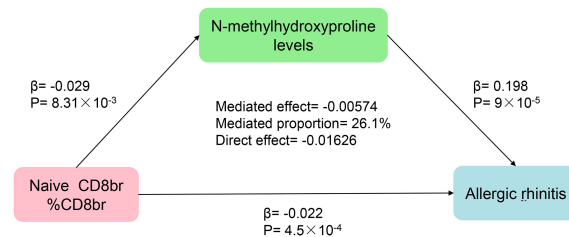


FIGURE 7

Schematic representation of the results from the mediation MR analysis.

It indicates that atopic dermatitis (AD), asthma, and Crohn's disease (CD) elevate the risk of AR, whereas Graves' disease (GD) and systemic lupus erythematosus (SLE) potentially mitigate this risk (36). Another MR investigation scrutinized 486 circulating metabolites and 55 targeted urinary metabolites concerning their association with atopic dermatitis, allergic rhinitis, and asthma. Meta-analysis unveiled several circulating and urinary metabolites potentially linked to the onset and advancement of allergic diseases (37). Undoubtedly, the findings of these studies carry substantial implications for formulating preventive and therapeutic interventions for allergic rhinitis. Building upon prior research, this study employed GWAS summary-level data comprising 731 immune cell phenotypes and 1400 metabolite levels. Initially, it conducted separate MR analyses to identify immune cell phenotypes and metabolite levels potentially causally linked to allergic rhinitis. Subsequently, via mediation MR analysis, it investigated and uncovered a potential pathway, thus holding significant implications for guiding clinical practice.

To our knowledge, this study is the pioneering use of mediation MR analysis to investigate the causal links among immune phenotypes, metabolites, and AR. Following multiple testing correction of P-values using the FDR method, our findings indicate a total of three immune cell phenotypes acting as protective factors for AR, alongside three metabolite levels identified as risk factors for the condition. Notably, Naive CD8+ T Cells were discerned to exert a protective effect on AR by mitigating the levels of N-methylhydroxyproline. The STROBE-MR checklist pertaining to this study is available in [Supplementary Table 5](#).

The human immune system comprises innate and adaptive components. Unlike the nonspecific actions of the innate immune system, the adaptive immune system involves T lymphocytes and B lymphocytes, acts with specificity, and induces immune memory, enabling rapid responses in subsequent encounters. T lymphocytes, vital in cellular immune responses, consist of two main subsets: CD4-positive (CD4+) T cells, known as helper T cells (Th), and CD8-positive (CD8+) T cells, known as cytotoxic T cells (CTL) (38). These cells play crucial roles in pathogen defense. Naive CD8+ T cells, stimulated by antigens, differentiate into effector and memory T cells, participating in respective immune processes (39). Furthermore, the existence of Naive CD8+ T cells empowers the organism to fend off novel, undetected infections and ailments (40). Our investigation identified a heightened ratio of Naive CD8+ T cells to the overall CD8+ T cell count, potentially diminishing the

susceptibility to AR. Naive CD8+ T cells constitute the reservoir of CD8+ T cells, having recently matured in the thymus without encountering antigens yet, and can promptly react, producing effector and memory T cells for immunological assaults upon exposure to the relevant allergen. Certain research has indicated that, under chronic stimulation or specific pathological conditions, such as chronic infections or cancer, CD8+ T cells may experience immune exhaustion, characterized by a gradual loss of function and activity in response to sustained stimuli (41). Regulatory CD8+ T cells have inhibitory effects during AR occurrences, impacting inflammatory responses (42–44). Though not primary participants in allergic reactions, CD8+ T cells may exhibit exhaustion in response to allergens. Thus, the reservoir of Naive CD8+ T cells might have a significant regulatory function in the immune response of AR patients. However, this represents a speculative hypothesis concerning the potential causes of chronic recurrent episodes in AR patients, and the precise mechanisms necessitate additional validation through clinical research.

The CD3 on CD39+ activated Treg refers to a distinct subset within CD4 regulatory T cells expressing both CD3 and CD39 on its surface. Our investigation demonstrates that an elevation in the CD3 on CD39+ activated Treg correlates with a decreased risk of AR. Treg, categorized within the CD4+ T cell subset, play a pivotal role in the immune response to AR. Study suggests that diverse Toll-like receptors play crucial immunomodulatory roles in the pathogenesis of AR (45, 46). Notably, TICAM-1, functioning as a vital adaptor protein in Toll-like receptor signaling domains, participates in the signal transduction of AR. Treg can exert significant immunomodulatory effects on AR through the TICAM-1 pathway (47). The findings of this study highlight that a specific cell subset within Treg may confer a protective effect against AR, holding substantial implications for guiding AIT strategies.

HVEM on CD45RA- CD4+ T cells denote a distinct subset of CD4+ T cells expressing HVEM on the cell surface while lacking CD45RA, belonging to the mature stage of T lymphocytes. Herpes virus entry mediator (HVEM) belongs to the tumor necrosis factor receptor superfamily (TNFRSF) and plays a pivotal role in immune response regulation, contributing to the preservation of mucosal immune homeostasis (48, 49). Several studies have underscored HVEM's role in maintaining T cell homeostasis within the intestinal epithelium and safeguarding against invasion by enteropathogenic bacteria (50, 51). However, current clinical research on the involvement of HVEM in AR remains limited. CD45RA, a

member of the CD45 antigen family, is a glycoprotein located on the surface of lymphocytes (52). Under specific antigenic stimulation, the expression of CD45RA on the surface of T cells is constrained, commonly regarded as an indicator of naive T cells (53). Consequently, HVEM on CD45RA⁺ CD4⁺ T cells signifies mature CD4⁺ T cells expressing HVEM. Our study discloses that a subset of mature CD4⁺ T cells may serve as a protective factor in AR. This finding is anticipated to offer valuable guidance for future AIT.

N-methylhydroxyproline is a derivative of proline. Extensive research has demonstrated that proline metabolism plays a crucial role in diverse biological processes, including cell signal transduction, stress response, and energy generation. Additionally, it may contribute to the pathogenic mechanisms of microbial invaders. Consequently, inhibiting the proline metabolism process emerges as a potential therapeutic strategy against the intrusion of specific pathogens into the body (54). The involvement of proline metabolism in the immune response triggered by allergens remains unclear. This investigation brings to light that N-methylhydroxyproline may pose a risk factor for AR, suggesting a promising avenue for future exploration in AR research.

N-acetylneuraminate, also known as sialic acid, represents a neuraminic acid embellished with an acetyl group, typically positioned at the terminal end of polysaccharide chains (55). Current research underscores that diverse pathogenic bacteria can exploit sialic acid for camouflage, eluding detection by the host's innate immune system, thus gaining entry to enact their pathogenic pathways. Moreover, sialic acid can function as a nutritional source for select pathogenic bacteria (56, 57). This study posits that N-acetylneuraminate may constitute a potential risk factor for AR. An elevation in N-acetylneuraminate metabolite levels could, to some extent, create a conducive environment for the infiltration of pathogenic bacteria. When certain pathogenic microorganisms act as allergens, they might instigate an immune response in the body, culminating in the onset of AR. Presently, there exists a research gap concerning the correlation between N-acetylneuraminate and AR, necessitating further clinical investigations to validate these findings.

1-stearoyl-2-arachidonoyl-gpc is a lipid molecule derived from the amalgamation of stearic acid and arachidonic acid, constituting a glycerophospholipid. This investigation postulates that 1-stearoyl-2-arachidonoyl-GPC may play a role in the onset of AR. Regrettably, there is presently a dearth of clinical research concerning this metabolite, necessitating subsequent exploration in the future.

Through mediation MR analysis, we have discerned that N-methylhydroxyproline levels mediate the correlation between Naive CD8⁺ T cells and AR. The protective impact of Naive CD8⁺ T cells on AR manifests through a reduction in the N-methylhydroxyproline levels. It is conjectured that Naive CD8⁺ T cells, following exposure to the corresponding allergen, may influence N-methylhydroxyproline through a specific pathway, thereby mitigating the risk of AR occurrence.

This study conducted MR analysis utilizing publicly available comprehensive GWAS summary-level data, incorporating a large sample size and employing diverse testing methods to mitigate

potential biases, thus demonstrating considerable statistical robustness. However, the study does have several limitations. Firstly, the absence of individual information on AR patients precludes further subgroup analysis. Secondly, the primary focus of this study is to investigate the potential mediated effect of metabolites between immune cell phenotypes and AR. Consequently, the impact of AR on immune cell phenotypes was not comprehensively analyzed. Thirdly, MR studies hinge on genetic variations for causal inference; therefore, to authenticate our research findings, additional clinical studies remain imperative. Furthermore, our study may serve as a guiding framework for identifying and validating biomarkers as therapeutic targets for AR in clinical practice, introducing new perspectives to future AIT, and fostering clinical advancements.

5 Conclusion

Our MR analysis explored the causal relationships between several immune phenotypes and metabolite levels with AR, shedding light on several factors potentially implicated in the occurrence of AR. Our study has alleviated the influence of confounding factors in clinical research, bolstering the reliability of the study results. Furthermore, employing mediation MR analysis, we unveiled a potential pathway to prevent the onset of AR, providing novel research directions for AIT.

In conclusion, this study represents a pioneering exploration into the genetic perspective of AR pathogenesis, providing fresh insights for future treatment and prevention strategies. Building upon this research, it holds promise for innovative clinical interventions for AR patients. Nonetheless, further investigations are warranted to validate these findings and extend their applicability to broader populations.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

ZC: Methodology, Project administration, Software, Writing – original draft. YS: Data curation, Visualization, Writing – original

draft. XD: Validation, Writing – original draft. XZ: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1396246/full#supplementary-material>

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Rheumatoid arthritis increases the risk of heart failure: results from the cross-sectional study in the US population and mendelian randomization analysis in the European population

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Objective: Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease. Among its various complications, heart failure (HF) has been recognized as the second leading cause of cardiovascular death in RA patients. The objective of this study was to investigate the relationship between RA and HF using epidemiological and genetic approaches

Methods: The study included 37,736 participants from the 1999–2020 National Health and Nutrition Examination Survey. Associations between RA and HF in the US population were assessed with weighted multivariate logistic regression analysis. A two-sample Mendelian randomization (MR) analysis was employed to establish the causal relationship between the two variables. The primary analysis method utilized was inverse variance weighting (IVW). Additionally, horizontal pleiotropy and heterogeneity were assessed to account for potential confounding factors. In cases where multiple independent datasets were accessible during MR analysis, we combined the findings through a meta-analytical approach.

Results: In observational studies, the prevalence of HF in combination with RA reached 7.11% (95%CI 5.83 to 8.39). RA was positively associated with an increased prevalence of HF in the US population [odds ratio (OR):1.93, 95% confidence interval (CI):1.47–2.54, $P < 0.0001$]. In a MR analysis utilizing a meta-analytical approach to amalgamate the results of the IVW method, we identified a significant

causal link between genetically predicted RA and a heightened risk of HF (OR = 1.083, 95% CI: 1.028–1.141; $P = 0.003$). However, this association was not deemed significant for seronegative RA (SRA) (OR = 1.028, 95% CI: 0.992–1.065; $P = 0.126$). These findings were consistent across sensitivity analyses and did not indicate any horizontal pleiotropy.

Conclusion: RA correlates with an elevated prevalence of HF within the US population. Furthermore, genetic evidence derived from European populations underscores a causal link between RA and the risk of HF. However this association was not significant in SRA.

KEYWORDS

rheumatoid arthritis, seronegative rheumatoid arthritis, heart failure, national health and nutrition examination survey, mendelian randomization

1 Introduction

Rheumatoid arthritis (RA) is the most prevalent autoimmune-mediated arthritis and is classified as a systemic inflammatory disease. Its prevalence is on the rise every year, making it a significant global healthcare challenge (1). RA can have long-term adverse consequences for patients, including physical disability and reduced quality of life. Furthermore, the presence of extra-articular manifestations can significantly increase the risk of death (2). In fact, there is a strong association between RA and cardiovascular disease (CVD), and RA has been identified as an independent risk factor for CVD, which contributes to the high mortality rate of RA (3, 4). Systemic inflammation and immune dysfunction serve as pathways that promote and accelerate CVD in patients with RA, ultimately leading to increased morbidity and mortality. It is important to address these underlying factors in order to improve outcomes for these patients (5, 6).

Heart failure (HF) is the second leading cause of cardiovascular mortality in patients with RA, with studies indicating that RA doubles the incidence of HF compared to those without the condition (7–9). Interestingly, the exact mechanism behind this heightened risk is still unclear. HF is a multifaceted clinical syndrome that involves the interaction and coexistence of multiple etiologies. Among these, hypertension and ischemic heart disease are considered to be the primary risk factors. However, despite the increased prevalence of ischemic cardiomyopathy with hypertension in the RA population, this

does not seem to impact the heightened risk of HF (10, 11). Compared to the typical symptom profile of the HF population, patients with both RA and HF may not exhibit the usual signs and symptoms and may have preserved ejection fraction, which could indicate a worse prognosis (12). The evidence presented above suggests a possible causal relationship between RA and HF, which could provide an explanation for the observed association.

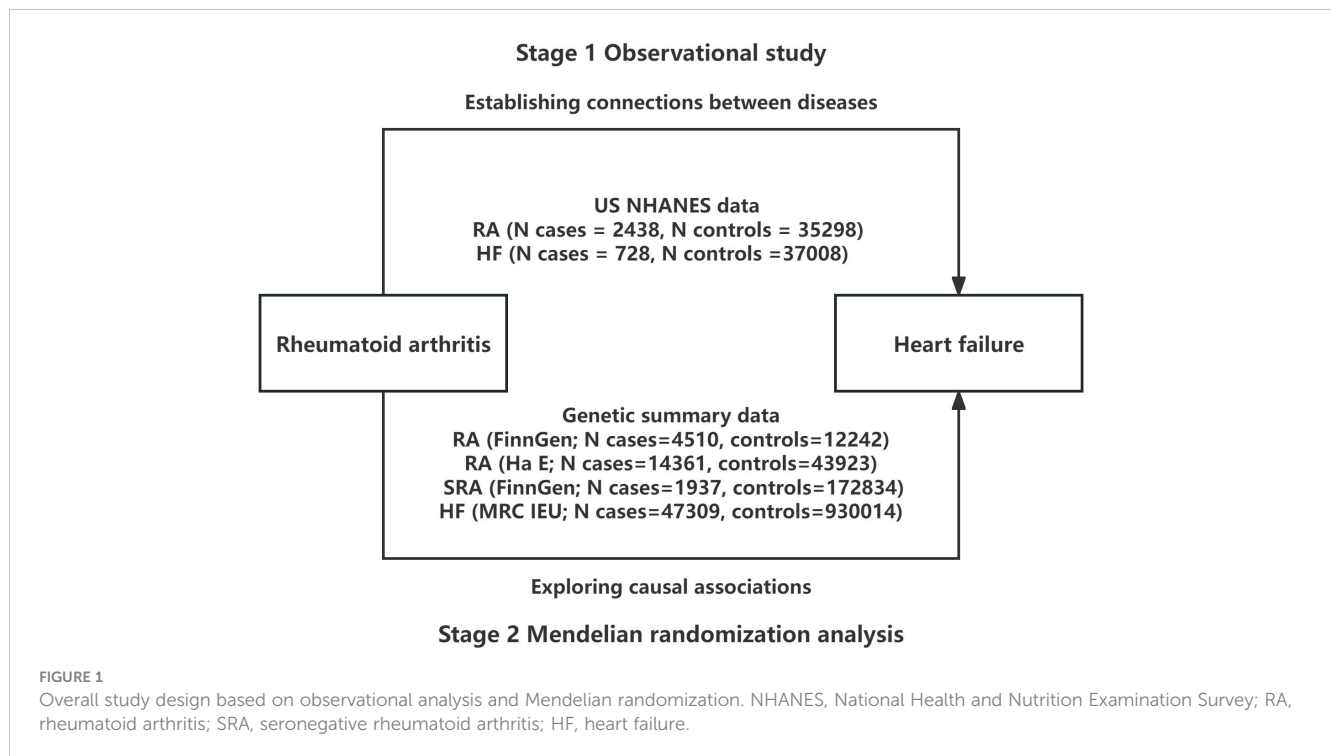
In observational studies, it can be challenging to eliminate the influence of residual confounding factors, which makes it difficult to draw causal inferences. However, by using Mendelian randomization (MR) analysis, we can overcome this limitation and obtain more reliable results based on available data (13). Our study aimed to investigate the potential link between RA and HF. To achieve this, we conducted a cross-sectional study using the National Health and Nutrition Examination Survey (NHANES) database, which has a national scope. Additionally, we utilized MR to further explore the causal relationship between the two conditions. Our hypothesis was that RA is associated with a higher prevalence of HF and that there is a positive causal effect between the two.

2 Materials and methods

2.1 Overall research design

As illustrated in Figure 1, this study comprises of two phases. In the initial phase, we aimed to investigate the association between RA and HF through a cross-sectional analysis utilizing data from the NHANES database. In the second phase, we obtained genetic instruments for RA from a meta-analysis of genome-wide association studies (GWAS) and used MR analysis to evaluate the causal relationship between genetically determined RA and HF. In cases where multiple independent datasets were accessible during

Abbreviations: RA, rheumatoid arthritis; HF, heart failure; MR, mendelian randomization; IVW, inverse variance weighting; OR, odds ratio; CI, confidence interval; SRA, seronegative rheumatoid arthritis; CVD, cardiovascular disease; NHANES, National Health and Nutrition Examination Survey; GWAS, genome-wide association studies; SNPs, single nucleotide polymorphisms; TNF, tumor necrosis factor.



MR analysis, we combined the findings through a meta-analytical approach.

2.2 Study population in NHANES

NHANES is a valuable resource for assessing the health and nutritional status of the US population. The survey uses a complex sampling design to ensure that the sample is representative of the non-institutionalized population, and collects data through interviews, physical examinations, and laboratory tests. The data collected from NHANES is used to inform public health policies and programs, as well as to monitor trends in health and nutrition over time. The NHANES survey was conducted in accordance with the ethical standards set by the National Center for Health Statistics Ethics Review Board. To learn more about the NHANES survey and access detailed information, please visit the official website of the Centers for Disease Control and Prevention at <https://www.cdc.gov/nchs/index.htm>. All participants provided written informed consent, and this study was based on a secondary analysis of publicly available data. The study was designed following the principles of the Guidelines for Strengthening the Reporting of Observational Studies in Epidemiology for reporting cross-sectional studies (14).

We enrolled individuals who were 20 years of age or older and had complete information on all variables for NHANES cycles from 1999–2020. We excluded participants with other forms of arthritis, cancer, and those who were pregnant. Please refer to Figure 2 for a detailed flow chart of our inclusion and exclusion criteria. The number and percentage of missing covariate data are shown in Supplementary Table S1.

2.3 RA and HF assessment in NHANES

In our observational study, we considered RA as an exposure variable and treated HF as an outcome variable. The study utilized a self-reported medical questionnaire to determine whether participants had RA or HF. Previous research has shown that self-reported disease in participants of large cross-sectional studies is a valid method of measurement (15, 16). The researchers used two questions to identify individuals with RA: “Have you ever been diagnosed with arthritis by a doctor or other health professional?”. If yes, “which type of arthritis were you diagnosed with?”. Because there is a lack of biomarker data for RA, its subtypes cannot be defined in detail in cross-sectional studies. They also used a question to identify individuals with HF: “Have you ever been informed by a physician or other healthcare provider that you have HF?”. Responding ‘yes’ to this question was indicative of HF.

2.4 Covariates used in NHANES

We made sure to include all relevant covariates in our study, based on previous research and data from NHANES (16). These covariates covered a wide range of demographics, lifestyle habits, medical history, laboratory tests, and physical measurements. Specifically, we included age, sex, race, education level, insurance coverage, body mass index (BMI), smoking, recreational activity, diabetes, hypertension, chronic renal failure, coronary heart disease, stroke, heart attack, angina, family history of heart disease, total cholesterol, triglycerides, and high-density lipoprotein. Socioeconomic factors were

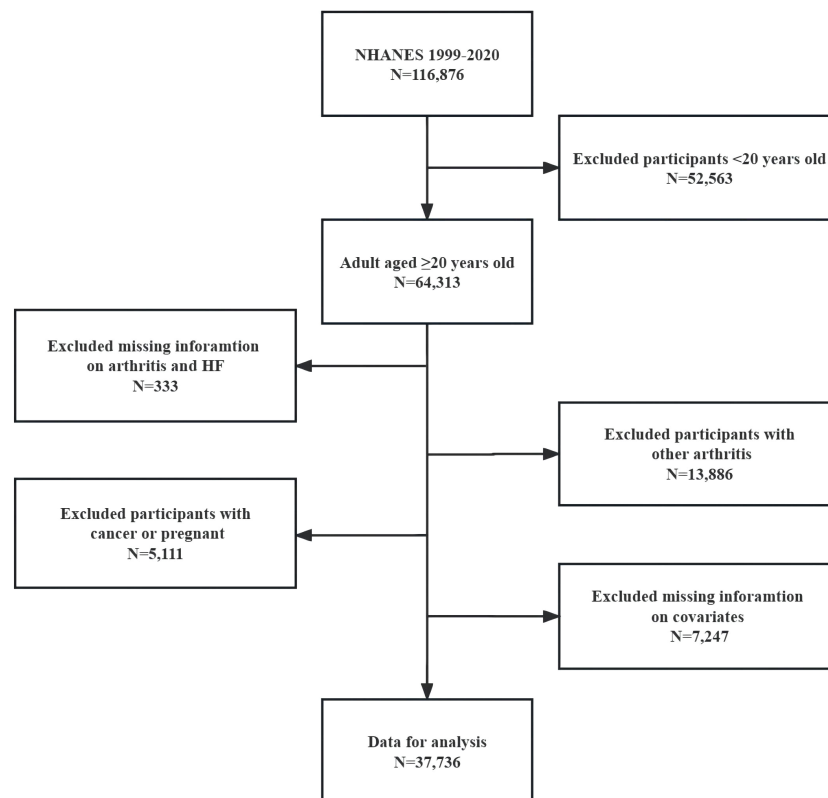


FIGURE 2

Flow chart of eligible NHANES participants included in this Study. NHANES, National Health and Nutrition Examination Survey; HF, heart failure.

assessed and collected during the home interview, based on the original survey records. Behavioral factors were obtained through self-reports. A never smoker is an individual who has never smoked more than 100 cigarettes in their lifetime. Former smokers are defined as those who have smoked more than 100 cigarettes in their lifetime but have since quit smoking. Current smokers are individuals who have smoked at least 100 cigarettes in their lifetime and continue to smoke on some days or every day. BMI was measured at a mobile examination center using standardized protocols. Subject had self-reported medical history of angina pectoris, coronary heart disease, heart attack, or stroke, which was confirmed by a physician. The definition of hypertension is a diagnosis made by a healthcare professional, based on an average blood pressure reading of $\geq 130/80$ mmHg or the use of hypertension medications. Diabetes was defined as a diagnosis made by a physician or other healthcare professional, glycated hemoglobin (%) greater than 6.5, random blood glucose (mmol/L) equal to or greater than 11.1, or use of diabetes medication or insulin. Chronic kidney disease is characterized by an estimated glomerular filtration rate < 60 mL/min/1.73 m² or a urine albumin–creatinine ratio of at least 30. A laboratory blood analyzer was used to collect the serum lipid profile, including total cholesterol, high-density lipoprotein, and triglyceride. For more detailed information about these covariates, please refer to [Supplementary Table S2](#).

2.5 Genetic instruments for RA in MR

For RA, we utilized GWAS data from the FinnGen database (<https://www.finnngen.fi/en>) and an independent study (17) as instrumental variables. Conversely, for seronegative RA (SRA), we relied on GWAS data solely from the FinnGen database. The GWAS analysis on RA sourced from the FinnGen database encompassed 4,510 cases and 12,242 controls. And data from an independent GWAS study comprised 14,361 cases and 43,923 controls. Conversely, the GWAS analysis on SRA involved 1,937 cases and 172,834 controls; The disease data utilized in this study offered clear and well-defined definitions and classifications. Specifically, the FinnGen Consortium employed International Classification of Diseases 9 and 10 codes to delineate RA and SRA. Furthermore, the inclusion and exclusion criteria for cases in the independent study were thoroughly elucidated in the original manuscript. All samples were from European populations, and all cases were included with reference to strict diagnostic criteria for confirmation.

We selected RA and SRA related single nucleotide polymorphisms (SNPs) at genome-wide significance ($P < 5 \times 10^{-8}$) as instrumental variables in secondary analysis to maximize specificity. Second, we request a number of bases between two SNPs (kb $> 10,000$) and further quality control was based on a minor allele frequency $> 1\%$ based on the population reference

data from 1,000 Genomes Project (18). In addition, we manually checked all the identified SNPs by PhenoScanner GWAS database and excluded variants for the linkage disequilibrium. SNPs are not associated with confounders by the PhenoScanner GWAS database. The strength of the included SNPs was evaluated using the F statistic, with a threshold set at an F value greater than 10.

2.6 Genetic summary data of HF

Data on genetic variants connected with HF were taken from a GWAS, which collected 47,309 HF samples and 930,014 controls samples. SNPs and HF association data are available for download from the MRC IEU Open GWAS dataset. Participants of European ancestry from 26 cohorts (with a total of 29 distinct datasets) with either a case-control or population-based study design were included in the meta-analysis. Cases included participants with a clinical diagnosis of HF of any etiology without inclusion criteria based on left ventricular ejection fraction; controls were participants without HF (19).

2.7 Statistical analysis

In a cross-sectional study utilizing the NHANES database, baseline data was categorized based on the presence or absence of RA. Continuous variables were compared between groups using t-tests, while categorical variables were compared using chi-square tests. Multivariate logistic regression analysis was conducted to assess the association between RA and HF using odds ratios (ORs) and 95% confidence intervals (CIs). Model 1 was a crude model, model 2 adjusted for age, sex, and race, and model 3 adjusted for all covariates included in the study. Furthermore, we assessed the variance inflation factors (VIF) for each covariate and found that all VIF values were less than 5. This suggests that there was no significant multicollinearity among the covariates. At the same time, subgroup analyses within fully adjusted models were performed stratified by age (<60/≥60 years), sex (Male/Female), and race (Non-Hispanic white/Non-Hispanic black/Mexican American/Others), and multiplicative interactions were assessed using likelihood ratio tests. During data analysis, we followed NHANES analysis guidelines exactly (20), using sample weights, stratification and clustering variables to account for complex sampling designs that allow results to be generalized to the entire US population.

In this study, the main method of analysis for the MR analysis was inverse variance weighting (IVW). We merged the findings from an independent study, along with the dataset from FinnGen, using a fixed-effects model for meta-analysis (21). Additionally, we employed median weighted MR (22), mode-based MR, MR-Egger, and MR-PRESSO analyses (23) to assess the robustness and stability of our findings. The MR-Egger regression was employed to identify horizontal pleiotropy (24). Furthermore, We utilized Cochran's Q test to evaluate potential heterogeneity among the SNPs. We

applied MR-PRESSO method and a leave-one-out analysis to identify potential pleiotropic outliers, and MR-PRESSO conducts a global test of heterogeneity to identify potential horizontal pleiotropy and MR-Egger method was performed, which can adjust for bias from directional pleiotropic effects (25). The statistical analyses mentioned above were conducted using R software (Version 4.2.1) and we conducted the meta-analysis using STATA software (Version 12.0) to synthesize the collected data from the MR studies.

3 Results

3.1 Baseline characteristics of the sample population in observational study

Table 1 presents the findings of the descriptive analysis conducted on the baseline characteristics of the sample. The observational study included a total of 37,736 participants who were 20 years of age or older and had complete variable data. Complex sample analysis indicated that this sample was representative of the 141,700,894 ambulatory population across the United States. Overall, participants had a mean age $\pm$ SD of 42.73 ± 0.16 years; 48.15% (95%CI 46.58 to 49.73) were women, and 64.13% (95%CI 60.53 to 67.73) were non-Hispanic white. The prevalence of HF in combination with RA reached 7.11% (95%CI 5.83 to 8.39). RA is more commonly found in the elderly, women, and individuals with lower levels of education and higher insurance coverage compared to their counterparts. Additionally, there are differences in the prevalence of RA among different races. In addition, We observed notable distinctions between individuals with and without RA in several factors, including smoking habits, participation in leisure activities, medical history, family history of CVD, and lipid profile (excluding high-density lipoprotein).

3.2 Association between RA and HF in observational study

Table 2 shows the relationship between RA and HF, as determined by a multivariate logistic regression analysis. As the model was adjusted from crude to fully adjusted, it was found that individuals with RA had a greater likelihood of developing HF compared to those without RA. The ORs (95% CIs) for the prevalence of HF across the model 1 to model 3 were 7.71 (6.26-9.49), 3.47 (2.77-4.33) and 1.93 (1.47-2.54), respectively. Table 3 displays the outcomes of our subgroup analyses, focusing on age, sex, and race. The likelihood ratio test for the interaction between age and sex in relation to RA did not yield statistically significant results. Therefore, we can conclude that the findings from the primary analysis remain consistent and reliable. However, when subgroup analysis based on race was conducted, it was found that Non-Hispanic Black have a lower risk of developing HF as a result of RA compared to Non-Hispanic White and Mexican American (P interaction = 0.012).

TABLE 1 General characteristics of included participants (n = 37,736) by the presence or absence of rheumatoid arthritis in the NHANES 1999–2020.

Characters	Overall (n=37,736)	Non-RA (n=35,298)	RA (n=2,438)	P-value
Age, year	42.73±0.16	42.05±0.16	56.53±0.38	< 0.0001
Body mass index (kg/m²)	28.40±0.07	28.31±0.07	30.32±0.21	< 0.0001
Sex				< 0.0001
Male	51.85(50.13-53.56)	52.26(51.71-52.81)	43.51(40.64-46.38)	
Female	48.15(46.58-49.73)	47.74(47.19-48.29)	56.49(53.62-59.36)	
Race				< 0.0001
Mexican American	9.90(8.83-10.96)	10.03(8.88-11.17)	7.25(5.84- 8.66)	
Non-Hispanic Black	11.55(10.61-12.49)	11.26(10.21-12.31)	17.44(15.12-19.76)	
Non-Hispanic White	64.13(60.53-67.73)	64.15(62.17-66.14)	63.69(60.16,67.22)	
Other Hispanic	6.64(5.81-748)	6.70(5.85-7.55)	5.45(4.14-6.76)	
Other race or multi-racial	7.78(7.12-8.44)	7.86(7.18-8.53)	6.17(4.61-7.73)	
Education				< 0.0001
Less Than 9th Grade	5.43(5.03-5.82)	5.23(4.83-5.62)	9.47(8.10-0.85)	
9-11th Grade	10.60(9.97-11.24)	10.36(9.76-10.97)	15.42(13.45-17.39)	
High School Grad	23.87(22.63-25.11)	23.56(22.73-24.40)	30.04(27.17-32.91)	
College degree	30.95(29.72-32.17)	30.91(30.08-31.75)	31.59(28.76-34.43)	
College or above	29.15(27.44-30.87)	29.93(28.51-31.35)	13.47(11.37-15.57)	
Insurance coverage	79.42(76.59-82.25)	79.00(78.03-79.97)	87.91(86.06-89.76)	< 0.0001
Smoking status				< 0.0001
Now	21.61(20.58-22.63)	21.40(20.62-22.19)	25.68(23.06-28.29)	
Former	21.66(20.57-22.74)	21.15(20.44-21.87)	31.80(28.95-34.66)	
Never	56.74(54.86-58.61)	57.44(56.47-58.42)	42.52(39.41-45.63)	
Vigorous recreational activity	33.98(32.45-35.51)	34.87(33.76-35.98)	16.04(13.66-18.42)	< 0.0001
Moderate recreational activity	50.27(48.31-52.24)	50.77(49.64-51.89)	40.35(37.64-43.06)	< 0.0001
Family history of heart disease	12.20(11.54-12.86)	11.67(11.19-12.15)	22.83(20.38-25.28)	< 0.0001
Diabetes	8.81(8.44-9.19)	8.20(7.85-8.55)	21.20(19.28-23.13)	< 0.0001
Hypertension	41.54(39.95-43.14)	40.24(39.37-41.11)	67.79(65.04-70.54)	< 0.0001
Chronic renal failure	10.63(10.15-11.12)	10.07(9.63-10.51)	22.06(20.02-24.10)	< 0.0001
Heart failure	1.27(1.13-1.42)	0.98(0.87-1.10)	7.11(5.83-8.39)	< 0.0001
Coronary heart disease	2.07(1.85-2.29)	1.81(1.62-2.01)	7.15(5.78-8.52)	< 0.0001
Angina	1.38(1.21-1.55)	1.13(0.98-1.28)	6.33(5.02-7.63)	< 0.0001
Heart attack	2.06(1.85-2.26)	1.71(1.55-1.88)	8.97(7.46-10.48)	< 0.0001
Stroke	1.63(1.47-1.79)	1.35(1.22-1.48)	7.25(5.84-8.66)	< 0.0001
Lipid Profile				
Total cholesterol (mg/dL)	194.13±0.37	194.00±0.38	196.75±1.24	0.028
High-density lipoprotein (mg/dL)	52.67±0.17	52.67±0.17	52.61±0.43	0.880
Triglycerides (mg/dL)	145.79±1.12	145.26±1.14	156.47±3.13	< 0.001

Values indicate the weighted mean±SD or weighted % (95% CI). P values are weighted. RA, rheumatoid arthritis; NHANES, National Health and Nutrition Examination Survey.

TABLE 2 Weighted multivariate logistic regression coefficients (ORs) and 95% CIs for the association between rheumatoid arthritis with heart failure: The United States, 1999–2020.

Rheumatoid arthritis	Case/participants	Model 1 OR (95%CI) P- value	Model 2 OR (95%CI) P- value	Model 3 OR (95%CI) P- value
No	519/35298	Reference	Reference	Reference
Yes	209/2438	7.71(6.26,9.49) P<0.0001	3.47(2.77,4.33) P<0.0001	1.93(1.47, 2.54) P<0.0001

Model 1 was a crude model; Model 2 was adjusted for the parameters in Model 1 plus age, sex, and race; and Model 3 was adjusted for the parameters in Model 2 plus education level, insurance coverage, body mass index, smoking, recreational activity, diabetes, hypertension, chronic renal failure, coronary heart disease, stroke, heart attack, angina, family history of heart disease, total cholesterol, triglycerides, and high-density lipoprotein.

3.3 Causal association between RA and HF in MR

In the MR analysis of RA and HF, 9 and 50 SNPs were utilized as IVs, respectively. Additionally, in the analysis of SRA as an exposure variable, 4 SNPs were also employed. As depicted in Figure 3; Supplementary Figure 1, in the main analysis employing a

TABLE 3 Subgroup analysis for the association between rheumatoid arthritis with heart failure.

Subgroup	Rheumatoid arthritis OR (95% CI), P- value		P for interaction
	No	Yes	
Age			0.380
≥60y	Reference	1.98(1.45,2.70) P<0.0001	
<60y	Reference	2.04(1.11, 3.74) P=0.021	
Sex			0.489
Male	Reference	1.92(1.25, 2.94) P=0.003	
Female	Reference	2.03(1.36, 3.03) P<0.001	
Race			0.012
Non-Hispanic White	Reference	2.43(1.68, 3.52) P<0.0001	
Non-Hispanic Black	Reference	1.65(1.03, 2.63) P=0.036	
Mexican American	Reference	2.48(1.10, 5.58) P=0.028	
Others	Reference	0.59(0.21, 1.67) P=0.594	

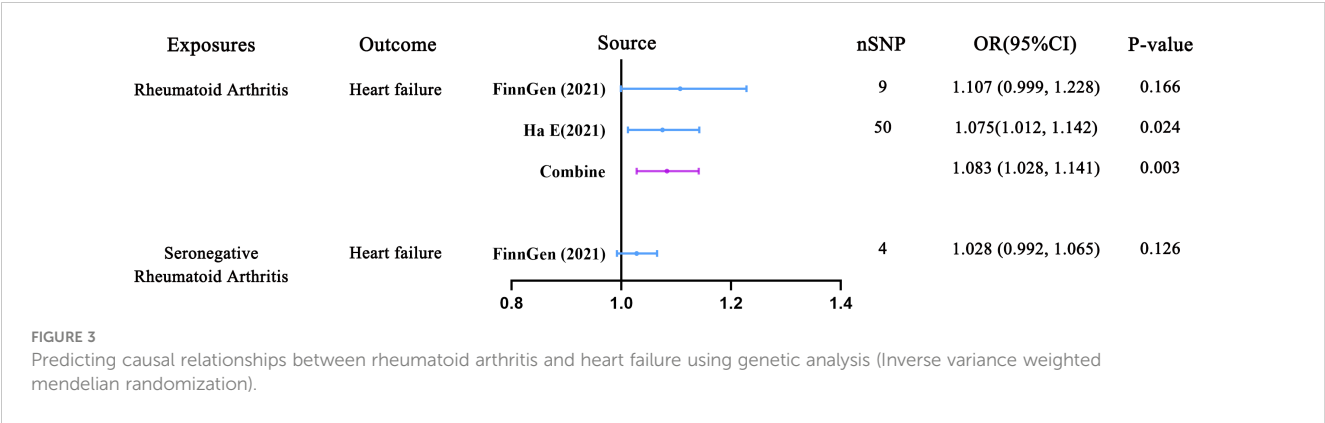
All presented covariates were adjusted (as Model 3) except the corresponding stratification variable.

meta-analytical approach to combine the outcomes of the IVW method, we uncovered a significant causal relationship between genetically predicted RA and an increased risk of HF (OR = 1.083, 95% CI: 1.028-1.141; P = 0.003). Notably, the FinnGen Consortium dataset did not yield any significant causal association in the IVW model (OR = 1.107, 95% CI: 0.999-1.228; P = 0.166) (Supplementary Figure 2). Conversely, findings from GWAS utilizing independent datasets consistently indicated a significant causal association between RA and HF (OR = 1.075, 95% CI: 1.012-1.142; P = 0.024) (Supplementary Figure 3). Additionally, the outcomes of nearly all supplementary analytical techniques (including weighted MR, mode-based MR, MR-Egger, and MR-PRESSO analyses) corroborate the associations noted in GWAS studies using independent datasets, which is illustrated in Supplementary Table 3. However, as depicted in Supplementary Figure 4, this association was not deemed significant for SRA (OR = 1.028, 95% CI: 0.992-1.065; P = 0.126).

Table 4 presents the results of the horizontal multiplicity and heterogeneity tests in the MR analysis conducted in this study. The MR-Egger regression analysis did not find any evidence of horizontal pleiotropy (All p > 0.05), indicating that the genetic variants used in the study do not have effects on the outcome through pathways other than the exposure of interest. Notably, while the Cochran Q test revealed heterogeneity among the included SNPs in the analysis, the meta-analysis did not yield significant heterogeneity(I² = 0%). This suggests that the genetic instruments employed consistently influenced the exposure variables. In addition, the MR-PRESSO method and leave-one-out analysis did not detect any potential pleiotropic outliers (Supplementary Tables 4–6). Finally, The F statistics of all included SNPs were >10, indicating no weak-instrument bias.

4 Discussion

To the best of our knowledge, this study is the first to investigate the association between RA and HF using a large-scale cross-sectional study conducted at the national level, along with a genetically related MR study. In addition, our study also includes the analysis of a specific subtype of RA known as SRA. Our findings are based on two parts: in the cross-sectional study, we found that RA was linked to a nearly twofold higher prevalence of HF in the US population. In the MR study, we discovered a causal association between RA and HF.



Several epidemiological studies have shown a link between RA and HF. A previous study conducted over a 46-year period further supports this association (7), suggesting that RA is associated with a higher risk of developing HF. This increased risk cannot be solely attributed to traditional risk factors for CVD and ischemic heart disease. Furthermore, Danish population studies have also substantiated these claims (26). RA is a chronic and progressive disease. Certain factors have been found to be associated with an increased risk of HF, such as the use of corticosteroids and the presence of severe extra-articular manifestations. However, the use of methotrexate has been shown to decrease the risk of HF in these patients. It is important to note that this association is not a direct link between the two diseases, but rather can be influenced by factors such as the specific treatment approach, disease control, and various other factors (5, 27, 28). It is crucial for clinical practitioners to recognize that repeated flare-ups of RA symptoms can lead to a higher likelihood of rehospitalization in patients with HF resulting from the disease (29). This association may indicate a poorer prognosis (9, 30). There are subtle differences in the signs of HF between patients with RA and those without. Patients with RA may experience atypical symptoms and exhibit fewer signs of HF. Additionally, these patients often have a poorer prognosis, with features such as preserved ejection fractions, and they undergo cardiac ultrasonography less frequently (12, 31).

RA is a condition that causes immune disorders and a systemic inflammatory response. This can lead to the development of diastolic dysfunction HF due to myocardial remodeling, fibrosis, and impaired left ventricular diastolic function (32, 33). A proteomic study has also supported this perspective, revealing that HF resulting from RA is linked to increased expression of

biomarkers associated with inflammation, such as tumor necrosis factor (TNF), fibrosis, and activation of the renin-angiotensin-aldosterone system (34). In RA, TNF plays a crucial role in promoting inflammation, along with other pro-inflammatory cytokines. It has been observed that increased levels of TNF are associated with disease activity (35). Consequently, the overexpression of TNF in RA may directly contribute to the development of HF, and this mechanism has been validated through animal experiments (36, 37). Cardiac biopsies from patients with inflammatory rheumatic diseases revealed a notable increase in the expression of adhesion molecules, pro-inflammatory cytokines, and human leukocyte antigens on both cardiomyocytes and endothelial cells (38). At the same time, research has demonstrated that elevated levels of C-reactive protein are linked to an increased risk of HF in individuals with RA. Conversely, the use of the anti-inflammatory medication methotrexate has been associated with a reduced risk of HF (5). In addition, there are theories suggesting that immune complexes that are highly specific to RA, such as anti-citrullinated protein antibodies, could potentially initiate autoimmune reactions in the heart, resulting in localized inflammation and restructuring of the myocardium (39, 40).

The association between RA and CVD has not only been demonstrated in epidemiological studies, but genetic susceptibility analysis further strengthens this association and indicates a causal relationship between the two conditions. A genetic study that examined the relationship between RA and cardiovascular and cerebrovascular diseases found positive associations with six outcomes: age angina, age heart attack, hypertension, abnormalities of heartbeat, stroke and coronary heart disease (41).

TABLE 4 Assessing heterogeneity and pleiotropy in mendelian randomization analysis of rheumatoid arthritis and heart failure.

Exposure	Pleiotropy test		Heterogeneity test			Global test
	MR-Egger		IVW			MR-PRESSO
	Intercept	p-value	Q	Q-df	P-value	P-value
RA (FinnGen)	-0.005	0.097	70.033	49	0.026	0.763
RA (Ha E)	0.014	0.300	34.124	8	<0.001	0.785
SRA (FinnGen)	0.041	0.365	3.328	3	0.344	0.439

RA, rheumatoid arthritis; SRA, seronegative rheumatoid arthritis; IVW, inverse variance weighting.

This suggests that there are shared genetic mechanisms between RA and these cardiovascular and cerebrovascular conditions. Furthermore, additional studies have investigated the potential link between RA and other CVD. The findings indicate a notable connection with ischemic heart disease and myocardial infarction, rather than atrial fibrillation and arrhythmias (42). Similar to our study, a study has also conducted MR analysis on the relationship between RA and HF, focusing on the biomarker NT-proBNP. The findings of that study are consistent with the results of our study (43). However, this study did not take into account SRA, and the clinical significance of the reported lower OR value remains to be elucidated. In order to address these limitations, our study addresses this gap by combining real-world national cross-sectional studies.

SRA may present with atypical manifestations, making it challenging to diagnose clinically and easily overlooked in patient management (44, 45). Unfortunately, there has been a lack of comprehensive research on the relationship between SRA and HF, both in clinical studies and fundamental research. A case report highlighting severe cardiac tamponade and right HF in a patient with RA, who remained seronegative, emphasizes the need for rheumatologists to be aware of this condition (46). A large cohort study has provided confirmation that individuals with SRA do not face an increased risk of HF. However, it revealed that patients who tested positive for RA antibodies had a 36% higher risk of developing HF (11). Our genetic analysis unequivocally demonstrates the absence of a causal relationship between SRA and HF. Nevertheless, additional studies are warranted to corroborate our results and delve deeper into the underlying mechanisms at play.

Our research methodology is a unique strength of our study, as we utilized both cross-sectional data and MR to examine the relationship between RA and HF. By utilizing national-level cross-sectional studies, we were able to derive ORs that are applicable to real-world situations. Additionally, the use of MR analysis helped address the limitation of the former approach by providing a means to infer causality. This approach also allowed us to overcome the limitations of previous studies and provide more clinically relevant findings (43). Furthermore, we expanded our analysis to include SRA as an exposure variable, which enhances the comprehensiveness and depth of our study conclusions.

However, it is important to acknowledge the limitations of this study. Firstly, it's worth noting that self-reported data from cross-sectional studies utilizing RA and HF lack details, such as disease duration and subtype. Due to the unavailability of data on biomarkers for RA, further investigation into these aspects is not feasible, despite the significance of RA biomarkers in diagnosis, disease prognosis, and management. Moreover, self-reported clinical data may be susceptible to various biases, including information bias and recall bias, among other potential limitations. Nevertheless, the currently published NHANES-based studies have demonstrated the significant epidemiological research value of self-reported diseases. In the study published in the *Lancet* sub-journal, which also utilized the NHANES database as the data source, the diagnosis of RA, similar to our study, was also obtained through self-report (47). In the US National Prevalence and Temporal Trends Study of Anemia

in HF Patients, participants were also diagnosed with HF through self-report (48). These cross-sectional studies encompass a large number of individuals on a national scale, making it impractical for researchers to determine disease status using strict clinical diagnostic criteria, which would require substantial time and effort. Moreover, additional confounding variables, such as undisclosed patient treatment information, may not have been taken into account, potentially influencing our results. Our findings indicate that genetically predicted associations were not influenced by the use of these drugs, and rheumatoid arthritis demonstrated a causal relationship with heart failure at the genetic level. In MR studies, we cannot completely eliminate the possibility of horizontal pleiotropy during analysis, which can introduce bias. However, it is worth noting that the statistical analysis in this study, specifically MR-Egger, did not indicate any signs of horizontal pleiotropy. Furthermore, it is important to mention that our study primarily focused on European populations, and caution should be exercised when generalizing the findings to other populations. At the same time, we noted relatively small ORs in our MR results, consistent with previous studies (43). It is important to recognize that these findings do not automatically imply that exposed populations are at low risk, as the variability in exposure factors may surpass that explained by genetic variation alone. Additionally, the primary objective of MR is not to determine the precise magnitude of genetic effects but rather to evaluate the causal impact of environmental exposure on outcomes. Fortunately, our cross-sectional surveys effectively captured the true effect size between the two diseases.

5 Conclusion

In summary, our study indicates that RA correlates with an elevated prevalence of HF within the US population. Furthermore, genetic evidence derived from European populations underscores a causal link between RA and the risk of HF. However this association was not significant in SRA. Therefore, additional research is imperative to corroborate these results and delve into the underlying mechanisms driving this association.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The studies involving humans were approved by Ethics Review Committee of the National Center for Health Statistics. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KK: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. DD: Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. XZ: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. HL: Data curation, Writing – original draft. LK: Data curation, Writing – original draft. JH: Data curation, Writing – original draft. XC: Data curation, Writing – original draft. TL: Conceptualization, Resources, Supervision, Writing – review & editing. FK: Conceptualization, Resources, Writing – review & editing. XL: Conceptualization, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1377432/full#supplementary-material>

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Targeting autoimmune mechanisms by precision medicine in Myasthenia Gravis

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Myasthenia Gravis (MG) is a chronic disabling autoimmune disease caused by autoantibodies to the neuromuscular junction (NMJ), characterized clinically by fluctuating weakness and early fatigability of ocular, skeletal and bulbar muscles. Despite being commonly considered a prototypic autoimmune disorder, MG is a complex and heterogeneous condition, presenting with variable clinical phenotypes, likely due to distinct pathophysiological settings related with different immunoreactivities, symptoms' distribution, disease severity, age at onset, thymic histopathology and response to therapies. Current treatment of MG based on international consensus guidelines allows to effectively control symptoms, but most patients do not reach complete stable remission and require life-long immunosuppressive (IS) therapies. Moreover, a proportion of them is refractory to conventional IS treatment, highlighting the need for more specific and tailored strategies. Precision medicine is a new frontier of medicine that promises to greatly increase therapeutic success in several diseases, including autoimmune conditions. In MG, B cell activation, antibody recycling and NMJ damage by the complement system are crucial mechanisms, and their targeting by innovative biological drugs has been proven to be effective and safe in clinical trials. The switch from conventional IS to novel precision medicine approaches based on these drugs could prospectively and significantly improve MG care. In this review, we provide an overview of key immunopathogenetic processes underlying MG, and discuss on emerging biological drugs targeting them. We also discuss on future direction of research to address the need for patients' stratification in endotypes according with genetic and molecular biomarkers for successful clinical decision making within precision medicine workflow.

KEYWORDS

Myasthenia Gravis, autoimmunity, B cells, neonatal Fc receptor, complement system, precision medicine

1 Introduction

Myasthenia gravis (MG) is a chronic T cell-dependent, B cell-mediated autoimmune disease caused by autoantibodies against proteins of the neuromuscular junction (NMJ) that impair neuromuscular transmission leading to disabling muscle weakness and fatigability (1).

Despite it is commonly considered a prototypic antibody-mediated autoimmune condition, MG is a complex and heterogeneous disorder, characterized by fluctuating symptoms, unpredictable disease course and wide clinical and immunopathological variability, including different autoantibody specificities, age at onset, distribution of muscle weakness, thymic histopathology and clinical response to therapies (1, 2). This variability likely reflects distinct pathophysiological states and differences in the mechanisms underlying the production of autoantibodies and their pathogenic effects, understanding of which is the prerequisite for the development of tailored and more specific approaches to treat different subgroups of MG patients.

Currently, MG treatment is mainly based on symptomatic agents and non-selective immunosuppression with corticosteroids and/or non-steroidal immunosuppressants, able to control symptoms but source of side effects and rarely leading to complete stable remission (3, 4). Moreover, about 10% of MG patients (~10%) are considered refractory to immunosuppressive (IS) drugs (4, 5). They represent a subgroup of patients treated with multiple drugs for long periods of time with considerable burden due to disability caused by uncontrolled MG and long-term immunosuppression (6). In this regard, the use of more specific therapeutic strategies is an urgent medical need for these patients. Biological drugs targeting disease effector mechanisms (3, 4, 7) are promising for the treatment of refractory MG patients as emerged from controlled studies. The introduction of these innovative drugs in the clinical practice could prospectively have a tremendous impact on therapeutic success in MG, especially when tailored treatments take into account the biological diversity among disease subgroups. Moreover, the introduction of innovative compounds early in the course of the disease could considerably reduce the risk of becoming refractory and limit the use of corticosteroids.

In this review, we provide an overview of the pathophysiological mechanisms targeted by innovative drugs in MG, and summarize data on their effectiveness in MG treatment. We also speculate on future directions of research to achieve precision medicine in MG.

2 MG heterogeneity, clinical subgroups and treatments

MG is a heterogeneous disease in which the variability allows patients' stratification in distinct disease subgroups according to the following features: i) autoantibody status; ii) distribution of weakness; iii) age at the disease onset; and iv) histopathology of thymus (1, 2). Stratification of MG patients according to clinical and immunological features such as autoantibody specificity is now

mandatory due to the different targets of innovative drugs, and therefore relevant to treatment decisions and outcomes, representing a first step towards personalization of therapies.

In most (80–85%) MG patients, the autoantibody target is the acetylcholine receptor (AChR) and its clustering in the post-synaptic membrane of NMJ is essential for muscle contraction. Less frequently, autoantibodies are directed to the muscle-specific tyrosine kinase (MuSK), or to the lipoprotein-related protein 4 (LRP4), two proteins implicated in AChR clustering. A very small proportion of patients have no specific autoantibodies (seronegative MG, or triple negative MG) and in these patients the neurophysiological confirmation of the diagnosis is mandatory (1, 2). Anti-AChR, -MuSK and -LRP4 autoantibodies have proven pathogenicity, since they are able to induce end-plate alterations and impairment of neuromuscular transmission (8–10).

Ocular symptoms are often observed at onset, then shifting to a generalized form (gMG) usually within two years, which involves skeletal and bulbar muscles (11).

Commonly, MG patients are stratified according to age at onset in patients with early- (EOMG, < 50 years) or late- (LOMG, > 50 years) onset disease (12). EOMG is more common in women than in men, and frequently shows thymic follicular hyperplasia, which is present in most (~80%) AChR-positive patients (1, 2, 13). LOMG is more frequent in males, who often have a normally involuted thymus and antibodies to other muscle proteins, mainly titin or the ryanodine receptor (RyR) (1, 14). An increase in MG incidence in the elderly population, particularly in subjects over 65 years, has been reported in recent years, likely due to improved diagnosis; these patients are mostly patients with anti-AChR antibodies and without thymoma (14, 15).

Thymomas are detected in approximately 10–15% of MG patients. Thymoma-associated MG can occur at any age and is characterized by the presence of anti-AChR antibodies and generalized disease in almost all patients, who frequently have additional autoantibodies directed to titin and RyR that are useful as biomarkers to diagnose thymoma in patients younger than 50 years (1, 16).

Disease severity is variable among patients. MuSK-MG and MG associated with thymoma have a higher risk of a severe clinical course compared to the other clinical subgroups (1, 2).

Several variables need to be taken into account for making clinical decisions in MG, including weakness severity, muscles involved, thymus pathology, autoantibody specificity and patient comorbidities.

International and national treatment guidelines are available for MG treatment (3, 17). The current therapeutic algorithm includes the following steps: 1) symptomatic therapy with cholinesterase inhibitors (first-line treatment); 2) corticosteroids, alone or combined with other IS agents (second-line treatment); 3) thymectomy in selected patients (young onset AChR-positive patients, or thymoma), and 4) plasmapheresis/immunoglobulins for acute exacerbations (2–4). The prognosis for MG patients has greatly improved over the past half century, with substantial reductions in mortality and morbidity. Nevertheless, inter-individual variability in drug effectiveness, adverse events related to conventional treatments, co-morbidities limiting corticosteroid

usage and treatment refractoriness (5, 6) are a challenge to clinicians, requiring the development of more specific and better tolerated therapeutic approaches.

In recent years, several biological drugs have been developed for MG treatment (4, 7, 18), namely biological compounds targeting B cells, the neonatal Fc receptor (FcRn) and the complement system (Tables 1–3, Figure 1), representing different tools able to interfere with key steps of the autoimmune process in MG (18). They proved to be effective for the treatment of MG patients/patients’ subgroups, in line with the crucial role of their biological targets in the disease pathogenesis, as discussed in the following paragraphs.

3 B cells: key players and therapeutic targets in MG

B cells are implicated in MG pathogenesis, since they produce the specific autoantibodies. Their contribution to MG has been widely investigated, and significant thymic and peripheral B cell dysfunctions in MG patients have been demonstrated (19).

B cells, as the major component of the humoral adaptive immune response, have always represented an attractive target for effective therapeutic intervention in autoimmune diseases, such as MG.

TABLE 1 B cell-targeted biological drugs in myasthenia gravis.

Biological drug	Drug molecule	Target	Drug function	Clinical studies	Treatment outcomes
Rituximab (RTX)	Chimeric murine-human IgG1 mab	CD20	Depletion of CD20+ B cells by apoptosis induction	BeatMG – Phase 2 study in AChR+ patients with mid-to-moderate gMG	No significant steroid-sparing effect ⁵⁹
				Long-term follow-up (31 months) of RTX-treated AChR+ and MuSK+ MG patients	Autoab decrease, remission/minimal manifestations in MuSK-MG, but not in AChR-MG, patients ⁶⁰
				RINOMAX – Clinical study in new-onset AChR+ patients with gMG	Increased probability of minimal manifestations and reduction of the need of rescue medications ⁶³
				REFINE – new trial on RTX efficacy in AChR+ MG patients	Ongoing trial (NCT05868837), expected to be completed in 2025
Inebilizumab	Human mab	CD19	Cytotoxicity and depletion of CD19+ B cells	MINT – new trial in AChR+ and MuSK+ gMG patients	Ongoing trial (NCT04524273), expected to be completed in 2029
Bortezomib	Pyrazine-containing small molecule	26S proteasome	Proteasome inhibition and plasma cell depletion	TAVAB – Phase 2 study in treatment-refractory patients with autoimmune diseases, including AChR+ MG patients	No results published yet (NCT02102594) ⁶⁹
TAK-079	Human mab	CD38	Cellular cytotoxicity, lysis and depletion of CD38-expressing cells, including plasmablasts and plasma cells	Phase 2 study in AChR+ and MuSK+ gMG patients	No publication available (NCT04159805)
Descartes - 08	Autologous CAR T cells engineered with RNA to target BCMA	BCMA	Inhibition of plasma cells	MG-001 – OLE phase 1b/2a study in seropositive (AChR+, MuSK+ or LRP4+) gMG patients	Decrease of MG-ADL, MGC, QMG, and MG-QoL-15r scores at up to 9 months of follow-up ⁷¹
Belimumab	Human mab	BAFF	Blocking of BAFF, inhibition of its interaction with BAFF-R, BCMA and TACI receptors and reduction of B cell survival	BEL115123 – Phase 2 study on gMG patients all AChR+, except 2 MuSK+, with mild-moderate disease	No significant QMG score change after 24 weeks ⁷⁴
Telitacicept	Fusion protein	TACI	Inhibition of BAFF and APRIL binding to the TACI receptor on B cells and reduced B cell survival	Phase III Study in AChR+ or MuSK+ patients with gMG	Ongoing trial (NCT05737160), expected to be completed in 2027
Satralizumab	Humanised mab	IL-6	Inhibition of IL-6 signaling	LUMINESCE – Phase 3 study in seropositive (AChR+, MuSK+, LRP4+) gMG patients	Modest improvement in MG-ADL and QMG scores from baseline to week 24 in AChR-MG patients (NCT04963270, Abstract, AAN 2024 ^a)

AChR, acetylcholine receptor; Autoab, autoantibodies; BAFF, B lymphocyte stimulator; BAFF-R, BAFF receptor; BCMA, B-cell maturation antigen; CAR T, chimeric antigen receptor T cells; gMG, generalized myasthenia gravis; IL-6, interleukin-6; LRP4, lipoprotein-related protein 4; Mab, monoclonal antibody; MG-ADL, MG-Activities of Daily Living score; MGC, MG Composite score; MG-QoL15r, MG Quality of Life 15-item Scale; MuSK, muscle-specific kinase receptor; OLE, open label extension; QMG, quantitative myasthenia gravis score; TACI, transmembrane activator and CAML interactor.
^aAAN: American Academy of Neurology, annual meeting 2024, abstract from: <https://medically.roche.com/global/en/neuroscience/aan-2024/medical-material/AAN-2024-poster-habib-LUMINESCE-a-phase-3-study-of-satralizumab-pdf.html> (access on 13 may 2024)

TABLE 2 Biological drugs targeting FcRn in myasthenia gravis.

Biological drug	Drug molecule	Target	Drug function	Clinical studies	Treatment outcomes
Efgartigimod (ARGX-113)	Humanized IgG1-derived Fc fragment	FcRn	Binding of FcRn with high affinity, competition with endogenous IgG, inhibition of IgG recycling and reduction of IgG levels, including autoabs	ADAPT – Phase 3 study in AChR +, MuSK+ and double (AChR/ MuSK)-seronegative patients with gMG	Reduction of IgG levels in AChR+ and seronegative patients. Sustained improvements of MG-ADL, QMG, MGC, and MG-QoL15r scores in AChR+ and seronegative patients ⁹²
				Retrospective study in AChR+ patients with gMG	MG-ADL reduction after the first cycle; minimal symptom expression in 25% of patients after 1 and in 25% after 2 cycles with Sustained benefits after cycle 2 ⁹³
				Study on the long-term effect (14 months) of the drug in AChR+, MuSK+, LRP4+, and triple negative gMG patients	MG-ADL, MGC and QMG improvement at the end of each cycle. No patient hospitalization during the treatment compared to the year before treatment ⁹⁴
				Study on drug efficacy in seropositive and seronegative gMG patients	MG-ADL, MGC and MG-QoL15r improvement in 34/36 patients after the first and subsequent cycles ⁹⁵
				ADAPT – OLE study (up to 3-year extension)	Confirmation of long-term efgartigimod safety, tolerability, and efficacy ⁹⁶
Rozanolixizumab (UCB7665)	Human IgG4 mab	FcRn	Binding of FcRn, competition with endogenous IgG, inhibition of IgG recycling and reduction of IgG levels, including autoabs	Mycarin G – Phase 3 study in AChR+ and MuSK+ patients with gMG	Significant MG-ADL reductions at day 43; improvements in MG-ADL, MGC, QMG and Myasthenia Gravis Symptoms PRO as early as day 8 up to day 99 ⁹⁷
Nipocalimab (M281)	Human IgG1 mab	FcRn	Binding of FcRn, competition with endogenous IgG, inhibition of IgG recycling and reduction of IgG levels, including autoabs	Vivacity-MG – Phase 2 study in moderate-to-severe AChR+ and MuSK+ patients with gMG and inadequate response to stable standard-of-care	Rapid improvement (within 2 weeks) in MG-ADL across 4 dosing arms, and reduction in total IgG and anti-AChR abs ⁹⁹
				Phase 3 study in seropositive patients with gMG	Ongoing trial (NCT04951622), expected to be completed in 2026
Batoclimab	Human mab	FcRn	Binding of FcRn, competition with endogenous IgG, inhibition of IgG recycling and IgG reduction, including autoabs	Phase 2 study with an OLE in AChR+ patients with gMG	Reduction in total IgG across IgG subclasses and anti-AChR abs, but no significant changes in G-ADL, QMG, MGC, and MG-QoL15r scores ¹⁰⁰
				Phase 3 study in AChR+ and MuSK+ patients with gMG	Significant increase in the rate of sustained MG-ADL improvement ¹⁰¹
				Phase 3 study in gMG patients	Ongoing trial (NCT05403541), expected to be completed in 2025

AChR, acetylcholine receptor; Autoab, autoantibodies; FcRn, neonatal Fc receptor; gMG, generalized myasthenia gravis; LRP4, lipoprotein-related protein 4; Mab, monoclonal antibody; MG-ADL, MG-Activities of Daily Living score; MGC, MG Composite score; MG-QoL15r, MG Quality of Life 15-item Scale – Revised score; MuSK, muscle-specific kinase receptor; OLE, open-label extension; QMG, quantitative myasthenia gravis score.

3.1 Role of B cells in MG pathogenesis

3.1.1 B cells in MG thymus

The thymus is a site of relevant B cell abnormalities in AChR-MG patients. B cell infiltration and development of ectopic germinal centers (GC) forming follicles are histopathological features of thymic follicular hyperplasia, which characterizes the majority of these patients (13, 19). B cells from hyperplastic MG thymus express markers of activation, including CD71, 4F2, CD23, and B8.7, and display functional signs of activation (20).

Ectopic lymphoid neogenesis is a feature commonly found in inflamed target tissues of patients with several autoimmune conditions, such as rheumatoid arthritis (RA) synovia, multiple sclerosis (MS) meninges, Sjogren’s syndrome salivary gland,

systemic lupus erythematosus (SLE) kidneys, and Hashimoto’s thyroiditis thyroid (21). The exact mechanisms leading to GC formation in these organs, and in MG thymus, is still unknown. GCs generally develop in the context of chronic inflammation. Indeed, the follicular hyperplastic MG thymus is characterized by a well-defined chronic inflammatory condition, and presents features of tertiary lymphoid organs, such as sustained over-expression of inflammatory cytokines and chemokines, GC development, and neoangiogenic processes (i.e. high endothelial venule and lymphatic vessel development) (22–25). The B cell-attracting chemokines CXCL13 and CCL21, key molecules over-expressed in follicular hyperplastic MG thymuses, contribute to the formation and maintenance of ectopic follicles by promoting active recruitment of peripheral B cells, as well as T cells, into the thymus (22–25).

TABLE 3 Biological drugs targeting the complement system in myasthenia gravis.

Biological drug	Drug molecule	Target	Drug function	Clinical studies	Treatment outcomes
Eculizumab	Humanized mab	C5	Inhibition of C5 cleavage into C5a and C5b and of MAC formation with reduction of the complement-mediated damage	REGAIN – Phase 3 study in treatment-refractory AChR+ patient with gMG and its OLE study	Significant MG-ADL and QMG reduction; rapid (i.e. by week 1) and sustained (i.e. at least 130 weeks) improvement in ocular, bulbar, respiratory, and limb muscle strength and in daily activities ^{125–127}
Ravulizumab	Humanized mab	C5	Inhibition of C5 cleavage into C5a and C5b and of MAC formation with reduction of the complement-mediated damage	CHAMPION-MG – Phase 3 study in AChR+ patients with gMG and its OLE study	Significant MG-ADL and QMG reduction within 2 weeks and maintained through 60 weeks ^{128,129}
Zilucoplan	Macrocyclic peptide	C5	Allosteric inhibition of C5 cleavage and of MAC formation with reduction of the complement-mediated damage	RAISE – Phase 3 study in AChR+ patients with moderate to severe gMG	Significant reduction in MG-ADL score from baseline to week 12 ¹³⁰
				RAISE-XT – OLE phase of RAISE	Ongoing trial, expect to be completed in 2026 (NCT04225871)
Pozelimab Cemdisiran	Pozelimab: human IgG4 (IgG4P) mab Cemdisiran: small interfering N-acetylgalactosamine-conjugated ribonucleic acid (siRNA)	C5	Pozelimab: inhibition of C5 cleavage and of MAC formation with reduction of the complement-mediated damage; Cemdisiran: Interference with the mRNA for C5, reduction of C5 expression and circulating levels	NIMBLE – Phase 3 study on the efficacy of a combined therapy based on the two drugs in AChR+ patients with gMG	Ongoing trial, expected to be completed in 2028 (NCT05070858)
Vemircopan	Oral inhibitor molecule	Factor D	Inhibition of C3 convertase formation and of MAC formation	Phase 2 study in AChR+ patients with gMG	Ongoing trial, expected to be completed in 2025 (NCT05218096)

AChR, acetylcholine receptor; gMG, generalized myasthenia gravis; Mab, monoclonal antibody; MAC, membrane attack complex; MG-ADL, MG-Activities of Daily Living score; QMG, quantitative myasthenia gravis score.

Peripheral B cells attracted by the two chemokines can be sensitized against AChR locally expressed in the thymus (i.e. thymic epithelial cells and myoid cells) (26–28). In this regard, there is evidence that the hyperplastic MG thymus contains plasma cells that are able to produce anti-AChR antibodies *in vitro* (29). Ectopic GCs are known to be the site of affinity maturation, clonal selection and differentiation of autoreactive B cells (19). In MG, ectopic GCs contain B cells undergoing antigen-driven clonal expansion, somatic hypermutation, and selection (30), indicating that they represent an immunological niche of autoreactive B cell differentiation and autoantibody production. Of interest, the GC number (i.e. degree of thymic hyperplasia) was found to correlate with autoantibody titers, and to decrease in MG patients undergoing corticosteroid treatment (31).

Pathogen infections are thought to play a role in chronic inflammation, type I interferon (IFN-I)-mediated anti-viral response and innate immune activation in hyperplastic MG thymuses (32, 33). IFN- β has been proven to play a major role in intra-thymic autosensitization to the AChR, since it is able to increase the expression of the AChR- α subunit by thymic epithelial cells, at the same time increasing the expression of CXCL13, CCL21 and BAFF (also known as B lymphocyte stimulator or BlyS), an important survival factor for B cells (34). Poliovirus-infected macrophages, and latently and lytically Epstein–

Barr virus (EBV)-infected B cells and plasma cells, were detected in MG but not in normal control thymuses (32, 33), supporting the hypothesis that IFN- β over-expression and chronic inflammation might be triggered by persistent viruses in MG thymuses. Due to EBV unique ability to induce abnormal B cell activation, proliferation and survival (35), EBV persistence and reactivation observed in hyperplastic thymuses of AChR-MG patients represent an important source of B cell dysfunction, possibly contributing to the chronicity of the intra-thymic inflammatory autoimmune process. MG GCs were found to be EBV-enriched (33), similarly to GCs in other target organs of B cell-mediated autoimmune diseases, such as RA synovia and MS brain (36, 37), thus supporting EBV involvement in GC formation and B-cell dysregulation in different autoimmune conditions, including MG.

Polyclonality of thymic B cells was observed in MG patients (38), in line with widespread B cell activation by EBV. Of interest, thymus-associated antigen-experienced B cell clones were detected in the circulation of AChR-MG patients after thymectomy (39), the persistence of which correlated with reduced clinical response to surgery, thus indicating that autoreactive B cells can migrate to other immunological compartments and perpetuate autoimmunity in the periphery.

B cell abnormalities can be also observed in MG thymomas. Neoplastic tissue of MG patients' thymomas is characterized by

increased B cell infiltration compared to thymoma tissue from patients without MG (40). GCs encircled by high endothelial venules, that are potentially able to recruit peripheral B cells, were identified in the adjacent tissues of a high proportion of MG thymomas, and their number positively correlated with anti-AChR titers (41). Of note, EBV latency markers were found in thymoma-infiltrating B cells in MG but not in non-MG thymomas, thus supporting the virus contribution to intra-thymic GC formation and B cell-mediated autoimmunity also in thymoma-associated MG (40).

3.1.2 B cells in MG peripheral blood

There is no evidence of increased frequency of B cells in peripheral blood of MG patients. However, circulating B cells of these patients, particularly when positive for anti-AChR antibodies, display increased expression levels of activation markers (e.g. CD23, CD71) (19, 42), as found for thymic B cells. In line with this observation, peripheral blood mononuclear cells (PBMCs) from MG patients have enhanced ability to secrete IgG, and anti-AChR

antibody secretion correlates with IgG secretion (43). The levels of several cytokines involved in B cell activation and antibody production are increased in serum of MG patients, and positive correlations were found between IgG and interleukin (IL)-6 levels (19, 43). Among B cell-stimulating cytokines, higher levels of serum BAFF (44), and higher frequency of circulating B cells expressing BAFF receptor (BAFF-R), have been reported in MG patients, in both AChR- and MuSK-MG (45, 46), indicating abnormal B cell survival. Regulatory B cells producing IL-10 (B10 cells), which play an important immunosuppressive role through potent inhibition of B- and T-cell responses, were found to be reduced in peripheral blood of both AChR-MG and MuSK-MG patients, and their reduction was associated with disease severity (46, 47). This finding represents an additional MG-associated B cell defect implicated in immune tolerance breakdown. By deep sequencing, large-scale abnormalities were found in both the naïve and memory B cell receptor repertoires of AChR- and MuSK-MG patients, indicating disturbed B cell repertoire as a fundamental MG component with distinct properties between the two disease

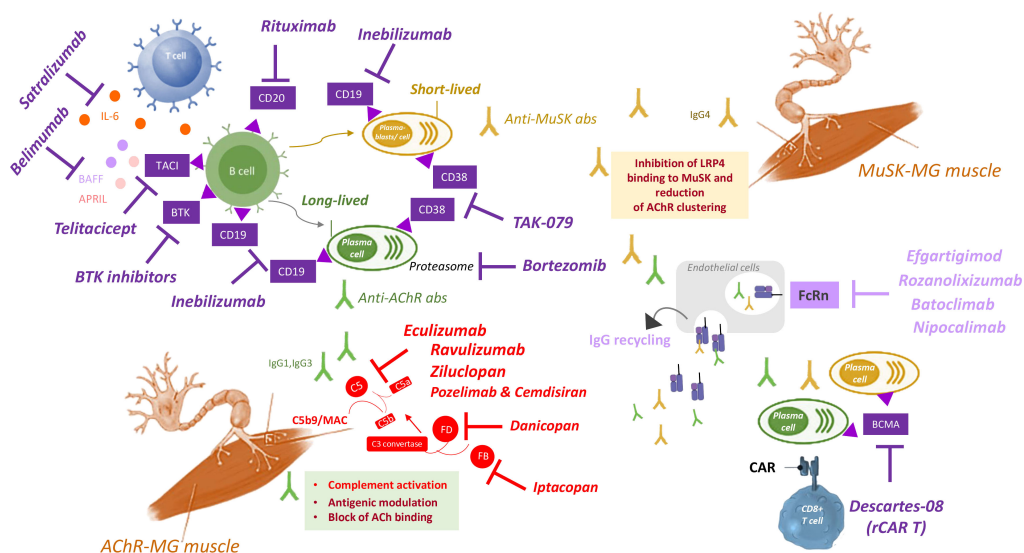


FIGURE 1

Summary of targeted therapies for MG treatment. Autoantibodies to AChR (green) produced by long-lived plasma cells (green) impair NMJ by the following mechanisms (green box): i) complement activation and MAC formation, ii) antigenic modulation, or iii) block of the ACh binding to the receptor. Autoantibodies to MuSK (yellow) produced by short-lived plasma blasts (yellow) inhibit LRP4 binding to MuSK, thus compromising AChR clustering (yellow box). Several biological drugs (purple, upper left quadrant) are available to directly target B or plasma cells, or to block B cell survival and activation, including: i) rituximab, that targets CD20 expressed on B cells; ii) inebilizumab, that targets CD19 expressed on B cells, plasmablasts and plasma cells; iii) BTK inhibitors, that target BTK expressed in B cells; iv) bortezomib, that inhibits proteasome to deplete plasma cells; v) TAK-079, that targets CD38 expressed on plasmablasts and plasma cells; vi) belimumab, that targets the B cell survival factor BAFF (also called Blys); vii) telitacicept, that targets the TACI receptor to inhibit the B cell survival factors BAFF and APRIL; viii) satralizumab, that targets IL-6 to inhibit B cell activation and differentiation. CAR T cells engineered with RNA (rCAR-T) has been recently developed as therapeutic strategy to block plasma cells, by specifically targeting BCMA expressed in these cells (lower right quadrant). Drugs targeting the FcRn (light purple, middle right quadrant) inhibit IgG recycling, thus reducing autoantibody levels. They include: efgartigimod, rozanolixizumab, batoclimab and nipocalimab. Drugs targeting the complement system (red, lower left quadrant) are effective to treat the disease in patients with anti-AChR antibodies, by inhibiting complement activation. They include eculizumab, ravulizumab, zilucoplan, and the combination of pozelimab and cemdisiran, all able to target C5, thus inhibiting its cleavage in C5a and C5b, and hence MAC (C5b9) formation. Additional targets of anti-complement drugs are Factor D, targeted by danicopan, and Factor B, targeted by iptacopan, two complement factors implicated in the formation (factor D) and amplification (factor B) of the process that leads to C3 convertase formation, that is essential for the formation of C5 convertase and hence for C5 cleavage and subsequent MAC formation. Abs: antibodies; ACh: acetylcholine; AChR: acetylcholine receptor; APRIL: a proliferation-inducing ligand, also known as B lymphocyte stimulator (Blys); BCMA: B-cell maturation antigen; BTK: Bruton's tyrosine kinase; CAR: chimeric antigen receptor; FcRn: neonatal Fc receptor; IgG: immunoglobulin G; IL-6: interleukin 6; LRP4: low density lipoprotein receptor-related protein 4; MAC: membrane attack complex; MuSK: muscle-specific kinase receptor; NMJ: neuromuscular junction; rCAR T: CAR T cells engineered with RNA; TACI: transmembrane activator and CAML interactor.

subgroups (48). Interestingly, the naïve B cell repertoire of MuSK-MG patients includes self-reactive clones capable of specific binding to MuSK with high affinity, suggesting that the MuSK antigen might trigger B cell activation and differentiation toward memory B cells and antibody-secreting cells that directly contribute to the disease (49).

AChR-MG patients are characterized by enhanced frequency of circulating plasma cells, which account for autoantibody production (50). Of note, anti-AChR antibody-producing plasma cells have been identified in the bone marrow (51), a well-recognized niche for long-lived plasma cells that are able to survive for prolonged periods of time and maintain long-term humoral immunity. Longevity and radio-resistance are typical features of persisting anti-AChR antibody-producing plasma cells (52). Since EBV-infected B cells are long-lived cells, presence of long-lived plasma cells in MG patients is in line with a possible contribution of EBV to chronic autoimmunity in MG. In contrast, short-lived plasmablasts are key autoantibody producers in MuSK-MG patients (53, 54), thus explaining the favorable outcome of B cell-depleting therapies in these patients compared to those positive for anti-AChR antibodies, as discussed below.

3.2 Targeting B-cell compartment as strategy “to strike at the heart of autoimmunity”

Due to their essential role in the autoimmune response, B cells are the main candidate target cells for effective therapies in MG to inhibit autoimmunity. Biological drugs targeting B cells can be categorized into drugs that directly target B or plasma cells, drugs that block the survival and differentiation of B cells, and drugs targeting cytokines involved in the differentiation and activation (Figure 1).

3.2.1 Drugs directly targeting B cells

Rituximab (RTX), a biological drug that directly and specifically targets B cells, has been approved in several B cell-mediated autoimmune conditions, such as RA and SLE (55–57). RTX is a chimeric murine-human IgG1 monoclonal antibody that depletes all B cell subsets (but not pro-B cells, plasmablasts and long-lived plasma cells) through specific binding to the transmembrane CD20 antigen and induction of apoptosis of the target cell, antibody-dependent cell-mediated cytotoxicity, or complement-dependent cytotoxicity (55).

RTX effectiveness in AChR-MG patients has been demonstrated in several uncontrolled studies and case reports (58), but a multicenter, placebo-controlled, randomized phase 2 trial (BeatMG Study) failed to achieve the primary steroid-sparing outcome (60% with RTX versus 56% with placebo), as well as secondary outcomes, in patients with mild-to-moderate gMG (Table 1) (59). Low disease activity, concomitant prednisone in a dose range of 15–60 mg/d and a consistent placebo response likely affected the negative results of this trial. Successful B-cell depletion was achieved in the treatment arm, but the drug did not affect

significantly anti-AChR antibody levels (59). Contrariwise, RTX has been proven to almost completely eliminate autoantibodies in MuSK-MG patients in line with long-lasting disease improvement (60) (Table 1). A systematic review of case reports and series from Tandan and colleagues (61) showed that MuSK-MG patients are more responsive to RTX than AChR-positive, showing markedly decreased post-treatment antibody levels, as early as 3 months after RTX-mediated B cell depletion, and a longer clinical improvement in most patients. The reason for the different response to RTX between AChR- and MuSK-MG patients is likely related to the different type of autoantibody-producing cells in the two disease subgroups: as reported above, autoantibodies against MuSK are produced by short-lived plasmablasts that are continuously regenerated from autoreactive CD20-positive B cells, thus depletion of these cells may be effective; autoantibodies against AChR likely derive from long-lived plasma cells, and hence total circulating IgGs remain constant in the long-term after CD20 depletion therapy (53, 54, 62).

Recently, RTX was found to increase the probability of achieving minimal disease manifestations despite low doses of corticosteroids in the short to medium term in new-onset AChR-MG patients with generalized disease (RINOMAX study), likely because early immune response may derive from plasmablasts and short-lived plasma cells, thus suggesting the use of RTX early in the disease course to reduce the risk of worsening or the need for additional therapies (63) (Table 1). However, the RINOMAX study lasted 48 weeks and the long-term benefit-risk balance was not addressed. Nevertheless, duration of RTX beneficial effects may be limited both in AChR- and MuSK-MG patients, since disease relapse can occur in patients who achieved drug-induced remission (54). This is likely due to resurgence of pathogenic B cells, highlighting the need for retreatment (64). Repopulation of pathogenic versus regulatory B cells is an important factor underlying clinical response to RTX. Indeed, it has been demonstrated that MG patients who respond well to the drug exhibit rapid repopulation of B10 cells compared to non-responder patients, who show a delayed B10 cell repopulation (65). A new trial on RTX efficacy in AChR-MG is ongoing (REFINE, <https://clinicaltrials.gov/study/NCT05868837>) and it is expected to be completed in 2025 (Table 1). In addition, a phase 3 clinical trial on Inebilizumab, a humanized antibody that binds to the B cell-specific antigen CD19, is ongoing (MINT, <https://clinicaltrials.gov/study/NCT04524273>) and should be completed in 2029 (Table 1). Inebilizumab looks promising for both AChR- and MuSK-MG as CD19 expression, compared to CD20, is maintained in plasmablasts and plasma cells, thus the drug is able to deplete also these pathogenic cells in MG patients.

Since B cells express Bruton's tyrosine kinase (BTK), an enzyme crucial for B-cell activation, growth and differentiation, another promising anti-B cell therapy could be based on BTK inhibitors. These drugs were found to be beneficial in treating some autoimmune diseases, such as SLE and RA (66), but clinical studies are needed to evaluate their efficacy in MG. Several clinical trials are also ongoing in MS (67).

3.2.2 Drugs directly targeting plasma cells

Therapies specifically targeting plasma cells might represent an appropriate therapeutic approach for AChR-MG. Bortezomib, a small-molecule proteasome inhibitor approved for the treatment of multiple myeloma and mantle cell lymphoma, depletes plasma cells and blocks specific autoantibody production in primary thymic cell cultures of EOMG patients (68). In the experimental autoimmune MG model (EAMG), the drug was effective in reducing anti-AChR antibody titers and prevent immune-mediated destruction of the neuromuscular junction (68). A phase 2 pilot study (TAVAB) on bortezomib in treatment-refractory patients with autoimmune diseases, including patients with AChR-MG, SLE and RA (n=6 for each disease) has been carried out, but the results have not been published yet (69) (Table 1).

Another drug directed against plasma cells is TAK-079, a high-affinity antibody specific for CD38, that is expressed on plasmablasts, plasma cells, but also natural killer cells and other non-immune cells (70) (Table 1). The drug has been evaluated in a phase 2 trial for AChR- and MuSK-MG (<https://clinicaltrials.gov/study/NCT04159805>) but no publication on this study is yet available.

Recently, chimeric antigen receptor (CAR) T cells engineered with RNA (rCAR-T) were developed to target the B-cell maturation antigen (BCMA) expressed on plasma cells (71). CAR molecules brings the extracellular target binding domain of an antibody directed toward a specific target together with the intracellular T-cell activation protein domains, enabling T-cell activation upon contact with the target antigen without antigen presentation by professional cells and regulatory checkpoints (72). Compared to cells engineered with the conventional DNA approach, in which CAR-expressing DNA is integrated permanently into the T-cell genome and replicates with each cell division, CAR-encoding mRNA does not replicate together with the activated and proliferating rCAR T cells, thus making rCAR-T safer than classical CAR-T therapy (71). A prospective open-label (OLE) phase 1b/2a study (MG-001) on the efficacy of Descartes-08, an autologous CD8+ T-cell product transfected with RNA to express the anti-BCMA targeting CAR protein, was carried out in seropositive (AChR-, MuSK- or LRP4-positive) gMG patients (n=14) by Granit and colleagues (71). They demonstrated that anti-BCMA rCAR-T therapy was safe and able to meaningfully decrease MG severity scales (i.e. MG-ADL, MGC, QMG, and MG-QoL-15r scores) at up to 9 months of follow-up (71). A more complete assessment of Descartes-08 efficacy is ongoing in a placebo-controlled study in gMG patients (<https://www.clinicaltrials.gov/study/NCT04146051>).

3.2.3 Drugs blocking survival of B cells

B cell targeting drugs can exert an indirect effect, by promoting inhibition of B cells via blockage of their stimulating factors, such as BAFF that is able to induce B-cell survival and differentiation, the levels of which are increased in MG patients' sera (44).

Belimumab is a human IgG1 monoclonal antibody that blocks both soluble and membrane-bound BAFF, thus inhibiting its interaction with the cognate receptors BAFF-R, B-cell maturation

antigen (BCMA), and transmembrane activator and CAML interactor (TACI) (73). The drug has been approved for the treatment of SLE and is promising for the treatment of patients with RA and Sjögren's syndrome (73). Conversely, a phase 2, double-blind, multicenter randomized trial carried out on gMG patients, who were all AChR-MG except for two MuSK-MG patients, showed no statistically significant differences in the primary endpoint (i.e. mean change in the Quantitative Myasthenia Gravis, QMG, score) between placebo and active treatment after 24 weeks (74) (Table 1). The trial failure could be explained by the inclusion of a small number of patients (n=40), who had mild-moderate disease, as well as by the presence of long-lived autoantibody-producing plasma cells in AChR-MG patients. No further trial on belimumab efficacy has been conducted in MG.

Telitacicept is another drug providing an option to block B cells in patients with autoimmune diseases by acting on B cell survival factors. It is a fusion protein binding the TACI receptor to inhibit both BAFF and APRIL, another cytokine sustaining B-cell survival (75). A clinical trial (<https://clinicaltrials.gov/study/NCT05737160>) on Telitacicept safety and efficacy in gMG patients is open and is expected to be completed in 2027.

Inflammatory cytokines play a key role in activation and differentiation of immune system cells, including B cells. Thus, therapies targeting these proteins can indirectly affect B cell populations. IL-6 has been implicated in autoantibody production, MG activity and severity (76–78), thus representing another therapeutic target. Satralizumab is a humanized IL-6 receptor monoclonal antibody developed to provide durable suppression of IL-6 signaling. The LUMINESCE phase 3 randomized, double-blind, placebo-controlled study evaluated satralizumab efficacy in MG patients (<https://clinicaltrials.gov/study/NCT04963270>) (Table 1). Only a modest improvement in MG-ADL and QMG scores from baseline to week 24 in AChR-MG patients has been reported in the abstract from: <https://medically.roche.com/global/en/neuroscience/aan-2024/medical-material/AAN-2024-presentation-habib-primary-and-secondary-results-of-LUMINESCE-pdf.html> (access on 13th may 2024).

3.2.4 Other strategies to deplete autoreactive cells

Transplantation with autologous hematopoietic stem cells (HSCT) represents a strategy to eliminate autoreactive immune system cells, including B cells. This procedure, preceded by immunoablative high-dose chemotherapy, has been evaluated as a potential intervention to restore self-tolerance in patients with refractory and severe MG (79). The treatment was found to lead to prolonged (29–149 months) and complete symptom- and treatment-free (CSR) remission in 7 MG patients (i.e. six AChR-positive; one negative for anti-AChR antibodies with unknown anti-MuSK and -LRP4 autoantibody status) (79). In this very small patients' cohort, HSCT was tolerable, and acute toxic effects were not observed. However, since the immunoablative conditioning regimen may cause important short-term complications, including opportunistic infections and rarer cardiac, renal, or other organ toxic effects, as well as late complications, such as

endocrine dysfunction, it should be carefully explored in selected patients in whom the risks of treatment are outweighed by potential benefits.

4 Antibody recycling by FcRn as physiological mechanism prolonging autoantibody effects

Current therapies for MG suppress the immune system without specifically targeting the autoantibodies, which play a central role in the disease development. Thus, biological drugs able to reduce autoantibody levels are promising to counteract MG, particularly AChR-MG for which anti-B cell drugs have limited efficacy due to the action of autoantibody-producing long-lived plasma cells. IgG have a longer half-life compared to other immunoglobulin classes (i.e. over 3 weeks *versus* 5–7 days) (80). Drugs promoting their clearance, or able to reduce their persistence in the body, represent an ideal strategy to treat MG. A key molecule ensuring prolonged half-life of IgG is the neonatal Fc receptor (FcRn): its biological function is to maintain IgG concentration in the circulation and interstitial fluids (80). In the era of biologicals, this function has offered a therapeutic potential, leading to the development of innovative drugs targeting this receptor to lower circulating IgGs in antibody-mediated autoimmune conditions, such as MG (Figure 1).

4.1 FcRn-IgG immunobiology

FcRn belongs to the heterogeneous family of MHC molecules, from which it differs for limited diversity and inability to present antigens. FcRn is a beta-2 macroglobulin-associated protein expressed in a wide variety of tissues, including epithelia, endothelia, intestinal cells, kidney, liver, and placenta (80). FcRn is critically implicated in the materno-fetal IgG transfer, a mechanism that protects offspring from infections in early life. The receptor continues to play a key immunological role beyond the neonatal period, being able to protect IgG from intracellular catabolism and recycle them back into the circulation. Intracellularly, the FcRn binds the Fc portion of IgG with high affinity within the pH acid microenvironment typical of endosomes, inhibits IgG lysosomal degradation and promotes their release outside the cells into the neutral pH circulation milieu (80, 81). This process, mainly occurring in the vascular endothelium, increases the IgG half-life, according to the specific binding affinity of different IgG isotypes to FcRn. IgG3 have been proven to have the lowest binding potential to the receptor, and indeed they have the lowest half-life in the circulation compared to other isotypes, likely as an effect of competition with IgG1 for FcRn (82).

FcRn is also widely expressed on the cell surface of several hematopoietic cells, including monocytes, macrophages, dendritic cells (DCs), neutrophils and B cells (83). Thus, the FcRn is involved in additional immune functions, other than IgG transport and recycling, including potentiation of innate immune responses to

IgG forming immune complexes with their antigens, that is important for immune surveillance against infections (81). In neutrophils, the receptor enhances phagocytosis of IgG-opsonized bacteria (84). In DCs and other antigen presenting cells, the FcRn participates in the presentation/cross-presentation of antigenic peptides by MHC class II and I to CD4+ and CD8+ T cells, by directing the internalization, trafficking and processing of antigen-bound IgG immune complexes (81, 84). In this way, the receptor contributes to the activation of adaptive immune responses. It has been demonstrated that production of pro-inflammatory cytokines (e.g., IL-6, TNF- α) by innate immune cells in response to IgG immune complexes strictly depends on FcRn, since it requires IgG binding to FcRn, and can be inhibited by FcRn antagonists (85). Thereby, pharmacological FcRn inhibition can result in an anti-inflammatory action via mitigation of pathogenic inflammatory responses in the context of inflammatory and autoimmune conditions.

FcRn expression is modulated in different ways depending on the different immunological microenvironments: the exposure of human PBMCs to TNF- α , LPS or CpG is able to induce a significant increase of FcRn expression via NF- κ B signaling pathways; contrariwise, activation of the JAK/STAT-1 signaling pathway by IFN- γ can down-regulate functional FcRn expression (86, 87). In addition, the promoter region of the FcRn-encoding gene, *FCGRT*, contains variable number of tandem repeats (VNTRs) that affect the receptor expression: subjects homozygous for VNTR3, which is the most common variant (~84% of human population), have higher FcRn expression, and increased IgG binding ability, than heterozygous subjects bearing the VNTR2 variant (VNTR2/VNTR3), which is the second most frequent variant (~13% of the human population) followed by VNTR1,4 and 5 (~3% of the human population) (88). These observations implies that the function of FcRn, and the magnitude of beneficial effects of its therapeutic blockage, could vary in different disease contexts and genetic backgrounds.

4.2 FcRn blockade in MG

The role of the FcRn as regulator of IgG homeostasis has made it an attractive target for precision medicine in MG to reduce pathogenic antibody levels in a specific manner compared to plasmapheresis or immunoglobulins.

Preclinical studies showed safety and efficacy of an anti-FcRn monoclonal antibody, 1G3, in a passive and active model of EAMG: in rats in which MG was induced by passive transfer of anti-AChR antibodies, 1G3 treatment resulted in dose-dependent improvement of symptoms and reduction of pathogenic antibody levels in serum; in rats immunized with the AChR, the treatment significantly reduced the severity of the disease symptoms and the levels of both total IgG and anti-AChR antibodies (89). Similarly, in a mouse model of MuSK-MG, obtained by injections with purified MuSK-MG patients' IgG4, FcRn blockade by efgartigimod reduced IgG4 levels and determined significant *in vivo* muscle function improvements, thus highlighting the potential of FcRn-targeted therapies to effectively improve MG (90).

Efgartigimod (ARGX-113) is the first FcRn antagonist approved (December 2021) in the USA by the US Food and Drug Administration (FDA). It is a humanized IgG1-derived Fc fragment able to bind FcRn with high affinity, thus competing with endogenous IgG and lowering their levels (91).

The phase 3 double-blind, multicentric randomized-controlled ADAPT study assessed the efficacy and safety of efgartigimod as add-on therapy in 167 patients with gMG on a stable dose of at least one IS treatment, including 129 (77%) AChR-MG patients, 6 (4%) MuSK-MG patients and 32 double (AChR/MuSK-) negative patients (92) (Table 2). Efgartigimod (10 mg/kg) or matching placebo was administered as four infusions per cycle (one infusion per week), repeated as needed depending on clinical response no sooner than 8 weeks after initiation of the previous cycle. Both in the AChR-positive and -negative patients, IgG levels significantly decreased with each cycle. A significantly higher proportion of AChR-MG patients in the drug group were MG-Activities of Daily Living (MG-ADL) responders (68%) than in the placebo group (30%) after the first cycle. Moreover, in cycle 1 the drug led to sustained improvements of the total mean scores for MG-ADL, QMG, MG Composite (MGC), and MG Quality of Life 15-item Scale - Revised (MG-QoL15r) rating scales (92). In patients who received a second cycle, a greater proportion of patients in the drug group (71%) were MG-ADL responders compared with the placebo group (26%), with similar rates observed after cycle 1. The drug was well tolerated and the safety profile was good. The results in the AChR-negative patients were similar to those in the AChR-MG population. Efgartigimod effectiveness in inducing clinically meaningful improvement in MG-ADL was recently reported by Katyal and colleagues (93), who analyzed the treatment outcomes in a cohort of 37 AChR-MG patients, with all except one having completed at least one cycle and 28 patients having completed at least two cycles. Clinically meaningful improvement in MG-ADL was achieved in 72% (26/36) of patients after the first cycle, including 3 of 4 patients with thymoma, and 25% (7/28) of patients achieved minimal symptom expression status after the second cycle (93) (Table 2). The long-term effect of efgartigimod along 14 months, and its impact on the disease course, was more recently reported in 19 MG patients (AChR-, MuSK-, LRP4-positive or triple negative MG). During the year before treatment 8 of 19 patients (42%) were hospitalized, and 15 of 19 (79%) needed treatment with plasma exchange or immunoglobulins; three of 19 (16%) were admitted to the intensive care unit. During efgartigimod, none of the patients was hospitalized and only one patient required plasma exchange and intravenous immunoglobulins (94) (Table 2). Positive results on the real world use of efgartigimod in 36 seropositive and seronegative MG have been recently reported from Japan (95) (Table 2). Long-term efgartigimod safety, tolerability, and efficacy has been confirmed by the OLE (up to 3-year extension) study of the ADAPT (96). Another FcRn blocker is Rozanolixizumab (UCB7665), a human IgG4 monoclonal antibody targeting the FcRn IgG binding region, whose safety and efficacy in gMG has been evaluated in a randomized, double-blind, placebo-controlled, adaptive phase 3

study (MycarinG) (97) (Table 2). The study included 200 gMG patients, who received subcutaneous infusions once a week for 6 weeks of either rozanolixizumab 7 mg/kg (n=66), rozanolixizumab 10 mg/kg (n=67), or placebo (n=67). Both AChR- (n=179) and MuSK-positive patients (n=21), under conventional IS treatment, were included. Significant MG-ADL reductions from baseline were observed at day 43 (primary efficacy endpoint) for both the two rozanolixizumab groups compared to the placebo group. Improvements in MG-ADL, MGC, QMG, and Myasthenia Gravis Symptoms PRO (98) scores were observed as early as day 8 from the treatment start, the first time-point at which efficacy was assessed, and returned towards baseline levels by day 99, according with rapid reductions in total IgG at day 8 and their gradual increase by day 99. MuSK-MG patients had greater score reductions than the overall population, and all of them achieved an MG-ADL response (97). The MycarinG study results thus provided further support to safety and efficacy of FcRn inhibition for the treatment of gMG.

Nipocalimab (M281) is a fully human IgG1 monoclonal antibody with high affinity for the IgG binding site on FcRn. A phase 2 multicenter, randomized, double-blinded placebo trial (Vivacity-MG) assessed the nipocalimab efficacy in moderate-to-severe gMG (99) (Table 2). The study included 68 MG patients randomly assigned to receive different intravenous doses of the drug (4 treatment groups) or placebo for 8 weeks. There were no treatment discontinuations due to side effects, and no difference between the treatment and placebo arms for adverse events (i.e. infections, headaches). A greater proportion of nipocalimab-treated patients exhibited rapid improvement (within two weeks of treatment) in MG-ADL across all the 4 dosing arms compared to the placebo arm. The drug led to substantial reductions in serum total IgG and anti-AChR autoantibodies that significantly correlated with MG-ADL improvement (99). Safety and efficacy of nipocalimab is under investigation in an ongoing phase 3 multicenter study in adult patients with seropositive gMG (<https://clinicaltrials.gov/study/NCT04951622>).

Recently, Nowak and colleagues published the results of a Phase 2 proof-of-concept, randomized, double-blind, placebo-controlled trial with an OLE, on the efficacy of batoclimab, a subcutaneous fully human anti-FcRn monoclonal antibody, in AChR-positive gMG patients (100) (Table 2). The drug was associated with significantly greater reductions in total IgG and anti-AChR antibodies from baseline to 6 weeks than placebo. MG-ADL, QMG, MGC, and MG-QoL15r scores showed improvements over time, but differences between the two treatment arms did not reach statistical significance, likely due to the small sample size (n=17) (100). A Phase 3 study performed in a higher number (n=132) of patients with gMG, including both AChR- and MuSK-positive patients (101), showed batoclimab ability to significantly increase the rate of sustained MG-ADL improvement, as early as week 2 for 4 or more consecutive weeks, with clinical effects and IgG reduction being similar to those previously reported for efgartigimod and rozanolixizumab. Currently, batoclimab therapeutic efficacy is under evaluation in another Phase 3 trial in AChR-positive gMG patients, expected to be completed in 2025 (<https://clinicaltrials.gov/study/NCT05403541>).

5 Complement system as key damage mediator and therapeutic target in AChR-MG

The complement system is the main mediator of the NMJ damage caused by anti-AChR autoantibodies (102), thus representing an ideal target of precision medicine therapies to specifically and effectively treat AChR-MG (Figure 1).

5.1 Role of complement system in AChR-MG

Anti-AChR autoantibodies belong to the IgG1 and IgG3 subclasses, that are the strongest activators of the complement system. Indeed, pathogenicity of these autoantibodies, but not that of anti-MuSK antibodies that belong to IgG4, is mainly due to complement fixation at the NMJ ultimately leading to postsynaptic membrane destruction and impairment of the neuromuscular transmission (102–104). Additional pathogenic mechanisms of anti-AChR antibodies are the direct functional block of the receptor and antigenic modulation, the last consisting in the cross-linking of two adjacent AChR molecules by the same antibody, causing AChR endocytosis and degradation (104).

The complement system, consisting of nearly 50 blood proteins, plays a key role in acute inflammation and defense against common pathogens as part of the innate immune response. Complement activation generates a robust proteolytic cascade that leads to opsonization for phagocytosis and osmotic/colloidal lysis of the pathogen, as well as generation of a potent inflammatory response through the production of pro-inflammatory molecules. Furthermore, the complement system acts to remove antigen-antibody complexes from circulation and is involved in adaptive immune responses, participating in the regulation of T and B cell activation (105).

In MG, complement-mediated damage of the NMJ occurs via the classical complement pathway, which starts with C1q binding to the Fc domain of the autoantibodies. Critical steps of the complement cascade are then the cleavage of C3 into C3a and C3b, and of C5 in C5a and C5b, the latter leading to the formation of the membrane attack complex (MAC, C5bC6C7C8C9 also known as C5b-9) that causes focal lysis of the post-synaptic membrane, disruption of post-junctional folds, and ultimately reduction of functional AChRs (102, 106, 107).

Several preclinical studies have demonstrated the role of the complement system in EAMG, as well as the efficacy of complement inhibition via recombinant proteins, chemicals, monoclonal antibodies and small interfering RNA (siRNA), in improving MG symptoms (108–113).

In muscle biopsies from MG patients, IgG and C3 deposition was localized at identical sites of post-synaptic membrane, and specifically on debris of junctional folds in the synaptic clefts and disintegrating junctional folds (114). Deposition of C9, the main component of the final and stable MAC, was also observed at the

MG end plate regions, with the most intense depositions associated with the destroyed NMJs, indicating that NMJ loss may be largely due to complement activation (114).

Complement regulators are present on host cell surfaces to prevent autologous destruction by the complement system. Mice deficient for both the decay accelerating factor 1 (DAF1 or CD55), which inhibits C3 and C5 convertases, and the MAC inhibitory protein (MAC-IP or CD59), which inhibits MAC formation, developed a significantly worse EAMG associated with crisis, further supporting the role of complement role in MG (115).

Recently, Obaid and colleagues developed an assay based on the EK293T cell line modified to disrupt complement regulator gene expression via CRISPR/Cas9 genome editing, that was used to measure patient-specific autoantibody-mediated MAC formation by flow cytometry (116). By means of this system, they observed a subset of AChR-MG patients with high complement activity, who are likely patients in whom complement activation is the major disease mechanism, whereas the remaining patients showed low activity, reflecting possible complement-independent autoantibody-mediated mechanisms of NMJ damage (116). Circulating complement protein profile may be used as a biomarker for evaluating patient-specific complement activation: (i) Romi and colleagues found reduced C3 and C4 serum levels in AChR-MG patients with high autoantibody titers, indicating increased *in vivo* complement consumption (117); (ii) Liu and colleagues showed lower C3 levels in serum of AChR-positive compared to AChR-negative gMG patients and healthy controls (118), again suggesting complement activation-dependent C3 consumption; (iii) C2 and C5 levels were significantly reduced, and C3, C3b and C5a increased, in plasma of AChR-MG patients compared to controls in our recent study, suggesting “C2, C3, C5, C3b, C5a” as a profile suggesting complement activation (119); (iv) increased levels of complement activation products (e.g. C5a, C3a, SC5b-9, C4a) were found in AChR-MG compared to controls by Staschei and colleagues (120), and (v) lower serum C5 levels, indicating consumption, associated with higher sC5b-9 levels, were found in AChR-MG compared to patients with non-inflammatory neurological disorders by Ozawa and colleagues, further indicating complement activation in AChR-MG (121). Discrepancy among the results from the different studies could be due to differences in the clinical features of the patients' cohorts (e.g. disease severity; IS treatments) or to technical issues related to the different methods used for the complement protein dosage, as well as to sample handling and storage and sample types (serum versus plasma). Recommendations for complement laboratory analyses should indeed be rigorously followed to avoid biased results, due to *in vitro* complement activation and consumption (122).

5.2 Complement inhibitors to block neuromuscular junction damage by anti-AChR-antibodies

Since complement activation results from a cascade of multi-step events, many potential targets are available as candidates for therapeutic intervention.

C5 is a central component of the complement cascade as its cleavage into C5a and C5b is critical for MAC formation that is initiated by C5b (106). Biological drugs targeting C5 have emerged as a successful strategy to treat complement-mediated diseases, including MG (123).

Eculizumab is a fully humanized monoclonal antibody binding C5, approved by FDA for the treatment of AChR-MG patients with gMG (124). By inhibiting C5 cleavage into C5a and C5b, the drug is able to block the MAC formation and abrogate complement-mediated damage. Preclinical studies showed that administration of an anti-C5 monoclonal antibody in a passive immunization rat model not only restored strength in the animals, but also inhibited deposition of C9, thereby preventing endplate disruption (110). Eculizumab efficacy and safety in refractory AChR-positive gMG patients were demonstrated in the phase 3 REGAIN trial, and the following OLE study, demonstrating that eculizumab is able to elicit a rapid (i.e. by week 1) and sustained (i.e. at least 130 weeks) improvement in muscle strength across ocular, bulbar, respiratory, and limb muscle groups and in daily activities (125–127) (Table 3).

Ravulizumab is another FDA-approved human anti-C5 monoclonal antibody with similar mechanism of action of eculizumab but with a longer half-life, requiring less frequent infusions (every 8 weeks compared to 2 weeks for eculizumab). The phase 3 CHAMPION-MG study showed the drug ability to induce a clinically meaningful response in terms of significant MG-ADL and QMG reduction in AChR-MG gMG patients, with a rapid improvement within 1 week of treatment initiation in the treatment versus the placebo group, and adverse events rates were comparable between the two groups (128). The OLE phase of this study showed a sustained efficacy and long-term safety of ravulizumab, administered every 8 weeks, with rapid improvements in MG-ADL and QMG scores (within 2 weeks) and maintained up to 60 weeks (129) (Table 3).

Zilucoplan is a macrocyclic peptide able to allosterically inhibit C5 cleavage. Compared to eculizumab and ravulizumab, that are administered intravenously, Zilucoplan offers the advantage to be administered subcutaneously at-home once daily by self-injections. Safety and efficacy of zilucoplan has been proven in patients with moderate to severe gMG in the phase III RAISE trial (130) (Table 3). Indeed, MG patients assigned to zilucoplan showed a greater reduction in MG-ADL score from baseline to week 12, compared with those assigned to placebo, with incidences of serious adverse events and infections similar in both groups. An OLE study is ongoing (RAISE-XT, <https://clinicaltrials.gov/study/NCT04225871>).

Other drugs developed to block C5 for treatment of complement-mediated disorders are pozelimab and cemdisiran. The first is a fully human IgG4 monoclonal antibody against C5 with a proline substitution to promote stabilization of the disulfide bonds between the two heavy chains (IgG4P); the second is a small interfering N-acetylgalactosamine-conjugated ribonucleic acid (siRNA) which interferes with mRNA for C5, thus offering the opportunity to reduce C5 hepatic synthesis and hence circulating levels. The combination of pozelimab and cemdisiran was recently found to inhibit complement activity more efficiently (i.e. durable and more complete suppression) than the monotherapy with either pozelimab or cemdisiran in non-human primates, thus opening the

way to clinical investigations on the potential of long-acting treatment with these two drugs for complement-mediated disease treatment (131). A phase 3 trial on the efficacy of a combination therapy based on intravenous pozelimab and subcutaneous cemdisiran is ongoing in gMG (NIMBLE, <https://clinicaltrials.gov/study/NCT05070858>).

Along with C5, additional complement cascade components are promising targets to counteract AChR-MG. Danicopan (ALXN2040) is an oral small molecule complement factor D (FD) inhibitor that showed improved benefit-risk profile as add-on therapy to ravulizumab or eculizumab in patients with complement-mediated paroxysmal nocturnal hemoglobinuria (PNH) compared to placebo (132). A phase 2 study on vemircopan, a similar oral factor D inhibitor molecule (ALXN2050) qualified for monotherapy in PNH, is ongoing in gMG patients (<https://clinicaltrials.gov/search?intr=NCT05218096>) (Table 3). Since FD is essential for complement activation being necessary for the formation of the C3 convertase (107), its blockage is a promising approach to counteract the complement-mediated damage of autoantibodies and hence to treat AChR-MG.

Another oral inhibitor of complement activity is Iptacopan (LNP023), a small-molecule factor B inhibitor approved for PNH. The drug is able to block the activation of the alternative pathway at the level of the C3 convertase; indeed, factor B is a serine protease that drives the central amplification loop of this pathway (133). Inhibition of factor B has been proven to prevent complement activation in different complement-dependent diseases, thus sounding promising also for the treatment of MG.

6 Future research directions to achieve precision medicine in MG

MG is a chronic autoimmune disease characterized by debilitating symptoms and unpredictable course. The development of biological drugs and the demonstration of their efficacy in clinical studies promise to profoundly change the treatment scenario for MG. Compared to conventional IS drugs, biological drugs are able to target specific disease effector molecules, thus minimizing the risk of adverse events. However, these drugs are used as add-on therapy, and hence their effect in monotherapy and in the early disease phase is unknown. Moreover, their high costs may limit their prescription as first-line therapy. As for IS drugs, response to biological drugs may vary among patients, according with individual differences in genetic, epigenetic, environmental and lifestyle factors and likely in the pathophysiological mechanisms underlying the disease.

Heterogeneity in MG clinical manifestations and inter-individual variation in the response to treatment highlight the need to adopt safer, more tailored and effective precision medicine approaches, still lacking in MG. The availability of different biological drugs highlights the importance of establishing criteria to select the better treatment option for individual patients or patients' subgroups. The identification of genetic, epigenetic or protein biomarkers able to predict unresponsiveness to conventional IS drugs and clinical benefits of the different

available biologicals is the key to individualization of MG care. When identified and properly validated, biomarkers of response to biological drugs may greatly facilitate introduction of these drugs in clinical practice, thus promising to improve therapeutic success in a cost-effective manner. Research on biomarkers and biomarker entry into clinical trials are pivotal steps for precision medicine in MG.

Research on biomarker identification may identify novel mechanisms associated with distinct pathological phenotypes, opening new hypotheses on MG pathogenesis and treatment.

An open research area is that of B10 cells. Therapeutic approaches aimed at restoring immunosuppressive B10 cell frequency could be successful for re-establishing B cell tolerance and reducing antibody-producing plasma cells. Moreover, since AChR-MG-associated long-lived plasma cells are resistant to most IS treatments and B cell-targeted therapies, they represent promising targets for therapies aimed at reducing autoantibody production in AChR-MG. Antigen-specific B-cell depletion has been recently described as a promising strategy for MuSK-MG patients (134). T cells expressing a MuSK chimeric autoantibody receptor with CD137-CD3 ζ signaling domains (MuSK-CAART) were engineered to target B cells expressing anti-MuSK autoantibodies. In an experimental autoimmune MG mouse model, MuSK-CAART reduced anti-MuSK IgG but not B cell counts or total IgG levels, indicating that MuSK-specific B cell depletion occurred in treated mice. Off-target cytotoxic interactions were not identified in mouse tissues or using high-throughput human membrane proteome array, thus suggesting MuSK-CAART as a safe and efficient strategy for the treatment of MuSK-MG (134).

FcRn blockage is particularly efficient in reducing circulating IgG and specific autoantibodies. In-depth investigation into the mechanisms underlying FcRn regulation of immune system cell (e.g. antigen presenting cell) functions could be useful to understand additional biological effects of drugs targeting this receptor, helpful for biomarker identification.

The involvement of the complement system has been widely demonstrated in AChR-MG patients and animal models. To date, it is unknown whether the prevailing autoantibody effector mechanism (e.g. complement-mediated damage vs antigenic modulation) is different among different MG patients, or whether different mechanisms co-exist in the same patient. As described above, by using a complement regulator-lacking HEK293T cell line-based model Obaidi and colleagues (116) measured the anti-AChR autoantibody-mediated MAC formation through flow cytometry in AChR-MG patients with a wide range in terms of disease severity and autoantibody titer. They identified a subset of patients lacking association between MAC formation and autoantibody binding or disease severity, indicating other complement-independent autoantibody-mediated mechanisms impairing the NMJ (116). The remaining patients showing a relationship between MAC formation, autoantibody binding and disease severity represent patients expected to benefit from complement inhibitor therapy (116). This and similar *in vitro* models able to predict the autoantibody-mediated effector mechanism in individual patients could be useful to pre-select AChR-MG patients as candidates for

anti-complement therapies. These patients could also be identified via complement-related protein profiling in serum, since complement components and activation products (119), or other proteins markers of the patient-specific degree of complement activity, could be easy-to-use biomarkers to predict responsiveness to anti-complement drugs. Common inherited variants in genes encoding complement components and regulators have been associated with complement-related diseases, suggesting that they can affect complement activity (135). These variants (i.e. haplotype) may contribute to the individual susceptibility to a higher or lower complement activation degree, thus representing additional candidate predictive biomarkers to be explored for their association with patient-specific response to anti-complement drugs and hence useful for treatment tailoring in each patient.

7 Conclusions

The advances in biological drugs' research promise to significantly change the therapeutic scenario for MG, since these drugs have the potential to overcome the limitations of conventional non-specific IS therapies and greatly increase therapeutic success. Different drugs have been developed, which specifically target different and distinct immune cells or mechanisms (Figure 1), thus highlighting the need to identify biomarkers (i.e. clinical, pathophysiological, molecular) of response predictive of the best option in individual patients. Despite stunning progresses in OMICs technology, the identification of OMIC-based biomarkers is still an open research area, and a top priority for research in MG. Biomarker profiling is indeed pivotal for patients' stratification in endotypes, offering the opportunity, or challenge, to discover endotype-associated pathogenetic features, factors and mechanisms to solve MG complexity and guide clinical trial design and therapy. In our vision, targeted therapies based on biological drugs and guided by biomarkers represent the new frontier for MG care to achieve precision medicine and improve patients' health and quality of life.

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PC: Writing – original draft. RM: Writing – review & editing. CA: Writing – review & editing.

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Conflict of interest

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The role of bilirubin as a biomarker of rheumatic diseases: a systematic review and meta-analysis

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The identification of novel, yet easily measurable biomarkers of inflammation and oxidative stress might assist in the diagnosis and management of patients with rheumatic diseases (RDs). We conducted a systematic review and meta-analysis of studies investigating the circulating concentrations of bilirubin, the end product of heme metabolism and a potent endogenous antioxidant with anti-inflammatory properties, in patients with RDs and healthy controls. The electronic databases PubMed, Scopus, and Web of Science were searched from inception to 31 December 2023 for relevant articles. We evaluated the risk of bias and the certainty of evidence using the Joanna Briggs Checklist and the Grades of Recommendation, Assessment, Development, and Evaluation Working Group system, respectively. In 17 eligible studies, all with low risk of bias, compared to controls, patients with RDs had significantly lower concentrations of total bilirubin (standard mean difference, SMD = -0.68, 95% CI -0.91 to -0.44, $p < 0.001$; $I^2 = 92.5\%$, $p < 0.001$; low certainty of evidence), direct (conjugated) bilirubin (SMD = -0.67, 95% CI -0.92 to -0.41, $p < 0.001$; $I^2 = 81.7\%$, $p < 0.001$; very low certainty of evidence), and the active antioxidant and anti-inflammatory indirect (unconjugated) form of bilirubin (SMD = -0.71, 95% CI -1.18 to -0.24, $p = 0.003$; $I^2 = 95.1\%$, $p < 0.001$; very low certainty of evidence). The results of the meta-analysis were stable in sensitivity analysis. In meta-regression, there were no significant associations between the SMD of total bilirubin and several clinical and demographic characteristics, including age, male to female ratio, number of participants, liver enzymes and erythrocyte sedimentation rate. In subgroup analysis, the SMD of total bilirubin was significant across a range of RDs, including rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren syndrome, and myositis. Therefore, the results of our systematic review and meta-analysis suggests that the reductions in bilirubin concentrations observed in patients with RDs reflect a state of impaired antioxidant and anti-inflammatory defence due to bilirubin consumption and highlight the promising role of this endogenous product as a biomarker of RDs.

Systematic review registration: <https://www.crd.york.ac.uk/prospero/>, identifier CRD42023500649.

KEYWORDS

bilirubin, oxidative stress, autoinflammatory diseases, mixed-pattern diseases, autoimmune diseases, rheumatic diseases, biomarkers, inflammation

Introduction

The term “rheumatic diseases (RDs)” includes a range of chronic, disabling conditions that are characterized by a pro-inflammatory and pro-oxidant state involving the musculoskeletal system and other organ and tissues. RDs are predominantly autoimmune (e.g., progressive systemic sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and Sjogren’s syndrome), mixed-autoimmune-autoinflammatory (e.g., ankylosing spondylitis, axial spondylarthritis, psoriatic arthritis, and Behcet’s disease), or autoinflammatory (e.g., familial Mediterranean fever) (1, 2).

The robust evidence of a dysregulation of inflammatory pathways and redox balance mechanisms in RDs has led to the routine use of circulating biomarkers of inflammation, e.g., C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and ferritin, to diagnose the presence of specific RDs (3–6). However, their limited diagnostic accuracy in several types of RDs has stimulated a significant body of research to identify better biomarkers (3, 7–9). Furthermore, the analytical challenges associated with the routine measurement of biomarkers of oxidative stress (10–12) has stimulated further research to identify the potential utility of other, more stable, and easily measurable endogenous compounds. One such compound is bilirubin, the product of heme metabolism which has been shown to exert potent endogenous antioxidant with anti-inflammatory effects in experimental and human studies (13–16). Physiologically, approximately 80% of bilirubin is generated from the breakdown of hemoglobin in senescent red blood cells and from erythroid cells undergoing premature destruction in the bone marrow (17). The rest is generated from the breakdown of several heme-containing proteins primarily located in the liver and muscle (17). In the blood, bilirubin is bound to albumin (unconjugated or indirect bilirubin) or conjugated with glucuronic acid (conjugated or direct bilirubin) and can be measured in either form as well as total bilirubin (17, 18). It is important however to emphasize that the reported anti-inflammatory and antioxidant effects of bilirubin are primarily attributed to the unconjugated or indirect form of bilirubin (19–22).

Given the increasing interest in the potential clinical and diagnostic utility of bilirubin in RDs, we conducted a systematic review and meta-analysis of studies investigating this endogenous antioxidant with anti-inflammatory properties in patients with RDs and healthy controls. We speculated that the presence of RDs was associated with a significant reduction in bilirubin concentrations, particularly the unconjugated or indirect form vs. healthy controls. We also investigated the presence of possible associations between the effect size of the between-group differences in bilirubin concentrations and several relevant demographic and clinical parameters, including specific types of RDs, age, male to female ratio, conventional biomarkers of inflammation (erythrocyte sedimentation rate, ESR), standard liver function enzymes (alanine aminotransferase, ALT, and aspartate aminotransferase, AST), study location, and study design.

Materials and methods

Search strategy and study selection

We conducted a systematic search of publications in the electronic databases PubMed, Web of Science, and Scopus from inception to 31 December 2023 using the following terms and their combination: “bilirubin” AND “rheumatic diseases” OR “rheumatoid arthritis” OR “psoriatic arthritis” OR “reactive arthritis” OR “ankylosing spondylitis” OR “systemic lupus erythematosus” OR “systemic sclerosis” OR “scleroderma” OR “Sjogren’s syndrome” OR “connective tissue diseases” OR “vasculitis” OR “Behcet’s disease” OR “idiopathic inflammatory myositis” OR “polymyositis” OR “dermatomyositis” OR “gout” OR “pseudogout” OR “systemic vasculitis” OR “ANCA-associated vasculitis” OR “Takayasu arteritis” OR “polyarteritis nodosa” OR “osteoarthritis” OR “fibromyalgia” OR “granulomatous polyangiitis” OR “Henoch-Schonlein purpura” OR “Wegener granulomatosis”. Each abstract was independently screened by two investigators. If relevant, the two investigators independently reviewed the full articles according to the following inclusion criteria: (i) the assessment of bilirubin concentrations in patients with RDs and healthy controls (case-control design), (ii) the inclusion of participants aged ≥ 18 years, (iii) the use of English language, and (iv) the availability of full-text. The references of each article were also hand searched for additional studies.

The following qualitative and quantitative variables were independently extracted and transferred to an electronic spreadsheet for analysis: year of publication, first author, study design (prospective or retrospective), study country, type of RD, sample size, age, male to female ratio, total bilirubin, direct bilirubin, indirect bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and erythrocyte sedimentation rate (ESR).

The risk of bias was assessed using the Joanna Briggs Institute (JBI) Critical Appraisal Checklist for analytical studies (23). The risk was considered low, moderate, and high for studies addressing $\geq 75\%$, $\geq 50\%$ and $< 75\%$, and $< 50\%$ of checklist items, respectively. The certainty of evidence was assessed using the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) Working Group system (24). The study complied with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 statement (Supplementary Tables 1, 2) (25). The study protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) registration number: CRD42023500649).

Statistical analysis

Standardized mean differences (SMDs) and 95% confidence intervals (CIs) were used to generate forest plots of continuous data

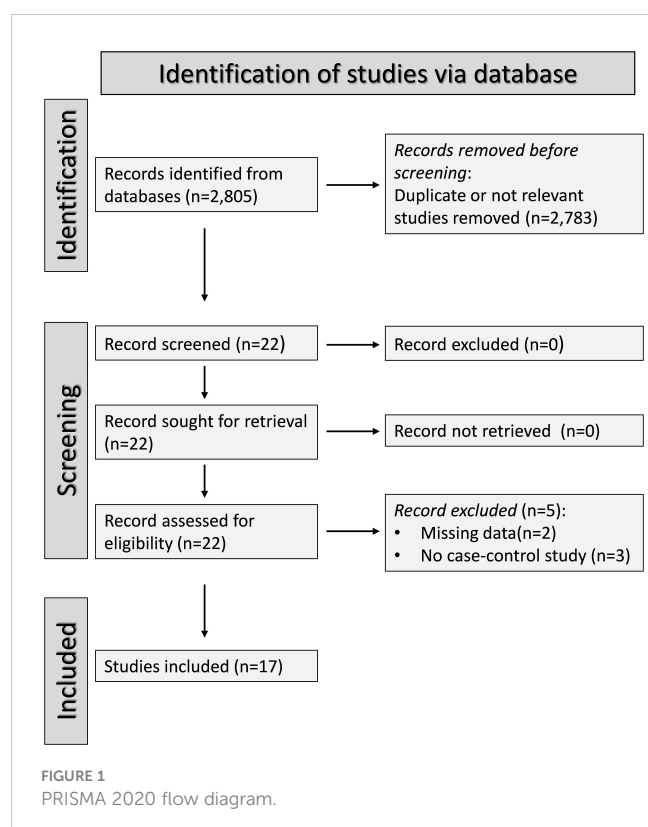
and to assess differences in bilirubin concentrations between patients with RDs and healthy controls. A p -value <0.05 indicated statistical significance. If required, the mean and standard deviation values were extrapolated from medians and interquartile ranges or medians and ranges using published methods (26). The heterogeneity of the SMD across studies was tested by using the Q statistic (significance level at $p<0.10$) and was interpreted as low when $I^2 \leq 25\%$, moderate when $25\% < I^2 < 75\%$, and high when $I^2 \geq 75\%$ (27, 28). A random-effect model based on the inverse-variance method was used in the presence of high heterogeneity. Sensitivity analysis was conducted to confirm the stability of the results (29). The presence of publication bias was assessed using the Begg's and the Egger's tests (30, 31), with a p -value <0.05 set as the level of significance, and the Duval and Tweedie "trim-and-fill" method (32).

Univariate meta-regression analyses were conducted to investigate associations between the effect size and the following parameters: year of publication, study design, country where the study was conducted, type of RD, sample size, age, male to female ratio, ALT, AST and ESR. Statistical analyses were performed using Stata 14 (Stata Corp., College Station, TX, USA).

Results

Study selection

A flow chart describing the screening process is described in Figure 1. From a total of 2,805 articles initially identified, 2,783 were



excluded because they were either duplicates or irrelevant. After a full-text review of the remaining 22 articles, a further two were excluded because of missing data and three because they did not have a case-control design, leaving 17 studies for further analysis (33–49) (Figure 1; Table 1). The risk of bias was considered low in all studies (Table 2). The initial certainty of evidence was also considered low because of the cross-sectional design of the studies selected.

Total bilirubin and rheumatic diseases

Sixteen studies including 17 group comparators investigated total bilirubin in a total of 2,102 patients with RDs (mean age 44 years, 80% females) and 2,492 healthy controls (mean age 45 years, 75% females) (33–47, 49). Eleven studies were conducted in China (34, 35, 38–41, 44–47, 49), two in Turkey (42, 43), one in the Czech Republic (33), one in Russia (36), and one in USA (37). Four study groups included patients with rheumatoid arthritis (39, 41, 43, 49), three with systemic lupus erythematosus (33, 34, 47), two with primary Sjögren syndrome (44, 45), one with psoriatic arthritis (36), one with systemic sclerosis (37), one with osteoarthritis (39), one with Takayasu arteritis (40), one with Behcet disease (42), one with axial spondyloarthritis (46), one with polymyositis (35), and one with both polymyositis and dermatomyositis (38). The study design was prospective in nine studies (33, 34, 36, 37, 40, 41, 46, 47, 49), retrospective in five (35, 39, 42, 44, 45), and unknown in the remaining two (38, 43).

The forest plot showed that the total bilirubin concentrations in patients with RDs were significantly lower when compared to controls (SMD = -0.68, 95% CI -0.91 to -0.44, $p<0.001$; $I^2 = 92.5\%$, $p<0.001$; Figure 2). Sensitivity analysis showed stability of the results, with the corresponding pooled SMD values ranging between -0.76 and -0.64 (Figure 3). There was no evidence of publication bias according to the Begg's ($p=0.59$) or the Egger's ($p=0.88$) test. The "trim-and-fill" method did not identify any missing study to be added to the funnel plot to ensure symmetry (Figure 4).

There were no significant associations between the effect size and age ($t=0.56$, $p=0.59$), male to female ratio ($t=1.51$, $p=0.16$), sample size ($t=-0.49$, $p=0.63$), AST ($t=0.19$, $p=0.85$), or ALT ($t=-0.39$, $p=0.71$) in univariate meta-regression analysis. A non-significant trend was observed between the effect size and ESR ($t=-2.23$, $p=0.053$, Figure 5A). This relationship was even more evident by cumulative analysis based on the ESR values of patients with RDs performed by metacum command (Figure 5B).

In subgroup analysis, the pooled SMD was significant in studies in patients with rheumatoid arthritis (SMD = -0.56, 95% CI -0.99 to -0.13, $p=0.01$; $I^2 = 90.9\%$, $p<0.001$), systemic lupus erythematosus (SMD = -0.98, 95% CI -1.47 to -0.50, $p<0.001$; $I^2 = 94.3\%$, $p<0.001$), primary Sjögren syndrome (SMD = -1.00, 95% CI -1.50 to -0.51, $p<0.001$; $I^2 = 83.8\%$, $p<0.001$) and myositis (SMD = -1.11, 95% CI -1.33 to -0.88, $p<0.001$; $I^2 = 29.3\%$, $p=0.234$, Figure 6), with a relatively lower between study variance in the myositis subgroup. The pooled SMD was statistically significant in studies conducted in China (SMD = -0.81, 95% CI -1.03 to -0.60, $p<0.001$; $I^2 = 89.0\%$,

TABLE 1 Characteristics of the studies investigating bilirubin in patients with rheumatic diseases and healthy controls.

Study	Healthy controls				Patients with rheumatic diseases				Disease type	Study design
	n	Age (Years)	M/F	Total bilirubin Direct bilirubin Indirect bilirubin (Mean ± SD)	n	Age (Years)	M/F	Total bilirubin Direct bilirubin Indirect bilirubin (Mean ± SD)		
Vitek L et al., 2010, Czech Republic (33)	180	39	93/87	12.53 ± 5.6 NR NR	218	39	32/186	7.03 ± 2.54 NR NR	SLE	P
Yang Z et al., 2012, China (34)	154	39	17/137	13.42 ± 5.43 2.75 ± 0.94 10.67 ± 4.49	179	36	22/157	8.25 ± 3.92 2.00 ± 1.49 6.00 ± 2.99	SLE	P
Peng YF et al., 2016, China (35)	108	45	34/74	15.2 ± 2.97 NR NR	77	47	22/55	11.7 ± 4.37 NR NR	PM	R
Smirnova SV et al., 2016, Russia (36)	30	NR	NR	12.13 ± 2.41 NR NR	98	NR	NR	15.77 ± 4.82 NR NR	PsA	P
van Bon L et al., 2016, USA (37)	20	NR	NR	0.62 ± 0.19 NR NR	41	NR	NR	0.47 ± 0.14 NR NR	SSc	P
Chen Z et al., 2017, China (38)	274	50	80/194	13.45 ± 4.7 3.81 ± 1.50 9.63 ± 3.65	110	51	34/76	8.31 ± 2.93 3.29 ± 1.22 5.03 ± 2.06	PM/DM	NR
Junping D et al. (a) 2017, China (39)	96	40	52/44	11.4 ± 3.01 4.17 ± 0.98 7.23 ± 2.26	35	59	8/27	7.57 ± 2.16 2.47 ± 1.08 5.13 ± 2.09	RA	R
Junping D et al. (b) 2017, China (39)	96	40	52/44	11.4 ± 3.01 4.17 ± 0.98 7.23 ± 2.26	81	56	47/34	10.73 ± 4.14 3.40 ± 1.43 7.50 ± 3.09	OA	R
Peng YF et al., 2017, China (40)	193	29	10/183	0.7 ± 0.22 NR NR	115	28	5/110	0.6 ± 0.31 NR NR	TA	P
Peng YF et al., 2017, China (41)	346	50	60/286	10.3 ± 4.16 NR NR	173	52	37/136	8.9 ± 3.99 NR NR	RA	P
Koca TT et al., 2018, Turkey (42)	75	46	44/31	0.53 ± 0.17 0.19 ± 0.61 0.33 ± 0.17	71	42	40/31	0.5 ± 0.25 0.0016 ± 0.008 0.39 ± 0.20	BD	R
Koca TT et al., 2019, Turkey (43)	75	52	40/35	0.52 ± 0.22 0.19 ± 0.01 0.37 ± 0.17	75	50	32/43	0.53 ± 0.22 0.15 ± 0.07 0.33 ± 0.17	RA	NR
Xie J et al., 2020, China (44)	100	51	17/83	13.67 ± 6.02 NR NR	97	54	12/12	9.67 ± 4.51 NR NR	pSS	R
Zhang Z et al., 2020, China (45)	138	48	17/121	10.88 ± 3.09 2.84 ± 1.15 7.93 ± 2.92	116	47	13/103	6.89 ± 3.3 2.32 ± 1.34 4.53 ± 2.53	pSS	R
Wang J et al., 2021, China (46)	78	40	55/23	16.15 ± 4.86 NR NR	78	41	51/27	9.97 ± 5.95 NR NR	axial SpA	P
Zhang W et al., 2023, China (47)	332	49	0/332	13.43 ± 4.62 NR NR	341	40	0/341	11 ± 4.1 NR NR	SLE	P

(Continued)

TABLE 1 Continued

Study	Healthy controls				Patients with rheumatic diseases				Disease type	Study design
	n	Age (Years)	M/F	Total bilirubin Direct bilirubin Indirect bilirubin (Mean ± SD)	n	Age (Years)	M/F	Total bilirubin Direct bilirubin Indirect bilirubin (Mean ± SD)		
Wang X et al., 2023, China (48)	73	44	38/35	10.1 ± 2.8 NR 10.1 ± 2.8	68	44	44/24	6.9 ± 3.7 NR 6.9 ± 3.7	PsA	R
Zhang H et al., 2023, China (49)	197	50	35/162	13.77 ± 3.66 NR NR	197	50	35/162	11.22 ± 3.92 NR NR	RA	P

BD, Behcet disease; DM, dermatomyositis; M/F, male to female ratio; NR, not reported; OA, osteoarthritis; P, prospective; PM, polymyositis; PsA, psoriatic arthritis; pSS, primary Sjögren syndrome; R, retrospective; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SpA, spondyloarthritis; SSc, systemic sclerosis; TA, Takayasu arteritis. Bilirubin values are reported as μmol/L or mg/dL.

$p < 0.001$) but not in other countries (SMD = -0.31, 95% CI -1.09 to 0.48, $p = 0.45$; $I^2 = 96.3\%$, $p < 0.001$; Figure 7). Furthermore, the pooled SMD was statistically significant both in prospective (SMD = -0.63, 95% CI -0.95 to -0.32, $p < 0.001$; $I^2 = 93.5\%$, $p < 0.001$) and retrospective studies (SMD = -0.77, 95% CI -1.18 to -0.37, $p < 0.001$; $I^2 = 90.1\%$, $p < 0.001$; Figure 8).

The overall level of certainty remained low (rating 2) after considering the low risk of bias in all studies (no change), the high but partly explainable heterogeneity (no change), the lack of indirectness (no change), the moderate effect size (SMD = -0.68) (50), and the absence of publication bias (no change).

Direct (conjugated) bilirubin and rheumatic diseases

Six studies including seven comparator groups investigated direct bilirubin concentrations in a total of 667 patients with RDs (mean age 46 years, 70% females) and 908 healthy controls (mean age 46 years, 67% females) (34, 38, 39, 42, 43, 45). Four studies were conducted in China (34, 38, 39, 45), and two in Turkey (42, 43). Two study groups included individuals with rheumatoid arthritis (39, 43), one with systemic lupus erythematosus (34), one with myositis (38), one with osteoarthritis (39), one with Behcet disease (42), and one with primary Sjögren syndrome (45). The study design was retrospective in three studies (39, 42, 45), prospective in one (34), and unknown in the remaining two (38, 43).

The forest plot showed that patients with RDs had significantly lower direct bilirubin concentrations when compared to controls (SMD = -0.67, 95% CI -0.92 to -0.41, $p < 0.001$; $I^2 = 81.7\%$, $p < 0.001$; Figure 9). The results were stable in sensitivity analysis, with the corresponding pooled SMD values ranging between -0.72 and -0.52 (Figure 10).

The assessment of publication bias, meta-regression, and subgroup analyses could not be conducted because of the limited number of studies. Consequently, the certainty of evidence was downgraded to very low (rating 1).

Indirect (unconjugated) bilirubin and rheumatic diseases

Seven studies including eight comparator groups investigated indirect bilirubin concentrations in a total of 735 patients with RDs (mean age 46 years, 67% females) and 981 healthy controls (mean age 45 years, 65% females) (34, 38, 39, 42, 43, 45, 48). Five studies were conducted in China (34, 38, 39, 45, 48), and two in Turkey (42, 43). Two study groups included patients with rheumatoid arthritis (39, 43), one with systemic lupus erythematosus (34), one with myositis (38), one with osteoarthritis (39), one with Behcet disease (42), one with primary Sjögren syndrome (45) and one with psoriatic arthritis (48). The study design was retrospective in four studies (39, 42, 45, 48), prospective in one (34), and unknown in the remaining two (38, 43).

The forest plot showed that patients with RDs had significantly lower indirect bilirubin concentrations when compared to healthy controls (SMD = -0.71, 95% CI -1.18 to -0.24, $p = 0.003$; $I^2 = 95.1\%$, $p < 0.001$; Figure 11). Sensitivity analysis confirmed the stability of the results, with the corresponding pooled SMD values ranging between -0.82 and -0.60 (Figure 12).

The assessment of publication bias, meta-regression, and subgroup analyses could not be performed because of the limited number of studies. Consequently, the certainty of evidence was downgraded to very low (rating 1).

Discussion

The reported significant reductions in circulating bilirubin concentrations in patients with RDs when compared to healthy controls, particularly the indirect (unconjugated) fraction which possesses anti-inflammatory and antioxidant effects *in vitro* and *in vivo* and the total fraction, which includes the unconjugated fraction, suggest a significant dysregulation in redox balance and inflammatory pathways in these patients. Notably, the results of our systematic review and meta-analysis also suggest that such

TABLE 2 Assessment of the risk of bias using the Joanna Briggs Institute critical appraisal checklist.

Study	Were the inclusion criteria clearly defined?	Were the subjects and the setting described in detail?	Was the exposure measured in a reliable way?	Were standard criteria used to assess the condition?	Were confounding factors identified?	Were strategies to deal with confounding factors stated?	Were the outcomes measured in a reliable way?	Was appropriate statistical analysis used?	Risk of bias
Vitek L et al. (33)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Yang Z et al. (34)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Peng YF et al. (35)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Smirnova SV et al. (36)	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Low
van Bon L et al. (37)	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Low
Chen Z et al. (38)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Junping D et al. (39)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Peng YF et al. (41)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Peng YF et al. (40)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Koca TT et al. (42)	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Low
Koca TT et al. (43)	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Low
Xie J et al. (44)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Zhang Z et al. (45)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Wang J et al. (46)	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Low
Zhang W et al. (47)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Wang X et al. (48)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low
Zhang H et al. (49)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Low

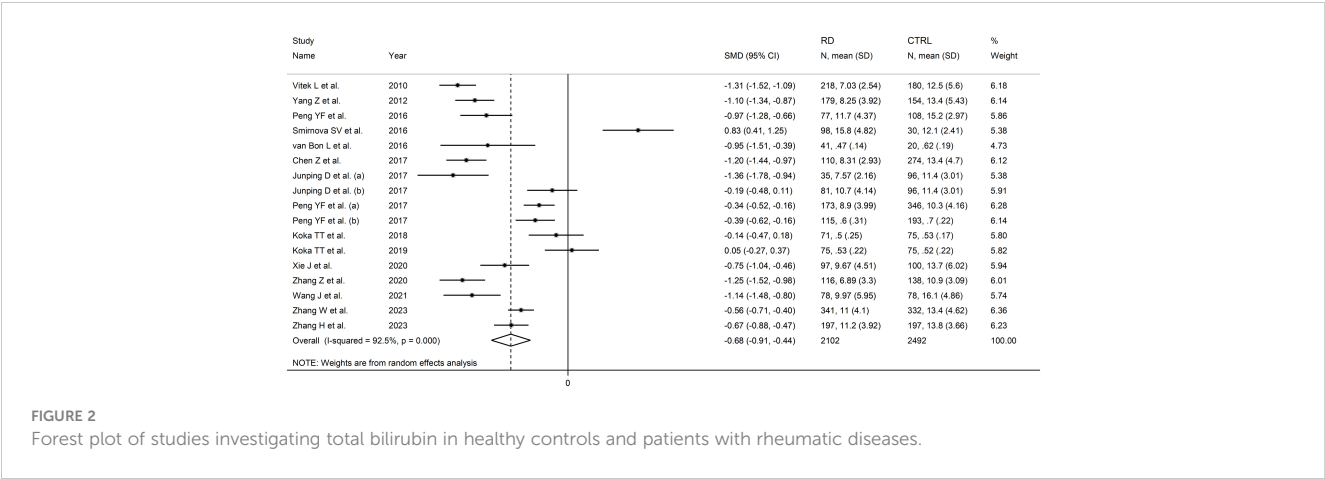


FIGURE 2 Forest plot of studies investigating total bilirubin in healthy controls and patients with rheumatic diseases.

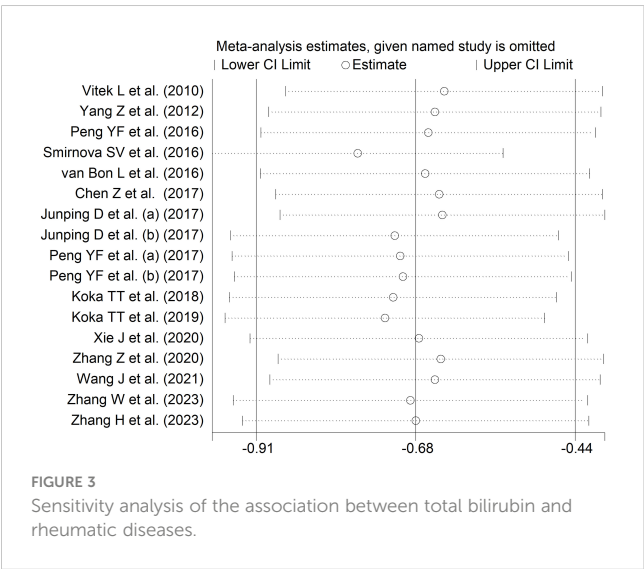


FIGURE 3 Sensitivity analysis of the association between total bilirubin and rheumatic diseases.

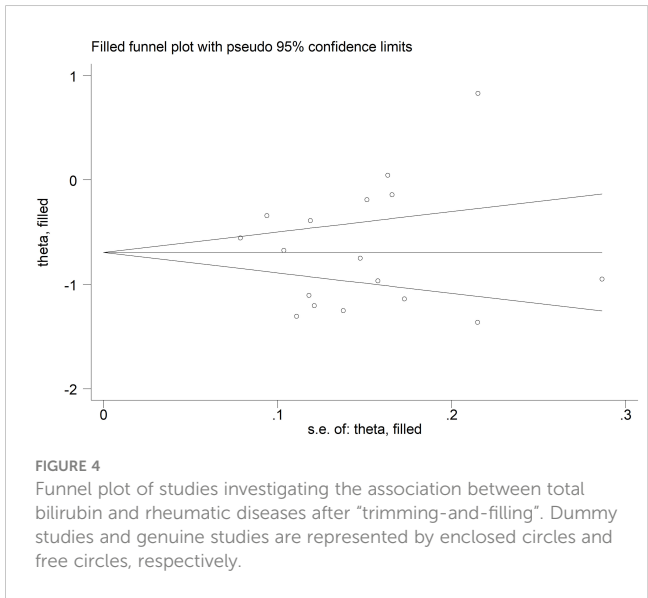


FIGURE 4 Funnel plot of studies investigating the association between total bilirubin and rheumatic diseases after "trimming-and-filling". Dummy studies and genuine studies are represented by enclosed circles and free circles, respectively.

alterations can be easily captured through the measurement of this endogenous end product of heme metabolism, which has been part of routine hematological and liver assessment in clinical practice for over 60 years (18, 51). Sensitivity analyses confirmed the stability of the results of the meta-analysis. Meta-regression analyses, only possible for total bilirubin, did not show any significant associations between the effect size and age, male to female ratio, sample size, AST, or ALT, and a non-significant trend with ESR. Subgroup analyses, also specifically conducted on total bilirubin, showed that the SMD was significant across several types of RDs belonging to the classic polygenic autoimmune type (e.g., rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren syndrome, and myositis). In further analyses, the pooled SMD was statistically significant in studies conducted in China but not in other countries but was similarly significant in prospective and retrospective studies.

One of the landmark experiments demonstrating the antioxidant effects of bilirubin was reported by Stocker et al. in 1987. The investigators speculated that bilirubin contains an extended system of conjugated double bonds and a reactive hydrogen atom with potential antioxidant properties. In their *in*

vitro experiments, bilirubin at micromolar concentrations was able to efficiently scavenge peroxide radicals generated in homogenous solution or multilamellar liposomes (52). However, prior to these experiments, there was evidence that bilirubin was able to prevent the oxidation of vitamin A and unsaturated fatty acids (53). Subsequent studies reported a protective effect of bilirubin against the development of atherosclerosis and inverse associations with biomarkers of inflammation, including C-reactive protein (54), monocyte chemoattractant protein-1 (55), tumour necrosis factor- α (56), and interleukin-6 (13, 56). There is also evidence that bilirubin can exert immunomodulatory effects, e.g., inhibiting the complement cascade by interrupting the binding of the C1 complex to the antibody (57). These effects were confirmed in *in vivo* experiments where the infusion of unconjugated bilirubin in rats primed for intravascular hemolysis through complement fixation reduced the lysis of blood cells (58). Unconjugated bilirubin has also been shown to interact with macrophages (59), inhibit the cell surface expression of MHC II class molecules on antigen-presenting cells (60), and inhibit lymphocyte proliferation (61). Therefore, the observation of a relative deficiency in

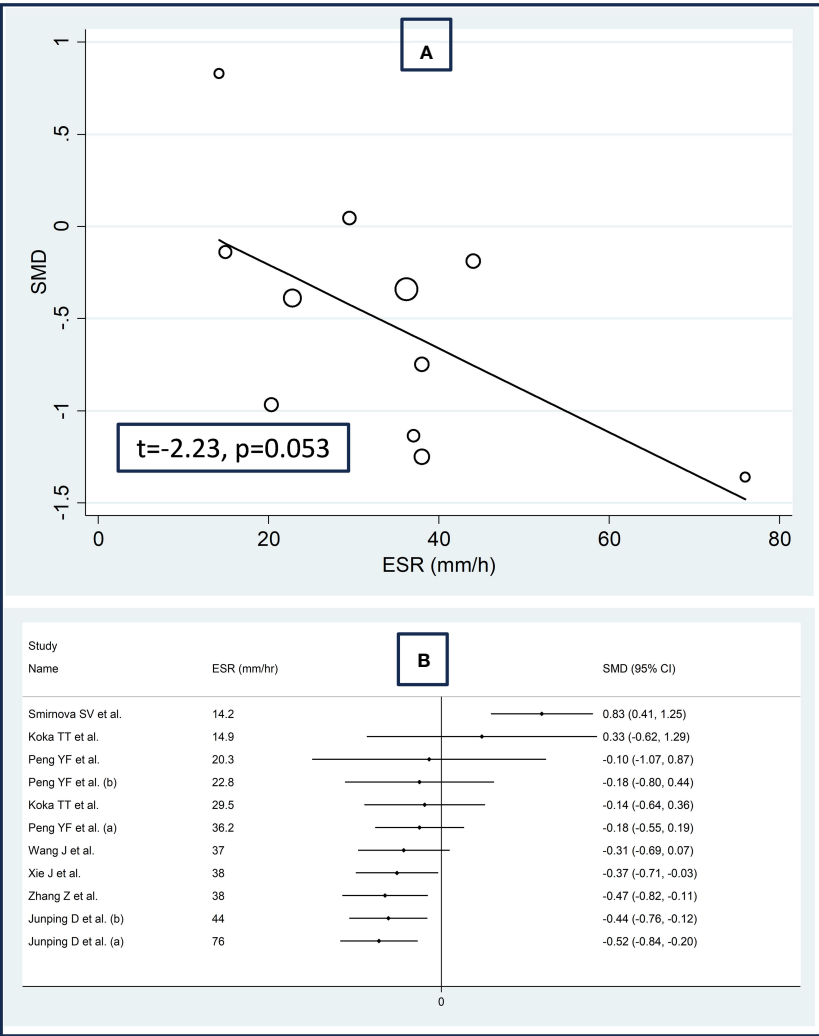


FIGURE 5 Bubble plot reporting the univariate meta-regression analysis between the effect size and erythrocyte sedimentation rate (A) and cumulative meta-analysis of total bilirubin concentrations based on the erythrocyte sedimentation rate (B).

unconjugated bilirubin in the presence of RDs might suggest a consumption process driven by scavenging peroxide radicals and/or inflammation although the exact mechanisms responsible require further investigation (15). While the results of our study suggest the potential utility of bilirubin as a biomarker of inflammation and oxidative stress in RDs, they also raise the intriguing possibility of using bilirubin therapeutically to combat the dysregulated redox and inflammatory pathways in these conditions. Pending further research to corroborate this hypothesis there is evidence that several interventions can increase bilirubin concentrations. For example, acute weight loss in overweight and obese subjects has been associated with a progressive increase in serum bilirubin concentrations, with each 1% weight loss associated with a mean 0.21 $\mu\text{mol/L}$ increase in men ($p<0.001$) and 0.11 $\mu\text{mol/L}$ increase in women ($p<0.001$) (62). Notably, the investigation of possible associations between the effect size of between-group differences in bilirubin concentrations and body mass index using meta-

regression could not be conducted in our study as only four articles reported information on this anthropometric parameter (36, 39, 41, 47). A higher intake of flavonoid-rich fruit and vegetables and intensive regular exercise have also been shown to increase bilirubin concentrations (63, 64). Finally, several pharmacological agents, e.g., non-steroidal anti-inflammatory drugs, statins, fibrates, and niacin have been shown by increase bilirubin following the activation of heme oxygenase-1 (65). Accurately designed prospective studies are now warranted to determine whether the elevations in bilirubin concentrations induced by these interventions translate into tangible antioxidant and anti-inflammatory effects and, consequently, into a clinical improvement in patients with RDs. An interesting observation in subgroup analysis for total bilirubin was the presence of significant between-group differences in studies conducted in China but not in those conducted in other countries, suggesting the presence of ethnic differences in bilirubin concentrations. Previous studies conducted

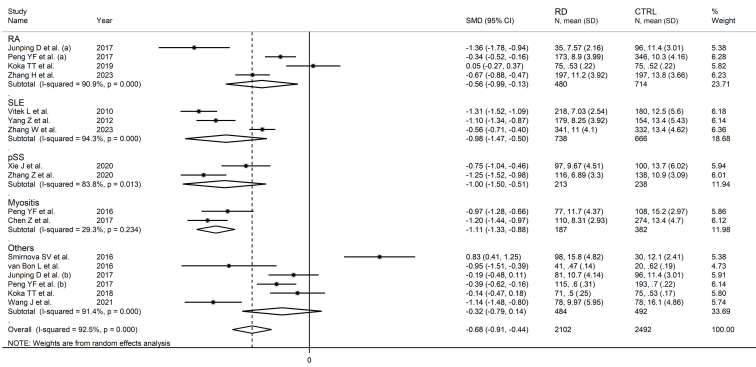


FIGURE 6
Forest plot of studies investigating total bilirubin according to the type of rheumatic disease.

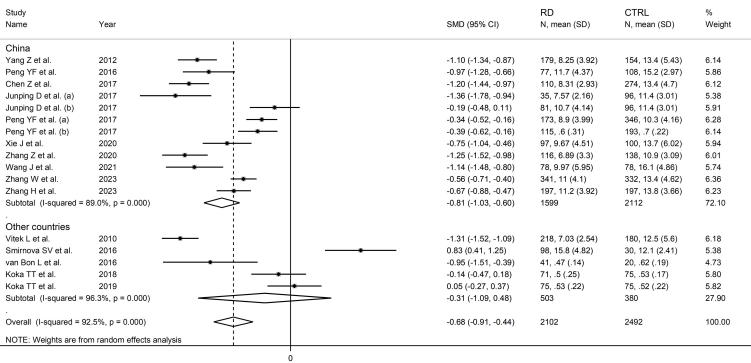


FIGURE 7
Forest plot of studies investigating total bilirubin according to the study country.

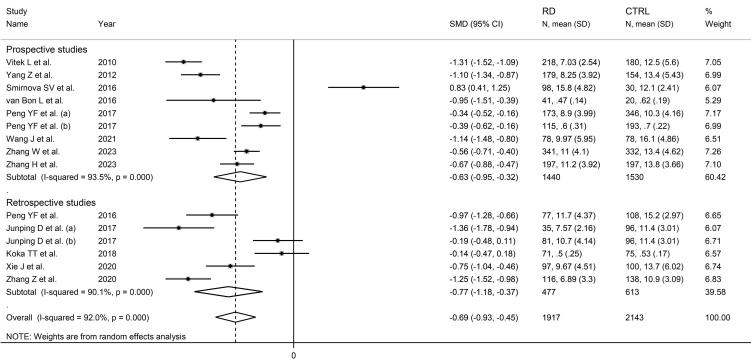


FIGURE 8
Forest plot of studies investigating total bilirubin according to study design.

in USA have reported significant ethnic-related differences in bilirubin concentrations, with African Americans having lower bilirubin concentrations when compared to white Caucasians and Mexican Americans (66). Clearly, more research is needed to confirm the reported reductions in bilirubin concentrations in RDs patients of different ethnicity and geographical location.

Our study has several strengths, including the assessment of bilirubin concentrations in different types of RDs within the autoinflammatory-autoimmune continuum including autoinflammatory, mixed-pattern, and autoimmune diseases (67, 68), the assessment of associations between the effect size and several study and patient characteristics, and a rigorous

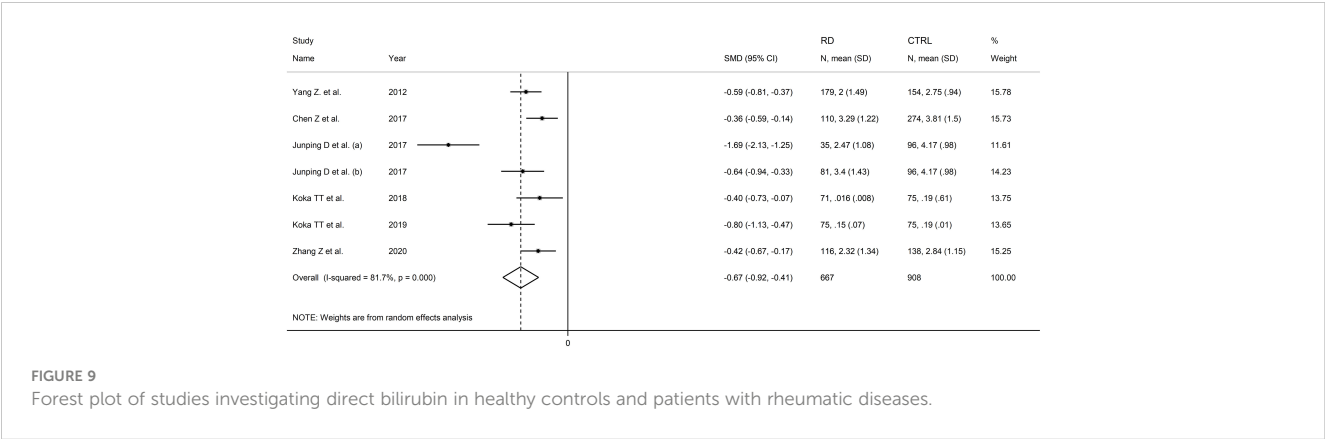


FIGURE 9
Forest plot of studies investigating direct bilirubin in healthy controls and patients with rheumatic diseases.

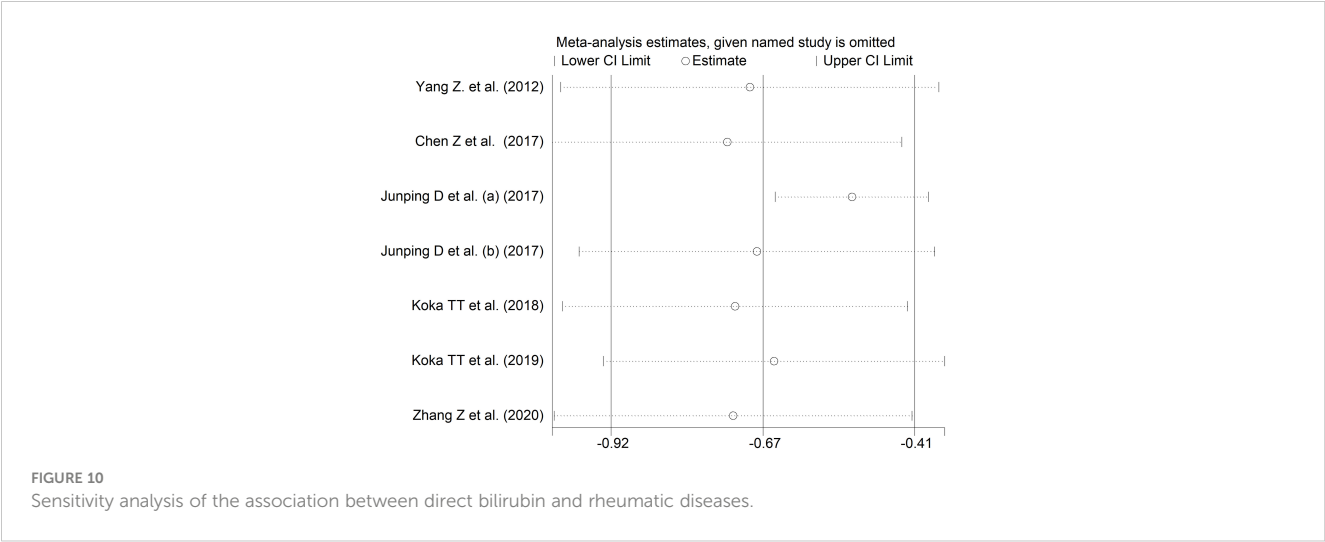


FIGURE 10
Sensitivity analysis of the association between direct bilirubin and rheumatic diseases.

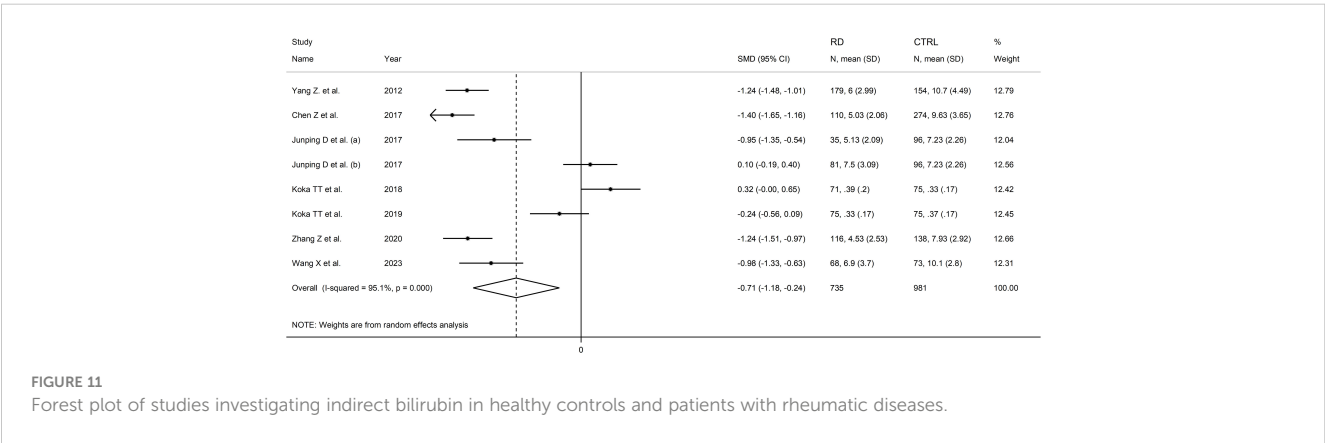
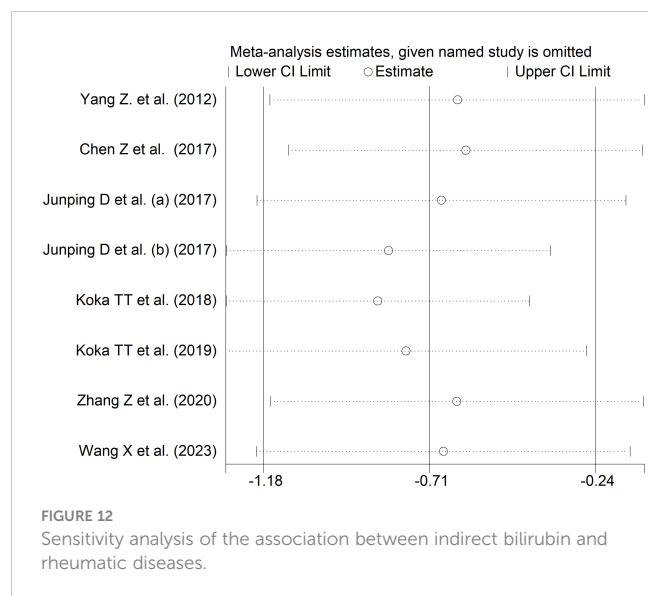


FIGURE 11
Forest plot of studies investigating indirect bilirubin in healthy controls and patients with rheumatic diseases.

evaluation of the risk of bias and the certainty of evidence. Furthermore, sensitivity analysis ruled out the effect of individual studies on the overall effect size. Important limitations include the focus of the studies identified in our search on a restricted number of RDs (systemic lupus erythematosus, polymyositis, dermatomyositis, psoriatic arthritis, systemic sclerosis, rheumatoid arthritis, osteoarthritis, Takayasu arteritis, Behcet disease, primary Sjögren syndrome, and spondyloarthritis), and the paucity of evidence from studies conducted in specific

geographical locations, particularly Europe and North and South America. These issues require further study given the established evidence of differences in inflammatory response across different types of RDs and ethnic groups (69–74). Additional issues worth investigating in future studies are the diagnostic accuracy of bilirubin concentrations in RD patients with co-existing liver disease, a phenomenon recently described in patients with autoimmune conditions (75), and the effects of fasting and specific dietary patterns (76–78).



In conclusion, our systematic review and meta-analysis has shown the potential utility of bilirubin as an easily measurable and widely available biomarker of RDs. However, additional research is required to confirm these observations and determine whether this endogenous antioxidant and anti-inflammatory agent can enhance the diagnostic capacity of current biomarkers and other clinical parameters in patients with different types of RDs and ethnicity and co-existing liver disease and, potentially, serve as a therapeutic strategy in this group.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Supplementary material

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Deciphering the molecular landscape of rheumatoid arthritis offers new insights into the stratified treatment for the condition

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Background: For Rheumatoid Arthritis (RA), a long-term chronic illness, it is essential to identify and describe patient subtypes with comparable goal status and molecular biomarkers. This study aims to develop and validate a new subtyping scheme that integrates genome-scale transcriptomic profiles of RA peripheral blood genes, providing a fresh perspective for stratified treatments.

Methods: We utilized independent microarray datasets of RA peripheral blood mononuclear cells (PBMCs). Up-regulated differentially expressed genes (DEGs) were subjected to functional enrichment analysis. Unsupervised cluster analysis was then employed to identify RA peripheral blood gene expression-driven subtypes. We defined three distinct clustering subtypes based on the identified 404 up-regulated DEGs.

Results: Subtype A, named NE-driving, was enriched in pathways related to neutrophil activation and responses to bacteria. Subtype B, termed interferon-driving (IFN-driving), exhibited abundant B cells and showed increased expression of transcripts involved in IFN signaling and defense responses to viruses. In Subtype C, an enrichment of CD8+ T-cells was found, ultimately defining it as CD8+ T-cells-driving. The RA subtyping scheme was validated using the XGBoost machine learning algorithm. We also evaluated the therapeutic outcomes of biological disease-modifying anti-rheumatic drugs.

Conclusions: The findings provide valuable insights for deep stratification, enabling the design of molecular diagnosis and serving as a reference for stratified therapy in RA patients in the future.

KEYWORDS

gene expression profiles, machine learning, rheumatoid arthritis, stratification, unsupervised clustering

1 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammatory polyarthritis, systemic inflammation, and autoantibody production. Uncontrolled RA results in progressive joint destruction and impaired functioning. Early and effective treatment using immunomodulatory medications is critical due to the potential for long-term chronic disease complications.

Nonsteroidal anti-inflammatory drugs, disease-modifying anti-rheumatic drugs (DMARDs), and glucocorticoids are commonly used conventional drugs for RA treatment. In recent years, DMARDs have assumed a more prominent role in managing RA and have been further categorized into conventional synthetic DMARDs (csDMARDs), biological DMARDs (bDMARDs), and target synthetic DMARDs according to the 2016 EULAR guideline (1). The 2021 ACR guidelines recommend starting treatment with csDMARDs in DMARD-naïve patients. As second-line therapies, bDMARDs such as Rituximab and Infliximab are commonly utilized (2). Despite various available treatments, managing RA remains complex due to variations in practice based on the latest EULAR and ACR guidelines and challenges in predicting treatment outcomes and choosing the most effective medications (2). This underscores the necessity to identify and characterize patient subtypes that share similar goals and biological markers.

Currently, the widely utilized rapid assessment of disease activity in RA patients is the Disease Activity Score 28 (DAS28) (3, 4). However, DAS28 may not be suitable for all patients across different stages of RA and cannot precisely guide treatment decisions. Consequently, there is growing interest in developing more specific evaluation criteria and identifying predictors of response to biologics in RA. Recent studies have focused on the stratification of RA patients to predict prognosis and drug response more accurately. For example, a study by Lewis et al. utilized deep phenotypic profiling of RA synovial tissue, identifying transcriptional subtypes linked to three distinct cell-specific pathobiological modules (5). Their integration of ultrasonographic and radiographic data revealed that the plasma cell-infiltrated synovial module was associated with antibodies

against citrullinated proteins (ACPA) positivity and a poorer prognosis in terms of radiographic damage at 12 months. Additionally, Jung et al. categorized RA synovial tissue gene expression into one inflammatory and two fibroblast subtypes, validating treatment responses in patients treated with a triple DMARD regimen (Methotrexate [MTX], Sulfasalazine, and Hydroxychloroquine) and Infliximab (6). These subgroups exhibited significant differences in ACPA positivity and treatment response. Moreover, Kraan et al. performed gene set enrichment analysis on peripheral blood data, identifying a high or low subtype based on the type I interferon (IFN) signature, and investigated the response to MTX treatment in this subtype (7). They found that IFN expression signature was a persistent trait in RA patients irrespective of MTX treatment. While these studies highlight the potential of stratifying RA patients for understanding disease pathogenesis and tailoring treatment more precisely, there remains a notable gap in research using large, publicly available peripheral blood databases, particularly concerning the response of stratified patients to bDMARDs. Therefore, it is imperative to leverage such publicly accessible data for RA stratification to refine individualized therapeutic strategies.

In the present study, we have constructed the largest genome-scale transcriptomic profiles of RA to identify patient subtypes sharing various pathways and signaling signatures. Additionally, we have evaluated the therapeutic outcomes of bDMARDs, providing a novel perspective for treating RA patients.

2 Methods

2.1 Data selection and processing

The whole workflow of our study is presented in Figure 1. We selected four recently compiled, independent microarray datasets (GSE97810, GSE110169, GSE74143, GSE45291) (8–11) of RA peripheral blood mononuclear cells (PBMCs) from the Gene Expression Omnibus (GEO) database as the training sets. These datasets consisted of a total of 1,138 RA patients and 97 HCs. Additionally, three RNA sequencing (RNA-seq) datasets (GSE138746, GSE129705, GSE120178) (12–14) from the GEO database were collected as test sets, comprising a total of 268 RA patients and 20 HCs. Given that our study focused on investigating RA patients, more RA patients were enrolled to align well with real-world conditions and enhance its clinical relevance (15, 16).

We included several DMARDs in the analysis to study the association between RA peripheral blood subtypes and therapeutic response. Specifically, MTX (GSE93272), Infliximab (GSE93272, GSE78068 and GSE58795), Tocilizumab (GSE93272, and GSE78068), Abatacept (GSE78068), and Rituximab (GSE54629) (17–19). Supplementary Table S1 provides more details on the study design and pre-processing of the microarray datasets.

The therapeutic response to conventional DMARDs such as MTX or biologic DMARDs (Infliximab, Tocilizumab [TCZ], Abatacept, and Rituximab) was evaluated by DAS28. Patients' response was defined according to the EULAR response criteria (20, 21). For example, patients with a DAS28 score below 2.6 were

Abbreviations: RA, rheumatoid arthritis; PBMCs, peripheral blood mononuclear cells; GEO, Gene Expression Omnibus; HCs, healthy controls; DEGs, differentially expressed genes; RNA-seq, RNA sequencing; DMARDs, Disease-modifying anti-rheumatic drugs; MTX, Methotrexate; TCZ, Tocilizumab; DAS28, Disease Activity Score 28; PCA, Principal components analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; CDF, cumulative distribution function; FDR, False discovery rate; log FC, Log fold change; GSEA, Gene-set enrichment analysis; ssGSEA, Single-sample gene-set enrichment analysis; ROC, receiver operating curve; AUC, area under the curve; MAF, minor allele frequency; cis-pQTL, cis-protein quantitative trait loci; HEIDI, heterogeneity in dependent instruments; PP, Posterior probabilities; GO-BP, Gene Ontology Biological Process; IFN, Type I interferon; IL, Interleukin; BCR, B cell receptor; TCR, T cell receptor; DC, Dendritic cells; RF, Rheumatoid factor; ACPA, Antibodies against citrullinated proteins; CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; SDAI, Simplified Disease Activity Index; TNF, Tumor necrosis factor.

considered responders, while those with higher scores were classified as non-responders.

To process the raw microarray data files from Affymetrix®, the robust multi-array average approach was used. It involved background correction, quantile normalization, and probe-set summarization using the ‘affy’ and ‘Simpleaffy’ R packages (22). The normalized matrix files for raw microarray data from Illumina were directly downloaded.

The expression profile of the RNA-seq data was converted to transcripts per kilobase millions format to allow for comparisons with the microarray datasets. To mitigate systematic, dataset-specific biases, the ‘ComBat’ function in the ‘sva’ R package was employed to adjust for residual technical batch effects resulting from integrating heterogeneous data (23). The ‘ComBat’ algorithm also showed its robustness when dealing with datasets from heterogeneous platforms, even the ones lacking HCs. Detailed validation information was presented in [Supplementary Tables S2, S3](#) and [Supplementary Figures S4, S5](#).

To ensure quality assurance and assess distribution bias, principal component analysis (PCA) was conducted on the identical datasets before and after normalization and batch correction.

2.2 Differentially expressed genes obtaining and functional enrichment analysis

We performed differential gene expression analysis between RA patients and healthy controls using the “limma” R package, incorporating a linear model and a modified t-test (24). We adjusted the p-values using the false discovery rate (FDR) correction to account for multiple hypothesis testing. The Benjamin-Hochberg method was used to control the proportion of false-positive results (25). A threshold of adjusted p-value < 0.05 and log fold change (log FC) > 0.32 was set to determine the significance of DEGs.

To gain insights into the biological functions of the up-regulated DEGs, we conducted functional enrichment analysis using Metascape. This analysis included Gene Ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, and Reactome. Enrichment terms with an adjusted p-value < 0.05 were considered significantly enriched (26).

2.3 Gene-set enrichment analysis

To investigate the potential biological processes or signaling pathways associated with the up-regulated DEGs between RA patients and healthy controls, we performed gene-set enrichment analysis (GSEA) using the GSEA software developed by UC San Diego and the Broad Institute (27–29). We obtained gene-set information on signaling pathways and biological processes from the KEGG and Reactome databases. Gene sets that were enriched among the up-regulated DEGs and had an FDR of less than 0.05 were identified.

2.4 Unsupervised clustering for gene expression-driven subtypes in RA patients

We utilized the R software “Consensus Cluster Plus” to perform hierarchical agglomerative clustering to subtype RA patients based on transcriptome signatures of PBMCs (30). The Partitioning Around Medoids algorithm, employing the Euclidean distance and Ward-D2 linkage, was applied for clustering the data. The core of Consensus clustering is to utilize resampling to generate subsamples from the original sample. These subsamples are then divided into a maximum of k groups and finally evaluated by analyzing the results obtained from multiple resampling returns. To ensure a robust stratification of subtypes and the consistency of the clustering results, we repeated the procedure across 1,000 reruns for k clusters. The number of clusters was determined using the cumulative distribution function (CDF). Unsupervised clustering results were also validated using PCA.

2.5 Molecular characterization by pathway and cell subset-driven enrichment analysis

In order to determine the activity levels of specific biological pathways within the three categorized subtypes, we employed single-sample gene-set enrichment analysis (ssGSEA) (31). This method utilizes an enrichment score to quantify the degree of enrichment in each sample for a given gene set within a dataset. We utilized publicly available resources, namely KEGG and Reactome databases, to identify RA-associated pathways. We also employed the “xCell” algorithm to calculate immune cell-type signature enrichment scores and determine cellular composition for the three subtypes (32). We utilized the Wilcoxon test to compare the enrichment scores, which represent pathway activity and cell-type signature, between any two of the three subtypes. Meanwhile, we employed the Kruskal-Wallis test for the comparison of all three subtypes. A p-value < 0.05 was considered statistically significant. Additionally, FDR correction was also applied to control the Type 1 error rate. Finally, the comparison of enrichment involving cell types and signaling pathways among subtypes would be considered significant at FDR < 0.05.

2.6 Development of an XGBoost classification model for subtype prediction

We constructed a decision tree using the XGBoost-tree approach in a multi-classification context with a softmax objective function to predict subtypes based on 404 gene characteristics (33). The receiver operating characteristic curve (ROC)’s area under the curve (AUC) was used to assess how well the prediction models performed. To train the classifier, 1,138 RA peripheral blood samples were divided into training (n = 799) and testing (n = 339) sets, with a respective ratio of 70% and 30%, utilizing the ‘caret’ R package. The subtype labels and expression values of the up-regulated DEGs were derived from the results of the unsupervised clustering approach. To mitigate

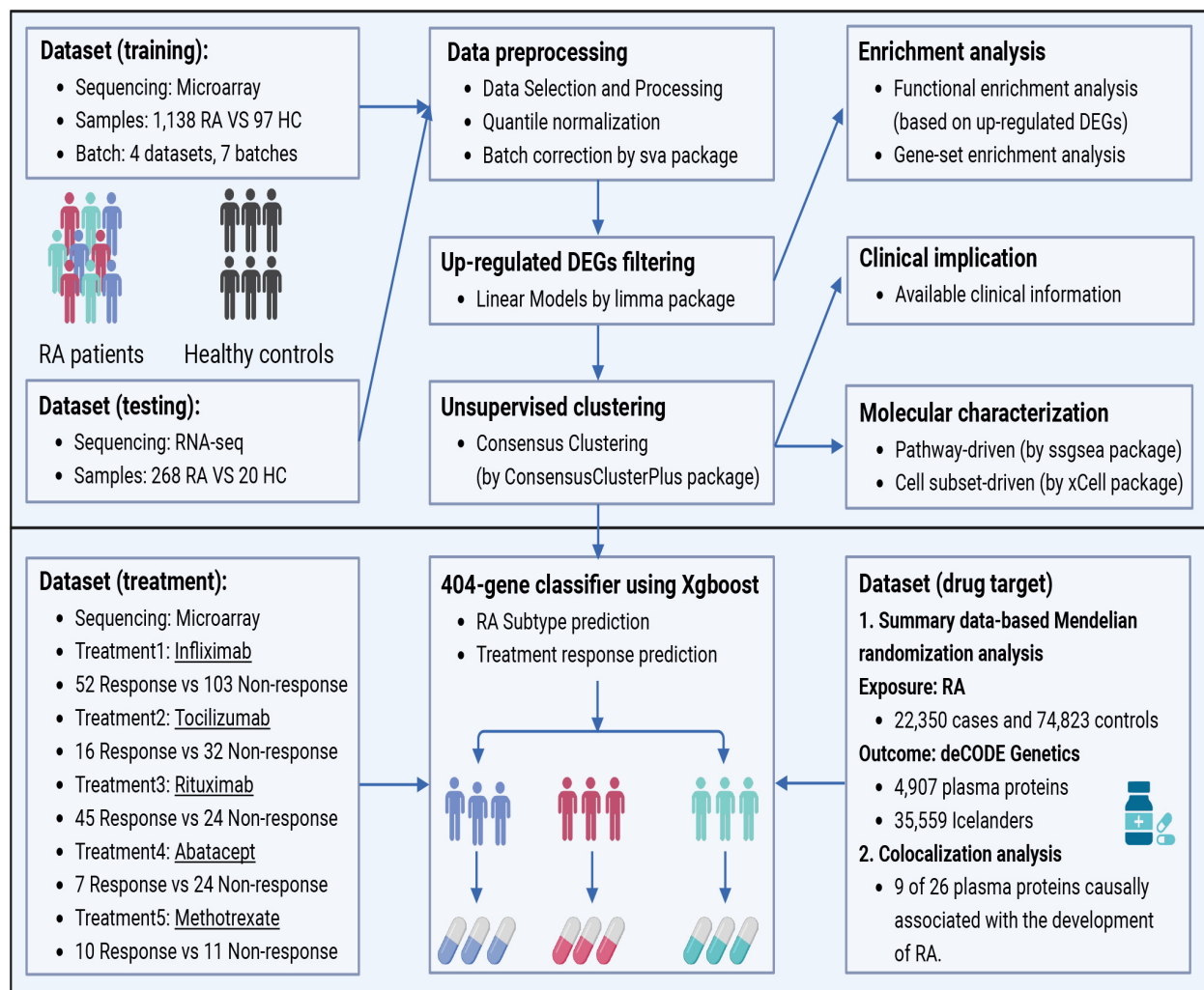


FIGURE 1

The workflow of data processing procedures in the study. Four microarray datasets containing 1,138 RA patients and three RNA-seq datasets including 268 RA patients were selected as training sets and test sets respectively from the public database. DEGs were filtered after the normalization, and unsupervised clustering was performed with enrichment analysis followed. Then, the XGBoost algorithm was contrived to predict the responses of stratified subtypes to commonly used five treatments. Finally, in search for more novel therapeutic targets of RA patients, drug prediction was carried out by utilizing SMR and the Open Targets platform. DEGs, differentially expressed genes; SMR, Summary data-based Mendelian randomization analysis.

overfitting, we used 10-fold cross-validation during the training process, and the fitted model was then utilized to assign subtypes to the testing sets using the 404-gene classifier.

2.7 SMR revealing the effects of plasma proteome on the risk of RA

To facilitate the discovery of RA drug targets, we used summary-data-based Mendelian randomization (SMR) and colocalization analysis for in-depth studies. Briefly, For the GWAS summary statistics for RA, we used the largest meta-analysis to date, comprising 22,350 cases and 74,823 controls of European origin (34). Quality control compared the RA GWAS data to the 1,000 Genomes Project Phase 3 European reference for the hg19 genome construction, excluded non-autosomal single

nucleotide polymorphisms (SNPs), filtered out SNPs without rsID or with duplicated rsID and only kept biallelic SNPs with minor allele frequency (MAF) > 0.01. Summary-level statistics of genetic associations with levels of 1,881 plasma cis-protein quantitative trait loci (cis-pQTL) were extracted from 4,907 plasma proteins in 35,559 Icelanders (35). For each protein, we contained cis-acting SNPs (i.e. SNPs located within ± 1 Mb of the gene body of the target gene). We used the cis-pQTLs as genetic instruments to evaluate the causal association between plasma proteins and the risk of RA.

SMR is a Mendelian randomization method that tests for shared causal variation between exposures and outcomes using summary-level data (36). In addition, we assessed whether the associations were in the presence of linkage disequilibrium using the heterogeneity in dependent instruments (HEIDI) test and removed single-nucleotide variants with $p < 0.01$. Based on the Benjamini-Hochberg method, FDR < 0.05 was considered statistically significant.

We performed further analyses using colocalization methods to determine whether there are potential shared causal variants between causal proteins and RA (37). A total of five posterior probabilities (PP) for mutually exclusive hypotheses were computationally generated (1). No genetic correlation for either trait (H0); (2) only trait 1 has a causal genetic variant (H1); (3) only trait 2 has a causal genetic variant (H2); (4) both traits have their own causal genetic variant, independent and different (H3); (5) both traits have the same shared causal genetic variation (H4). A colocalized locus was declared when the posterior probability of H4 (PP.H4) was greater than 0.7.

Finally, we searched the plasma proteins (PP.H4 > 0.7) using the Open Targets database to determine the potential druggability of the identified proteins (38).

2.8 Statistical analyses

For categorical variables, two groups were compared using the Wilcoxon test, and the Kruskal-Wallis test for comparisons involving more than two groups. To control the overall Type 1 error rate, FDR correction was also applied and FDR less than 0.05 was considered

significant. As the variables for RA subtypes and clinical measures are numerical, we used the chi-square test or Fisher's exact test to analyze the correlation between the two. Statistical significance was defined as $p < 0.05$ for a two-tailed test. All statistical analyses were performed using R software (version 4.0.3).

3 Results

3.1 Screening and functional enrichment analysis of DEGs

Upon comparing the gene expression profiles of peripheral blood samples from RA patients and HCs, we identified 404 up-regulated DEGs. These DEGs were visualized using volcano and heat maps (Figures 2A, B). Functional enrichment analysis of the DEGs revealed gene ontology biological process terms that align with the current understanding of RA pathophysiology. Specifically, GO analysis indicated significant enrichment of biological processes such as response to viruses and bacteria, inflammatory response, organelle inheritance, and cellular macromolecule biosynthetic process (Figure 2C).

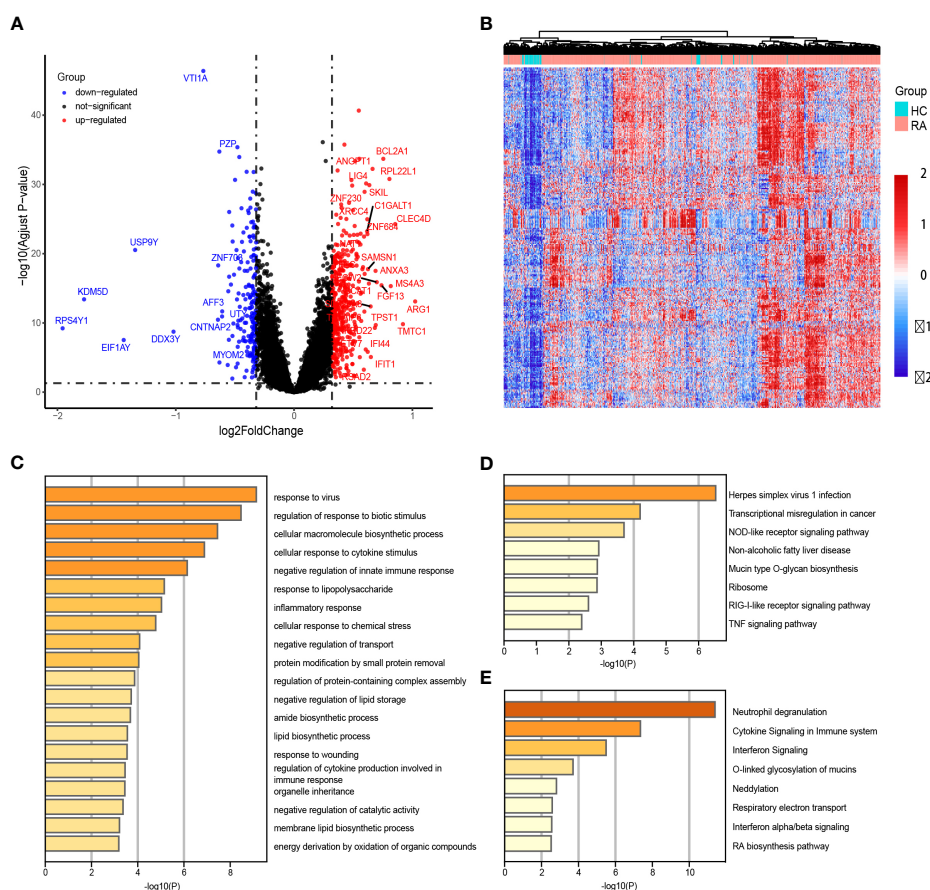


FIGURE 2

Identification of differentially expressed genes (DEGs) between patients with rheumatoid arthritis (RA) and healthy controls (HCs). (A, B) The heatmap and volcano plot of DEGs in RA patients versus HCs. (C–E) GO enrichment, KEGG and Reactome analyses of 163 up-regulated DEGs.

Additional KEGG analysis indicated that these elevated DEGs mostly enriched the TNF signaling, RIG-I-like receptor signaling, and NOD-like receptor signaling pathways (Figure 2D). Additionally, Reactome analysis demonstrated significant enrichment of the Interferon alpha/beta signaling pathways and Respiratory electron transport (Figure 2E).

3.2 Unsupervised cluster analysis identifies RA peripheral blood gene expression-driven subtypes

We employed unsupervised cluster analysis to classify RA patients with different peripheral blood phenotypes based on the expression patterns of DEGs. This analysis was repeated 1,000 times to determine the optimal number of clusters, ranging from $k = 2$ to 6, using the CDF value and delta area as evaluation criteria. Our results indicated that $k = 3$ was the optimal number of subtypes (Figures 3A–C).

3.3 Molecular processes and biological functions of three subtypes in RA

To elucidate the potential pathological mechanisms underlying RA subtypes, we examined the molecular processes and their biological functions within each subtype (Figures 3D–F). By comparing, with HCs, the specific up-regulated DEGs signatures in the three subtypes, we identified 161 significantly up-regulated DEGs in subtype A, 109 in subtype B, and 166 in subtype C (Figure 3G). Next, using the GO Biological Process (GO-BP) and Reactome databases in Metascape, we explored the signaling pathways and most notably dysregulated biological processes in each subtype.

Subtype A was mainly enriched in biological processes related to response to bacteria, neutrophil-mediated immunity, and inflammatory response (Figure 3D). Subtype B exhibited significant activation of pathways involved in viral processes, including antiviral innate immune response, response to viruses, and interferon signaling (Figure 3E). Notably, subtype C was associated with protein synthesis processes, such as peptide chain lengthening, cytoplasmic translation, and transcription of DNA-templated genes (Figure 3F).

3.4 Molecular and cellular characterization of three RA subtypes

The three clustered subtypes were labeled as subtype A ($n = 399$), subtype B ($n = 356$), and subtype C ($n = 383$). By comparing the enrichment scores of cell subsets and key RA-related pathways, we observed distinct immune-inflammatory characteristics among the three subtypes. The enrichment scores of 12 RA-related signaling pathways from literature, KEGG, and Reactome databases exhibited significant differences among the three subtypes after FDR correction (Figure 4A).

Specifically, subtype A was characterized by neutrophil activation, including pathways such as NOD-like receptor, Toll-like receptor, Interleukin (IL)-17, and various Interleukins (ILs) signaling pathways. On the other hand, subtype B exhibited prominent enrichment in IFN activation, including IFN- α/β and IFN- γ pathways. Subtype C did not show significant molecular characterization differences compared to the other two subtypes, however, subtype B and subtype C demonstrated greater significance than subtype A in the B cell receptor (BCR) and T cell receptor (TCR) signaling pathway.

Furthermore, using the xCell software and a machine learning framework, we estimated the enrichment of different cell types and validated differential activation across the three subtypes (Figure 4B). Subtype A displayed substantial infiltration of neutrophils, macrophages M2, and eosinophils. In contrast, B-cells, plasma cells, dendritic cells (DC), and macrophages M1 were more prominent in subtype B. Subtype C exhibited moderate enrichment in most cell types but had high expression of CD8⁺ T-cells.

3.5 Clinical implication of gene-driven subtypes in RA

To delve deeper into the association between molecular subtypes of RA and clinical attributes, we scrutinized the variations across the three subtypes concerning autoantibodies and disease activity. Autoantibodies serve as a distinctive feature of RA, notably rheumatoid factor (RF) and ACPA. Seropositive patients with the three subtypes of RA exhibited positivity for RF (A: 87.0%; B: 94.0%; C: 94.6%) and ACPA (A: 88.3%; B: 88.0%; C: 80.4%) (Figure 5A).

Moreover, we discovered that the levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in subtype A and subtype B were typically higher than in subtype C. Notably, CRP, commonly utilized as an indicator of systemic inflammation in RA, was abundantly expressed in subtype A (Figure 5B). Tools such as the Health Assessment Questionnaire (HAQ), DAS28, and Simplified Disease Activity Index (SDAI) are widely employed to evaluate disease activity. These tools corroborated that the disease activity index was notably higher in the subtype A of RA. However, these differences lacked statistical significance, possibly due to the constrained sample size.

Subsequently, we observed an elevated expression of neutrophils and lymphocytes in subtype A and subtype C, respectively, while the expression of basophils and eosinophils in subtype B showed a decrease (Figure 5C). These findings further corroborate the variances in clinical characteristics across the three subtypes.

3.6 Verification of classification results using RNA-seq datasets

We integrated three RNA-seq datasets from PBMCs, all comprehensively adjusted for batch effects and biases (Supplementary Figures S1A–D). To validate our classification results, these datasets encompassed 288 individuals, including 268

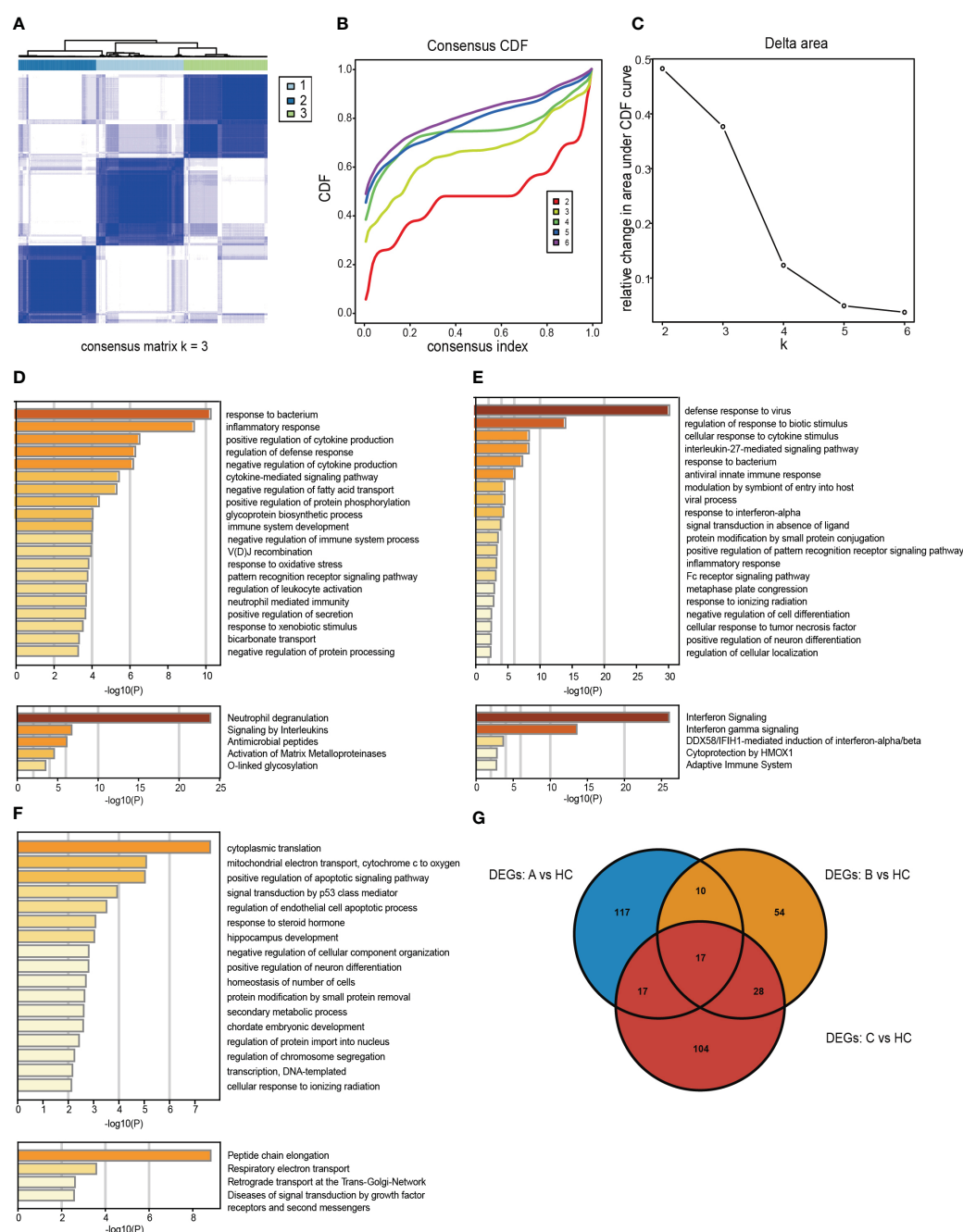


FIGURE 3

Identification and gene expression characterization of rheumatoid arthritis (RA) subtypes. **(A)** The consensus score matrix for RA samples when $k = 3$. A higher consensus score between the two samples indicated they were more likely to be assigned to the same cluster in different iterations. **(B)** Consensus clustering for the cumulative distribution function for $k = 2-6$. **(C)** Relative changes in the area under the cumulative distribution function curve for $k = 2-6$. **(D-F)** Molecular pattern distribution of three subtypes of RA in different biological processes and pathways. The top 20 most significantly enriched biological processes in each subtype of GO BP database and the top 5 most important signaling pathways in the Reactome database. **(G)** A Venn diagram showing up-regulated DEGs in subtype A, subtype B and subtype C compared with HCs.

RA patients and 20 HCs. Using the gene expression profiles of 213 up-regulated DEGs, we segregated the patients into three subtypes: subtype A ($n = 95$), subtype B ($n = 95$), and subtype C ($n = 80$) (Supplementary Figures S2A-E).

We further examined the enrichment scores of RA-related pathways and cell subsets across the three subtypes (Supplementary

Figures S3A, B). The consensus from our observations indicated that subtype A was principally enriched in neutrophil activation-related pathways and responses to bacteria. In contrast, subtype B exhibited an abundance of transcripts in IFN signaling and defense responses to viruses. Meanwhile, subtype C was notably associated with CD8+ T-cells.

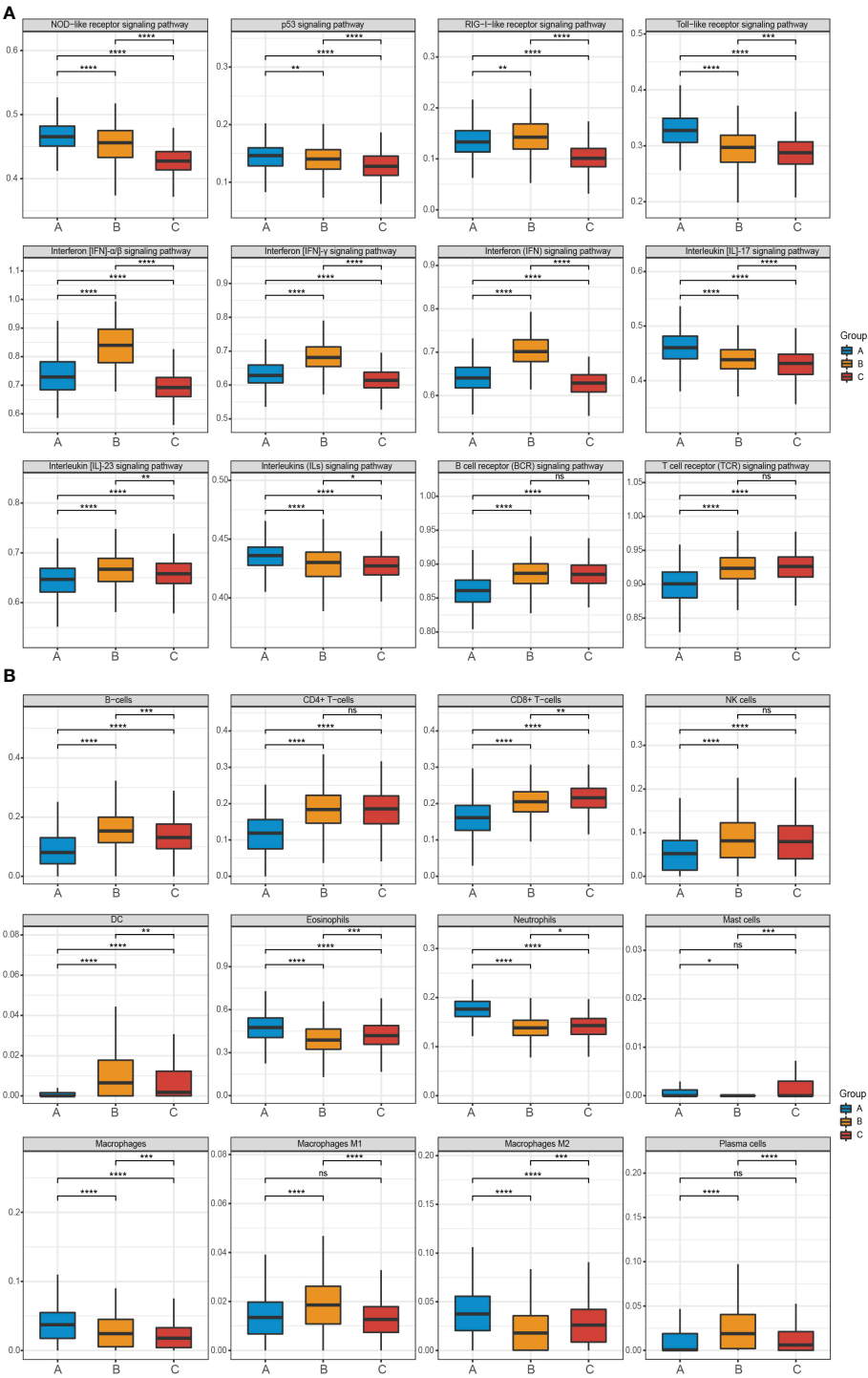


FIGURE 4 Pathway and cell subset-driven characterization in RA subtypes. **(A, B)** Enrichment scores for pathways and cell subsets for each RA subtype. Box plots for the enrichment scores of pathways and cell subsets for each RA subtype. Wilcoxon test was used to analyze the differences across three subtypes. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. FDR, false discovery rate.

3.7 Construction of a RA gene classifier and treatment responses of gene-driven subtypes

We devised a 404-gene classifier using an XGBoost machine learning algorithm to validate our RA subtyping scheme. We applied

this classifier to a training set of 799 RA samples and a testing set of 339 RA samples. Our results attest to the practicality and robustness of this classifier, which successfully categorized the training set with an average area under the curve (AUC) value of 100%. Moreover, testing set validation achieved an accuracy of 89.68%, with an average AUC value of 90.99%. Hence, we concluded that the classifier serves

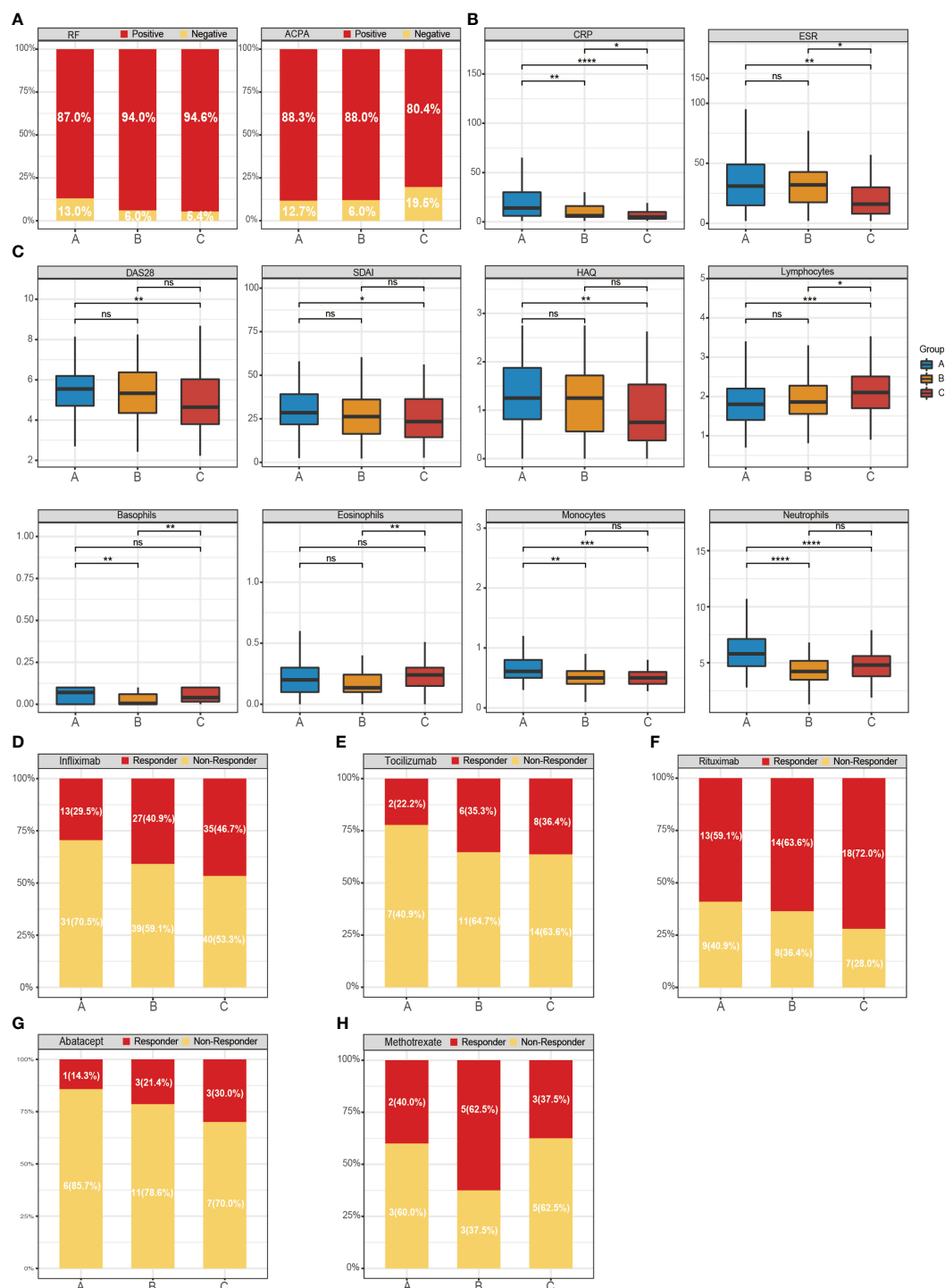


FIGURE 5

Distribution of gene-driven subtypes and multiple biologics treatments response to the RA subtypes. (A–C) The variations across the three subtypes concerning autoantibodies, disease activity and four immune cells. The box plots show the disease activity scores of the three subtypes and the enrichment scores of immune cells. Responder: responded to the biologics; non-responder: did not respond to the biologics. (D) Responder/non-responder to infliximab: 29.5%/70.5% in subtype A, 40.9%/59.1% in subtype B and 46.7%/53.3% in subtype C. (E) Responder/non-responder to Tocilizumab: 22.2%/77.8% in subtype A, 35.3%/64.7% in subtype B and 36.4%/63.6% (0/5) in subtype C. (F) Responder/non-responder Rituximab: 59.1%/40.9% in subtype A, 63.6%/36.4% in subtype B and 72.0%/28.0% in subtype C. (G) Responder/non-responder to Abatacept 14.3%/85.7% in subtype A, 21.4%/78.6% in subtype B and 30.0%/70.0% in subtype C. (H) Responder/non-responder MTX: 40.0%/60.0% in subtype A, 62.5%/37.5% in subtype B and 37.5%/62.5% in subtype C. Wilcoxon test was used to analyze the differences across three subtypes. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

as a viable and potent strategy for assessing RA subtypes in clinical trials.

Assessing the response to biological agents across different RA patients is crucial for elucidating the disease's pathological specificity. Next, the 404-gene classifier was applied to predict the treatment response. We evaluated the response of Infliximab, TCZ, Rituximab, Abatacept, and MTX across the three RA subtypes (Figures 5D–H). Our findings indicated that subtype B (62.5%) exhibited a superior response to MTX compared to subtype A (40%) and C (37.5%). The three RA subtypes showed favorable responses to Rituximab, a B-lymphocyte-depleting agent, with subtype C achieving a response rate as high as 72%.

However, the response rates to Infliximab (a TNF inhibitor), TCZ (a humanized IL-6 receptor-inhibiting monoclonal antibody), and Abatacept (a T-cell co-stimulation modulator) across the different subtypes were comparatively low. In particular, subtype C consistently showed higher proportions of positive responses to Infliximab (46.7%), TCZ (36.4%), and Abatacept (30.0%) compared to subtypes A and B. In summary, the efficacy of certain targeted biological agents is intimately linked to RA patients with specific subtypes.

3.8 Causal proteins determined by SMR and colocalization analysis

We performed an in-depth study of MR associations between 1,881 proteins with cis-pQTL and the risk of RA outcome using the SMR approach. We identified 197 unique proteins (SMR $p < 0.05$), of which 26 passed the HEIDI Test and FDR correction. Further colocalization analysis identified 9 proteins that may have significant roles in RA disease progression (PP.H4 > 0.7), including FCRL3, IL1RN, CCN4, NMB, MAPK3, HAPLN4, CILP2, ICOSLG, and TMEM9 (Figure 6). Among them, MAPK3 was identified as the highest-risk protein (FDR adjusted $P = 2.56 \times 10^{-4}$).

3.9 Druggability of the causal proteins

To further determine the potential new therapeutic targets of RA patients, we investigated the druggability of nine plasma proteins highlighted by colocalization analysis. Specifically, we identified six drugs targeting MAPK3 and ICOSLG proteins. For instance, AMG-557, an inducible co-stimulator (ICOS) ligand inhibitor, attenuates inflammation by inhibiting the accumulation of polyfunctional T helper 1 and T helper 17 cells. Ravoxertinib, Ulixertinib and MK-8353 serve as MAP kinase ERK1 inhibitors, and Temuterkib and KO-947, are ERK1/ERK2 inhibitors. They are all involved in the inflammatory response and tissue destruction in RA through mitogen-activated protein kinase (MAPK) and play a crucial role in the pathogenesis of RA. Therefore, whether the drugs that regulate these proteins can be reused for the treatment of RA requires further clinical experimental research.

4 Discussion

In this study, we explored differential expression patterns, significant pathways, and cellular components utilizing the most comprehensive microarray and RNA-seq datasets for RA to date. Employing an unsupervised cluster analysis, we identified three distinct subtype clusters. Subtype A was found to be enriched in neutrophil activation-related pathways and responses to bacteria. Subtype B, abundant in B cells, demonstrated an increased number of transcripts involved in IFN signaling and defense responses to viruses. Subtype C was discovered associating with CD8+T cells. These subtypes exhibited distinct clinical characteristics, including RF, ACPA positivity, and clinical assessments such as DAS28 scores.

Many preceding studies employing the stratification method have illustrated RA subtypes that can help predict potential prognoses for RA patients. Kraan et al. conducted a large-scale expression profiling by cDNA microarrays on peripheral blood, highlighting the identification of

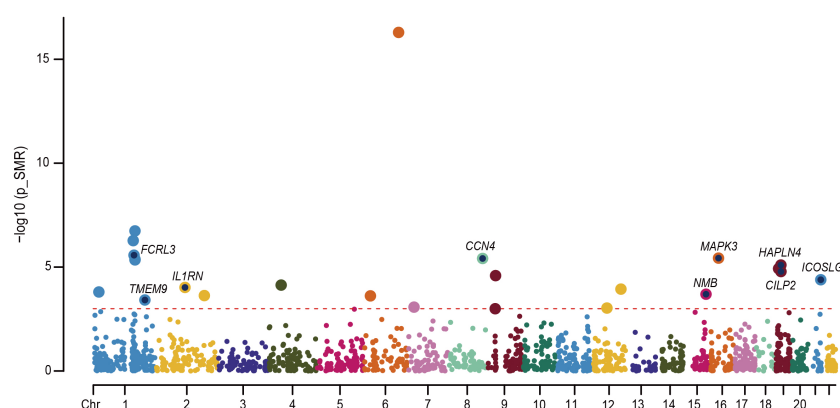


FIGURE 6

Manhattan plot for the 9 proteins identified in RA. Each point in the plot indicates a single association test between a plasma protein and RA as the $-\log_{10}(P)$ of a z-score test result which is ordered by genomic position on the x-axis and the association strength on the y-axis. The red horizontal line represents the significant threshold for the P value of FDR less than 0.05 under Bonferroni correction.

subtypes using complex IFN markers. They demonstrated that RA patients had much higher levels of IFN type I-regulated gene expression than healthy people. IFN-response genes showed increased expression in approximately half of the patients (IFN high patients). The IFN high group significantly varied from the IFN low group, according to pathway analysis, showing elevated pathways related to fatty acid metabolism, complement cascades, and coagulation (7). Our findings showed that subtype B aligns with the IFN cluster from the previous study. Furthermore, the enrichment of IFN signaling was also noted in other autoimmune disorders akin to subtype B of RA. For instance, Lanata et al. utilized an unsupervised clustering approach to stratify patients with systemic lupus erythematosus (SLE) and identified significant enrichment of genes associated with IFN signaling, antiviral responses, and inflammatory pathways (39). Mi et al. also identified two distinct IFN-1 subtypes in SLE patients. Surprisingly, they pointed out that IFN-1 might be a critical susceptibility factor for SS, potentially elucidating the pathogenesis of SLE patients who also develop SS (40). These findings strongly supported that identical signaling pathways exist in autoimmune diseases however they could also be clues for investigating the possible mechanism of the comorbidity of autoimmune diseases such as RA and SLE, proving the salience of investigating the stratification of patients.

Our study unveils that subtype A is associated with the inflammatory response, particularly the response to bacteria. The pathway of neutrophil degranulation, likely related to innate immunes such as the NOD-like receptor signaling pathway and ILs signaling pathway, is enriched in subtype A. We postulate that the delayed apoptosis, incited by NF- κ B signaling activated by RA neutrophils, could exacerbate inflammation (41). With external pathogen irritation, the abnormal activation of the innate immune system and NOD-like receptor signaling may promote the secretion of ILs, which could trigger autoinflammatory and autoimmune responses. Subtype B contrasts with type A patients by displaying an abundance of interferon signaling, including both type-I and type-II interferon, CD +4 T cells, and B cells (42, 43). Naive CD4+ T cells become activated and differentiate into diverse T helper cell subsets that produce interferons in response to antigenic stimulation and cytokine signaling (44). IFN and IFN-related signaling pathways partly promote the inflammatory and adaptive response in RA patients. We also observed that both ACPA and RF show a relatively high positivity in subtype B patients. IL-21 produced by follicular helper CD4 T cells acts directly on B cells via their IL-21 receptors (IL21R), leading to the production of autoantibodies, including ACPA and RF (45–47). B cells are capable of producing both proinflammatory and anti-inflammatory cytokines, which might make the inflammatory state in peripheral blood for RA worse (48).

In addition to the above neutrophil activation and IFN signaling subtypes, CD8+ T cell factors also contribute to RA development. Due to ACPA+ RA being associated with major histocompatibility complex class II (MHC class II) HLA-DRB1 alleles, T cell studies in RA have focused primarily on CD4+ T cells. However, RA also exhibits genetic associations with alleles in the MHC class I HLA-B locus, highlighting the probable importance of CD8+ T cells (49, 50). It has been demonstrated that IL21R signaling in CD8+ T cells controls CD8+ T cell development and affects cellular metabolism, especially under conditions of persistent antigen presentation (46, 51, 52). In patients with subtype C, the abundant IL-21

produced by CD4+T cells plays a crucial role in RA development by acting on the IL-21R of CD8+T cells. Prior research and our results suggest that joint inflammation and destruction in RA are not exclusively antibody-driven and that IL-21/IL21R signaling may further drive autoimmune pathogenesis through autoreactive CD8+T cells (46).

To unravel and understand the disease heterogeneity of RA, we researched the response of biological agents to different RA subtypes by analyzing the notable therapeutic effect of Infliximab, TCZ, Rituximab, Abatacept, and MTX on patients of different subtypes. Treatment responses of gene-driven subtypes indicate that patients with specific subtypes may benefit more from some targeted biological agents than patients with other subtypes. Humby et al. has compared three non-tumor necrosis factor (TNF) α inhibitors drugs, including Rituximab, a B-lymphocyte depleter, Abatacept, which targets T-cell co-stimulation, and TCZ, an IL-6 receptor inhibitor. It was found that among adults with refractory rheumatoid arthritis, the results of Rituximab and TCZ were superior to those of Abatacept two years later, which aligns with our findings (53, 54). Furthermore, Rituximab, directed against CD20, affects the B cell population and reduces antibody production, therefore, demonstrating high therapeutic efficacy for the three subtypes. Infliximab exerts therapeutic effects on RA patients by inhibiting TNF- α binding to its target receptors and preventing the production of other proinflammatory cytokines, including IL and GCSF (55). Gerlach et al. observed that many CD8+T cells upregulate CX3CR1 upon pathogen challenge (56). Moreover, a study concluded that the CX3CL1-CX3CR1 system in patients with active RA might be sensitive to anti-tumor necrosis factor-alpha therapy and confirmed that CX3CL1 plays a critical role in the pathogenesis of RA, which may validate better therapeutic effects of Infliximab on C-type patients characterized by CD8+T cells compared to other types (57). MTX remains a cornerstone in treating rheumatoid arthritis and other rheumatic diseases. Previous studies showed that T cells isolated and activated ex vivo from RA patients treated with MTX have a diminished capacity to produce IFN γ , IL-4, IL-3, TNF, and granulocyte-macrophage colony-stimulating factor (58, 59). Over-activation of the interferon pathway, a characteristic pattern of mRNA expression, has been demonstrated in RA patients, and the same results have been observed in subtype B. Our work demonstrates great efficacy in some patient subtypes and indicates a potentially important result in efficacy that is now buried in unstratified analysis.

Furthermore, our SMR analysis based on cis-pQTL identified a total of six drugs including AMG-557, Ravoxertinib, Ulixertinib, MK-8353, Temuterkib and KO-947. Of these, AMG-557 as a class of autoimmune disease drugs has already completed the phase II clinical trial of Sjogren's syndrome (NCT02334306) and phase I clinical trial of systemic lupus erythematosus (NCT02391259, NCT00774943 and NCT01683695) (60). Whereas Ravoxertinib, Ulixertinib, MK-8353, Temuterkib and KO-947 are currently used primarily to treat tumors. It is notable that both MAPK3 and ICOLSG, the protein targets of the above drugs, have now been shown to play a vital role in rheumatoid arthritis disease progression (61, 62). In the future, further in-depth studies are needed on the application of these drugs in RA subtyping therapy. Therefore, for this aspect, the pQTL analysis proposed in this study to identify drug

targets as a more precise and personalized drug selection contributes to the new concept of precision medicine.

This study does have some limitations. First, additional meta-data would have been ideal, albeit this may be difficult given that this study was carried out in several clinical settings with various characteristics. Secondly, the Consensus clustering mainly relied on the subsampling which could lead to a reduction in data size and may result in potential clustering bias. Thirdly, the stability and potential changes in the proposed subtypes over time remain unknown due to the absence of longitudinal assessments within the descriptions of the subtypes in this review. Finally, a critical objective is to establish the correlation between clinically defined subtypes and biomarkers that reflect the underlying disease biology.

5 Conclusions

This endeavor holds significant potential for guiding the advancement of therapies tailored to specific subtypes. RA is a major medical challenge that requires more precise treatment. It is crucial to concentrate on the use of cutting-edge machine learning tools, as demonstrated in this study, in order to promote a better understanding of RA at a system level. We extensively analyzed the largest transcriptomic compendium for RA, utilizing the most comprehensive microarray and RNA-seq dataset available to date. These findings can serve as valuable guidance for developing molecular diagnostic approaches and as a future reference for tailored therapy in RA patients.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval for this study was not required in accordance with the local legislation and institutional requirements. Written informed consent for participation in the original study was provided by the participants.

Author contributions

MC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. QF: Writing – original draft, Conceptualization. JH:

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Conflict of interest

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Supplementary material

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Precision therapy for ulcerative colitis: insights from mitochondrial dysfunction interacting with the immune microenvironment

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Background: Accumulating evidence reveals mitochondrial dysfunction exacerbates intestinal barrier dysfunction and inflammation. Despite the growing knowledge of mitochondrial dysfunction and ulcerative colitis (UC), the mechanism of mitochondrial dysfunction in UC remains to be fully explored.

Methods: We integrated 1137 UC colon mucosal samples from 12 multicenter cohorts worldwide to create a normalized compendium. Differentially expressed mitochondria-related genes (DE-MiRGs) in individuals with UC were identified using the “Limma” R package. Unsupervised consensus clustering was utilized to determine the intrinsic subtypes of UC driven by DE-MiRGs. Weighted gene co-expression network analysis was employed to investigate module genes related to UC. Four machine learning algorithms were utilized for screening DE-MiRGs in UC and construct MiRGs diagnostic models. The models were developed utilizing the over-sampled training cohort, followed by validation in both the internal test cohort and the external validation cohort. Immune cell infiltration was assessed using the Xcell and CIBERSORT algorithms, while potential biological mechanisms were explored through GSVA and GSEA algorithms. Hub genes were selected using the PPI network.

Results: The study identified 108 DE-MiRGs in the colonic mucosa of patients with UC compared to healthy controls, showing significant enrichment in pathways associated with mitochondrial metabolism and inflammation. The MiRGs diagnostic models for UC were constructed based on 17 signature genes identified through various machine learning algorithms, demonstrated excellent predictive capabilities. Utilizing the identified DE-MiRGs from the normalized compendium, 941 patients with UC were stratified into three subtypes characterized by distinct cellular and molecular profiles. Specifically, the metabolic subtype demonstrated enrichment in epithelial cells, the immune-inflamed subtype displayed high enrichment in antigen-presenting cells and pathways related to pro-inflammatory activation, and the transitional subtype exhibited moderate activation across all signaling pathways. Importantly, the

immune-inflamed subtype exhibited a stronger correlation with superior response to four biologics: infliximab, ustekinumab, vedolizumab, and golimumab compared to the metabolic subtype.

Conclusion: This analysis unveils the interplay between mitochondrial dysfunction and the immune microenvironment in UC, thereby offering novel perspectives on the potential pathogenesis of UC and precision treatment of UC patients, and identifying new therapeutic targets.

KEYWORDS

ulcerative colitis, mitochondria, immune infiltration, metabolism, bioinformatics analysis, machine learning, unsupervised clustering

1 Introduction

Ulcerative colitis (UC) is an idiopathic chronic inflammatory bowel disease (IBD) characterized by mucosal inflammation, which starts in the rectum and generally extends continuously to proximal segments of the entire colon (1). The global incidence and prevalence of UC have been increasing, affecting millions of patients across the globe (2). Despite the exact etiology is not fully understood, it is regarded to be an interaction of multiple factors, involving genetic predisposition, epithelial barrier defects, microbiota, leucocyte recruitment, and dysregulated immune responses (3, 4). The diagnosis of UC is confirmed by clinical symptoms, endoscopic biopsy, and histological evaluation, and there is currently no gold standard (4). UC has been classified as proctitis, left-sided colitis or extensive pancolitis depending on the Montreal classification (5). Levels of disease severity - mild, moderate, or severe - is usually determined by the Mayo score (6) or Lichtiger score (7). Nevertheless, neither of above two clinical classifications, whether based on the inflammation extent or severity, takes into account the molecular mechanism of UC, which is a vital step forward in the drive towards precision medicine. Currently, the mainstay therapeutic agents for UC include 5-aminosalicylates, corticosteroids, immunomodulators and biologics, among which, biological agents, such as the tumor necrosis factor (TNF)- α inhibitors, infliximab (IFX) and Golimumab (GLM) (8, 9), anti- $\alpha 4\beta 7$ integrin antibodies, vedolizumab (VDZ) (10) are the most classical and widely used medications for patients with UC, all of which can induce and maintain remission to promote mucosal healing (11). Additionally, another new drug, ustekinumab (an IL-12/IL-23 inhibitor) (12) has demonstrated efficacy in achieving clinical remission following the failure of anti-TNF- α therapy (11). Despite expanding therapeutic options, 10–20% of refractory patients still require proctocolectomy due to adverse drug reactions and secondary loss of response (4). The key to breaking through this therapeutic ceiling might be the combination of therapeutics with personalized precision therapy

based on the identification of molecular subtypes that are unique to individual patients. Therefore, there is a pressing need to detect reliable diagnostic biomarkers and develop novel molecular stratification to approach more effective therapeutic strategies of UC patients.

Emerging research has recently revealed that mitochondrial dysfunction is implicated as a key factor in UC pathogenesis (13). Mitochondrial dysfunction can lead to energy deficiency to cause abnormal energy metabolism, such as tricarboxylic acid (TCA) cycle, fatty acid catabolism, and amino acid biosynthesis, often impair the epithelial barrier function by increasing susceptibility to TNF- α -induced cell death, reducing secretory barrier function (especially Paneth cells and goblet cells), and responding to regenerative capacity for damaging stimuli (14–16). Thus, the colonic epithelia of UC patients might have a uniquely high susceptibility to mitochondrial dysfunction, which can be revealed by the immune microenvironment of the colonic mucosa to affect the response to immunotherapy. Mitochondrial damage is able to affect the phenotype and activation of infiltrating immune cells, especially dendritic cells (DCs), macrophages, and B cells, via metabolic reprogramming, which contributes to the maintenance of an inflammatory milieu for abnormal innate and adaptive immune responses, then in turn further exacerbates mitochondrial dysfunction in the colonic mucosa (17, 18). A comprehensive comprehension of the mechanisms underlying mitochondrial action in UC may provide new insights into unraveling the complexity and heterogeneity of UC, facilitate the identification of optimal treatment strategies for UC patients for better outcomes, and aid in the discovery of novel therapeutic targets.

In our study, the most comprehensive colonic mucosal tissue transcriptomic data by integrating the publicly available UC transcriptome datasets to date were utilized to explore the mechanisms underlying mitochondrial action in UC through comprehensive bioinformatics analysis. Based on a variety of machine learning algorithms, UC genes diagnostic model was developed. Unsupervised clustering was applied to identify genes subclassification in UC patients to comprehensively characterize the

molecular and clinical characteristics of the subtypes. Furthermore, the patients in different UC subclassification showed different performance in the efficacy of the four biological agents, infliximab (IFX), ustekinumab (UST), vedolizumab (VDZ) and golimumab (GLM), respectively. These may provide new ideas for the clinical precision therapy of UC patients from the perspective of disease heterogeneity.

2 Materials and methods

2.1 Data acquisition and processing

Figure 1 depicts a comprehensive flowchart of the research procedure. The microarray and RNA-seq datasets analyzed in this study were obtained from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) database. The final participants included 17 independent UC datasets, comprising a total of 1891 samples, with 1695 UC samples and 196 healthy control samples. The training cohort consisted of 941 UC patients from 12 microarray datasets (GSE66407, GSE87466, GSE75214, GSE47908, GSE48634, GSE212849, GSE92415, GSE206285, GSE73661, GSE16879, GSE12251, GSE23597), while the validation cohort included 754 UC patients from 5 RNA-seq datasets (GSE174159, GSE193677, GSE165512, GSE128682, GSE72819). Detailed explanations for all datasets are presented in [Supplementary Table S1](#). The mitochondria-related genes (MiRGs)

were collected from MitoCarta 3.0 database (<https://www.broadinstitute.org/mitocarta/mitocarta30-inventory-mammalian-mitochondrial-proteins-and-pathways>) (19) and the gene set enrichment analyses (GSEA, <http://www.gsea-msigdb.org/gsea/index.jsp>) (Supplementary Table S2) (20, 21). For genes represented by multiple probes, only the probes with maximal signal were used for the further analysis. All microarray data were normalized using the “limma” R package (22). The raw count data from RNA-seq experiments were first transformed into the transcript per million (TPM) formats, followed by $\log_2$ (TPM+1) transformation, to achieve normalization. To remove batch effects of different data sources, the “ComBat” function within the “sva” R package (23) was utilized for batch correction.

2.2 Identification of differentially expressed mitochondria-related genes in UC

The “limma” R package was applied to identify differentially expressed genes (DEGs) between UC and HC samples (22). All analyses were adjusted for false positive results using False Discovery Rate (FDR) correction. Adjusted P-value < 0.05 and $|\log_{FC}| > 0.58$ were set as the threshold. Volcano plots and heat maps generated by the “ggpubr” and “pheatmap” packages, respectively, were used to visualize the screened DEGs. Additionally, Venn diagram was exploited to display common genes in both DEGs groups and MiRGs (24).

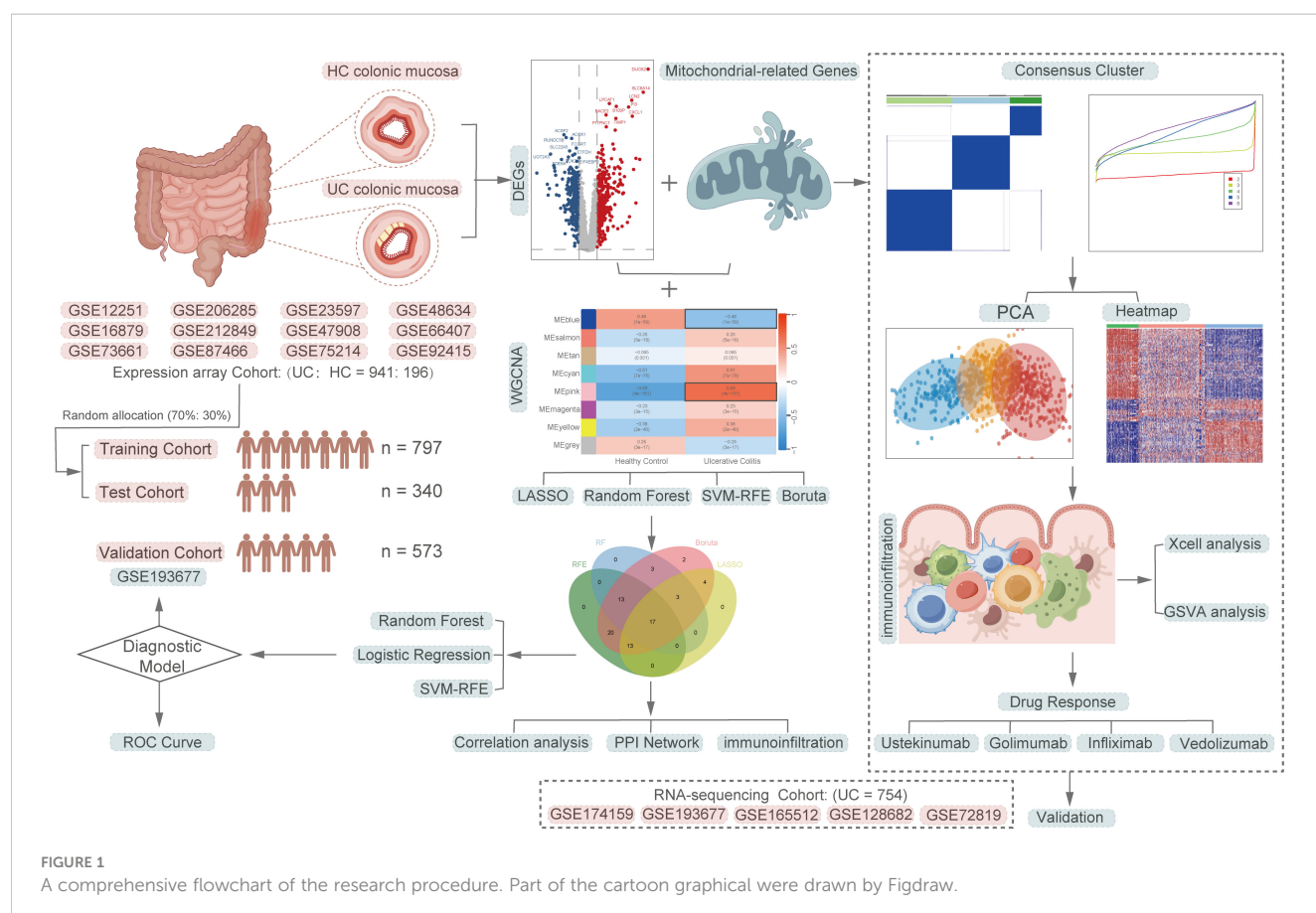


FIGURE 1

A comprehensive flowchart of the research procedure. Part of the cartoon graphical were drawn by Figdraw.

2.3 WGCNA and machine learning algorithms identified the diagnostic feature genes

Weighted correlation network analysis (WGCNA) is commonly used to reveal disease-related gene networks and identify co-expressed gene modules. To screen for co-expressed gene modules associated with UC, we performed WGCNA analysis using the “WGCNA” R package (25) for the top 25% of differentially ranked expressed genes. A soft threshold of $\beta = 10$ was selected for scale independence. The cluster height of module feature genes was set at 0.25, indicating a similarity greater than 0.75. Module and phenotypic data were utilized to evaluate gene significance (GS) and module significance (MS), and to investigate the correlation between modules and models. The most significant module genes, positively and negatively correlated with UC, were chosen for further analysis. To achieve the most effective feature subset, four machine learning algorithms were employed for feature selection: the Boruta algorithm, least absolute shrinkage and selection operator (LASSO), recursive feature elimination (RFE), and random forest (RF). The “Boruta” R package was used for the Boruta feature selection analysis, with 1000 significant runs (maxRuns = 1000). The LASSO algorithm, implemented through the “glmnet” R package (26), utilizes λ (lambda) as a penalty value or shrinkage operator to generate a penalty function. This function compresses the regression coefficients of variables in the model, effectively addressing the covariance issue and mitigating overfitting. The optimal composition of signature genes is determined by selecting the level of minimum cross-validation prediction error (lambdam.min). The backward feature elimination technique, known as RFE, is employed to eliminate feature vectors generated by SVM in order to identify the most optimal variables (27). In this study, we utilized the “caret” R package to implement the RFE algorithm for feature gene ranking and dimensionality reduction. Additionally, the “randomForest” R package (<https://cran.r-project.org/web/packages/randomForest/>) was utilized to construct the random forest tree of the feature genes, calculate their importance scores, and rank them accordingly. Only genes identified by the aforementioned four machine learning feature selection algorithms were deemed as key genes for subsequent analysis.

2.4 Construction and validation of a UC diagnostic model based on key MiRGs

A total of 1137 samples (UC: HC = 941:196) were randomly divided into a training set (UC: HC = 659:138) and a test set (UC: HC = 282:58) using the “caret” R package at a 70% to 30% ratio. The training cohort was balanced using the SMOTE algorithm from the “smotefamily” R package to address data imbalance and prevent overfitting. SMOTE is an oversampling method that creates new minority instances based on existing minority instances using the k-nearest neighbor algorithm. Logistic regression (LR), RF, and support vector machine (SVM) were employed to develop a diagnostic model in the training cohort. The LR algorithm was

implemented using the “glm” function from the R package “glmnet”. To mitigate the risk of overfitting, a 10-fold cross-validation approach was employed. Furthermore, the model’s robustness was assessed by validating it in both the test cohort and an independent RNA-seq dataset, serving as an external validation cohort.

2.5 Molecular subtypes of UC driven by genes associated with mitochondria

To enhance our comprehension of the molecular attributes of genes associated with mitochondria in UC, we attempted to uncover the molecular heterogeneity associated with mitochondria in UC. Unsupervised consensus clustering algorithm analysis of 1000 iterations based on the identified MiRGs in the training cohort via R package “ConsensusClusterPlus” (28). The clustering algorithm was configured to employ the “km” method, while the similarity between samples was assessed based on the euclidean distance. The determination of the optimal number of clusters was based on the cumulative distribution function (CDF) and the relative change in the area under the CDF curve. Principal component analysis (PCA) plots were generated using the “ggord” R package to verify the consensus clustering outcomes. The expression of MiRGs in different subtypes was visualized using the R package “pheatmap”.

2.6 Cibersort and Xcell immune cell infiltration analysis

The immune infiltrating cells within the immune microenvironment of the colonic mucosa of patients with UC and HC were analyzed using the Cibersort algorithm (29). The infiltrating abundance of 22 immune cells infiltrating were evaluated and compared through the utilization of the Wilcoxon test. Additionally, the Spearman correlation was employed to investigate the associations between the expression levels of the identified core genes and the Cibersort scores of the 22 immune infiltrating cell types. To investigate the infiltration of cells in the immune microenvironment of the colonic mucosa in patients with UC, we employed Xcell analysis (30). This analysis utilized xCell’s standard 64 cell type signatures and was conducted through the “xCell” R package. The objective was to ascertain the abundance score of infiltrating immune cells and stromal cells in colonic mucosal tissues of distinct ulcerative colitis subtypes driven by mitochondrial genes.

2.7 Enrichment analyses and gene set variation analysis

In order to ascertain potentially enriched pathways for DEGs linked to mitochondrial disorders in colonic mucosal tissue from patients with UC compared to healthy individuals, the pathway and process enrichment analyses were employed to assess the cellular component (CC), biological process (BP), molecular function (MF),

and Kegg pathways associated with UC using the Metascape platform (Metascape, <http://metascape.org>) (31). To identify the MiRGs-driven UC subtypes and their corresponding biological differences, we utilized the MSigDB database (<https://www.gsea-msigdb.org/gsea/msigdb/mgs/>) and employed the C2: CP: KEGG gene sets and C5: GO gene sets as input files for Gene set Variation Analysis (GSVA) (32). Furthermore, we utilized the single-sample gene set enrichment analysis (ssGSEA) to assess the relative enrichment score of biological pathways across different subtypes. The specific parameters used in this analysis were method = “ssgsea” and kcdf = “Gaussian”.

2.8 Protein-protein interaction network construction and module analysis

Utilizing the outcomes of machine learning feature selection, the chosen feature genes were employed to establish a protein-protein interaction (PPI) network using the string database (33), aiming to elucidate the intricate regulatory associations among the corresponding proteins of the feature genes. The visualization of the co-expression network was accomplished utilizing Cytoscape software (V3.10.1) (34). The module for Molecular Complex Detection (MCODE) algorithm was employed to identify hub genes (35). Genes were depicted as nodes, with the size of the shape indicating the MCODE value, while the lines represented the interactions between the genes.

2.9 Statistical analysis

Statistical analyses were conducted using R software (version 4.3.2, <https://www.r-project.org>) and associated R packages. The Kruskal-Wallis test was employed to assess the statistical significance among the three groups, while the Wilcoxon test was utilized to evaluate the statistical significance between the two groups. A significance level of $p < 0.05$ was deemed statistically significant.

3 Results

3.1 Identification of DE-MiRGs, functional annotation, and pathway enrichment in UC

Principal Component Analysis (PCA) was employed to visually represent the relative distances between datasets, thereby exposing discrepancies between microarray and RNA-seq datasets utilized in this study (Figures 2A, C). Additionally, Figures 2B, D demonstrates the effectiveness of the “ComBat” algorithm in eliminating batch effects and data heterogeneity from the integrated microarray datasets and integrated RNA-seq datasets, respectively. Differential expression analysis of the integrated microarray dataset identified a total of 823 DEGs that exhibited significant differential expression between UC and HC mucosa samples, including 358 down-regulated genes and 465 up-regulated genes in the training cohort ($p < 0.05$, $|\log FC| > 0.58$);

results are presented in a volcano plot (Figure 2E). The heatmap shown visualizes the expression patterns of the top 20 up-regulated and the top 20 down-regulated DEGs (Figure 2F). Subsequently, a combined analysis of 2,030 MiRGs and the 823 DEGs was performed to filter out 108 different expressed MiRGs (DE-MiRGs) in UC mucosa samples, the intersection of which was displayed as a Venn diagram (Figure 2G). We used Metascape to carry out GO analysis and KEGG pathway enrichment analysis to explore the potential biological roles of DE-MiRGs in UC. The results of GO enrichment analysis indicated significant involvement in mitochondrial molecular characteristics (mitochondrial membrane and mitochondrial matrix), mitochondrial metabolism of biological process (monocarboxylic acid metabolic process and small molecule biosynthetic process), and oxidoreductase activity (Figure 2H). In addition, an investigation of KEGG and the Reactome pathway primarily suggested that these genes were also significantly enriched in immune- and inflammatory-related signaling pathways. These included the peroxisome proliferator-activated receptor signaling pathway and interleukin-4 (IL-4) and interleukin-13 signaling but not mitochondrial energy metabolism, including metabolism of lipids, pyruvate metabolism, and the metabolism of amino acids and derivatives (Figure 2I). The terminologies were in alignment with established concepts of UC pathophysiology, that suggests that MiRGs expression patterns participate in the development and progression of UC.

3.2 Construction of MiRGs diagnostic models based on machine learning algorithms

Utilizing the WGCNA for module categorization, a total of eight modules were identified (Figures 3A, B). We selected the pink module ($r = 0.69$, $p = 4e-161$), which showed the most positive correlation with UC, and the blue module ($r = -0.46$, $p = 1e-59$), which showed the most negative correlation with UC as key modules (Figure 3C). A total of 978 significant module genes were screened for subsequent analysis, which were overlapped with the 823 DEGs and 2030 MiRGs by the Venn diagram, ultimately yielding 75 candidate genes (Figure 3D). Next, four machine learning algorithms [Boruta (Figure 4A), LASSO (Figures 4B, C), SVM-RFE (Figure 4D), and RF (Figure 4E)] were used for feature selection in these 75 genes. Finally, we obtained 17 signature genes by taking the intersection of the results of the above four algorithms (Figure 4F). Given the current limitations in tools for clinical detection of UC, we constructed diagnostic models using RF, SVM, and LR algorithms utilizing signature genes co-selected by the abovementioned machine learning. The microarray datasets were randomly partitioned into a training cohort and internal test cohort at a ratio of 7:3, while RNA-Seq datasets were used as an external validation cohort. The performance of RF, SVM, and LR models was evaluated in all cohorts using diagnostic ROC curves. In the training cohort, the prediction accuracy of the feature genes screened by the RF algorithm was 99.98%, which was better than those of SVM algorithm (98.29%) and the LR algorithm (98.48%) (Figure 4G). Similarly, consistent results are observed in the test

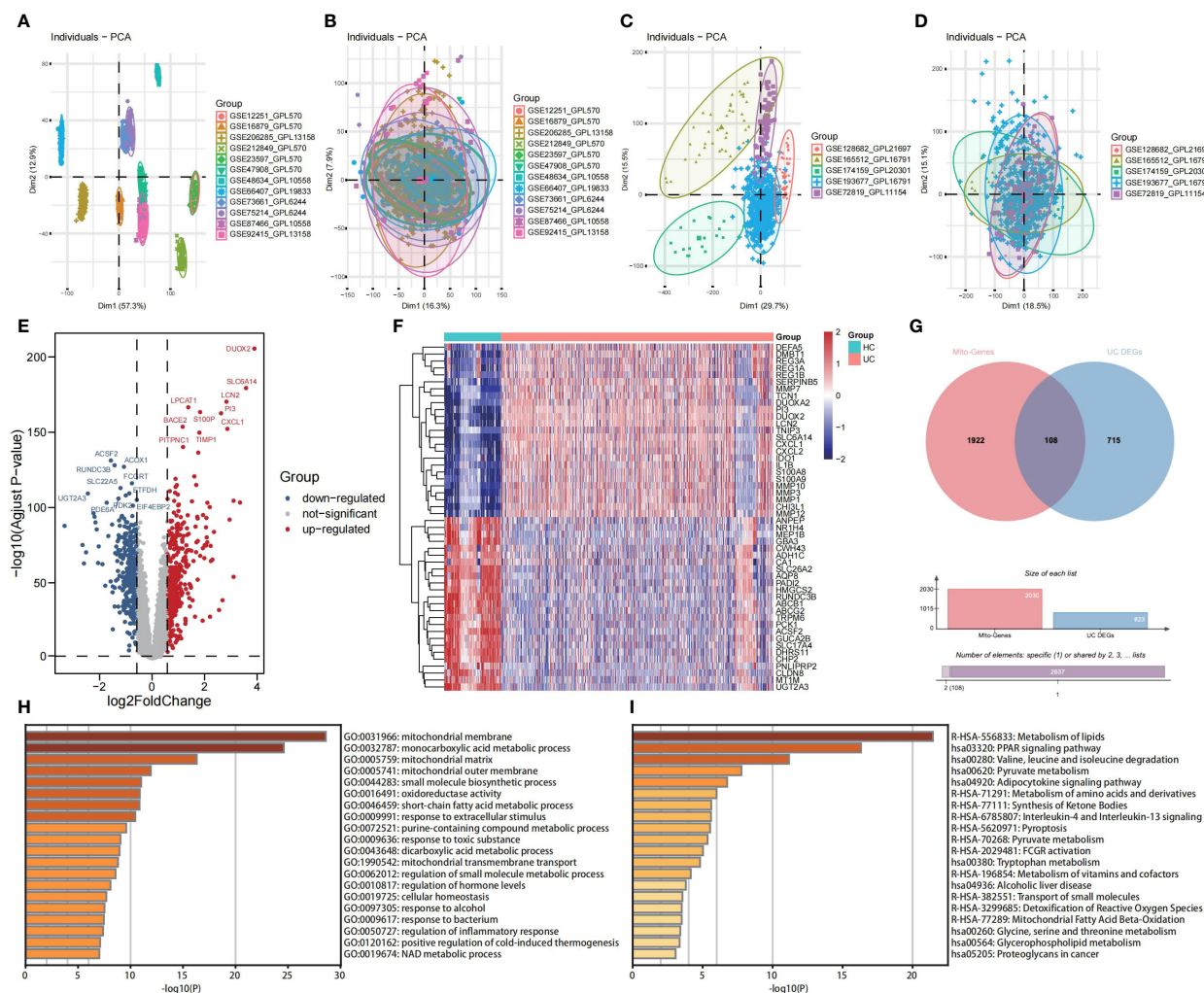


FIGURE 2
Screening of DE-MiRGs between UC and HC samples, and function enrichment analysis. PCA shows the batch effects of the twelve microarray datasets before (A) and after (B) the removal of batch effects using the 'ComBat' function from the 'sva' package in R. PCA shows the batch effects of the five RNA sequencing datasets before (C) and after (D) the removal of batch effects using the 'ComBat' function from the 'sva' package in R. Volcano plot (E) and heatmap (F) show the DEGs in combined GEO microarray datasets of UC and HC in colonic mucosa samples. (G) The Venn diagrams to demonstrate the extent of overlapping of DEGs and MiRGs. (H) GO enrichment analysis results of DE-MiRGs in BP, CC, and MF were performed with Metascape. (I) KEGG and Reactom enrichment analysis results of DE-MiRGs were performed with Metascape.

cohort that the RF algorithm achieved an AUC value of 98.76%, while SVM and LR models achieved AUC values of 97.93% and 97.71% (Figure 4H). Nevertheless, in the validation cohort, AUC values were 75.22% and 82.36%, 81.61%, respectively (Figure 4I). Overall, the three machine learning models demonstrate excellent predictive performance, with the SVM model exhibiting the best performance and robustness, which suggests a satisfactory diagnostic effectiveness of 17 MiRGs for a clinically precise diagnosis of UC.

3.3 Immune-infiltrating landscape of UC

The CIBERSORT algorithm was employed to characterize the abundance of 22 immune cell infiltration in the colonic mucosa of UC and HC groups. Figure 5A shows the distribution of immune

cell types in each sample for both groups, while Figure 5B illustrates differences in the abundance of infiltrated immune cells between the two groups. Compared with HC groups, higher levels of memory B cells, follicular helper T cells, γ & δ T cells, M0 and M1 macrophages, activated dendritic cells, and activated mast cells and neutrophils were found to be infiltrating the colonic mucosa of patients with UC. This indicated a significant difference in the immune microenvironment between UC and HC groups. Spearman's correlation analysis was used to investigate the relationship among the 17 signature genes. We found that SLC25A20, ACADM, ETVFDH, SLC19A3, CPT1A, BSG, and other genes exhibited predominantly positive correlations, while STOM, PDPN, PLAUR, PLA2G2A, and OLFM4 showed mainly negative correlations (Figure 5C). Subsequently, we constructed a PPI network of these signature genes using a STRING database to reflect their interaction. The result was visualized by Cytoscape,

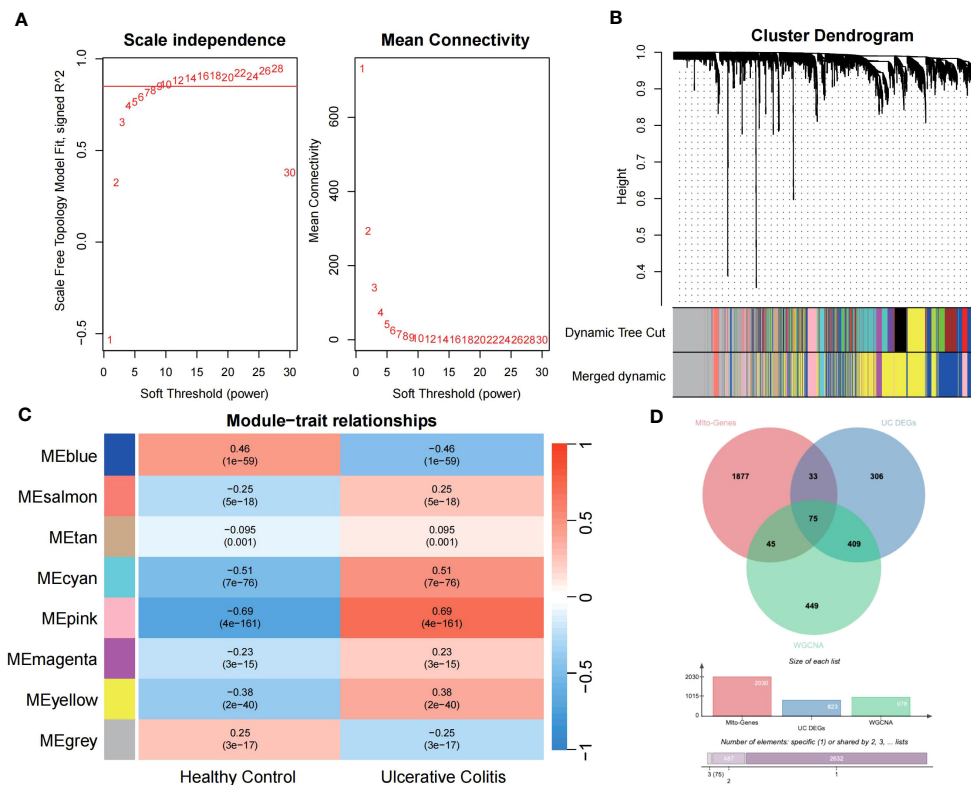


FIGURE 3

WGCNA reveals modules of co-expressed genes related to UC disease. (A) The scale-free fit index for various soft-thresholding powers (β) and the mean connectivity for different soft-thresholding powers determine the final soft threshold of the WGCNA. (B) The heatmap of the WGCNA module-trait association. (C) The dendrogram displays the clustering of dissimilar genes according to their topological overlap, along with the corresponding merged module colors. (D) The Venn diagram shows the common intersection genes of DEGs, module genes identified by WGCNA, and MiRGs.

as shown in Figure 5D, in which four highly related hub genes were identified, including SLC25A20, ACADM, CPT1A and ETFDH. To clarify the potential association between hub genes and UC, we assessed the correlation between the expression levels of four hub genes and 22 immune-infiltrating cells in the colonic mucosa of patients with UC. The result demonstrated that all four hub genes showed a variety of correlation with immune infiltration. Such hub genes exhibited a close negative link with most immune cells, such as activated mast cells, memory B cells, M0 and M1 macrophages, and activated DCs. The hub genes were positively associated with M2 macrophages, activated NK cells, resting CD4 memory T cells, resting mast cells, and CD8 T cells (Figures 5E–H). This finding is consistent with our previous studies, and may facilitate understanding the immunological features and molecular mechanisms of UC.

3.4 Identification of three molecular subtypes of UC with distinct cellular and molecular features driven by MiRGs

In order to establish a more comprehensive definition of mitochondria-related gene expression-driven subgroups in UC, we performed an unsupervised cluster analysis on 941 UC colonic

mucosal samples based on 108 DE-MiRGs. As a result, $k = 3$ was identified as the optimal number of clusters to ensure the robustness of clustering results, based on the CDF values and delta area (Figures 6A–C). Consequently, we finally obtained three MiRGs driven subtypes of UC, designated as UC-M ($n = 192$), UC-T ($n = 399$), and UC-I ($n = 350$) subtypes. The heatmap showed obvious heterogeneity in the gene expression profiles of these 108 DE-MiRGs among the three subtypes (Figure 6D). The PCA results further confirmed a distinct separation between the three UC subgroups (Figure 6E).

To explore the possible molecular attributes and physiological roles of three subtypes in the colonic mucosa of patients with UC, we characterized different MiRGs related to the immune state across 64 cell signatures and immune-related pathways. The results of differential abundance of immune cell infiltration revealed that the UC-I subtype was highly infiltrated by most immune cells, especially antigen-presenting cells (including DCs, monocytes, macrophages, and B cells), while the UC-M subtype was distinctly enriched in epithelial cells (Figure 7). GSVA analysis was performed to assess differences in functions and pathways enriched in mitochondria gene expression-driven subtypes. Figure 8 shows that the UC-M subtype had a relatively high enrichment score in metabolism-related pathways. For instance, these included the citrate cycle (tricarboxylic acid [TCA] cycle), butyrate CoA ligase activity, fatty

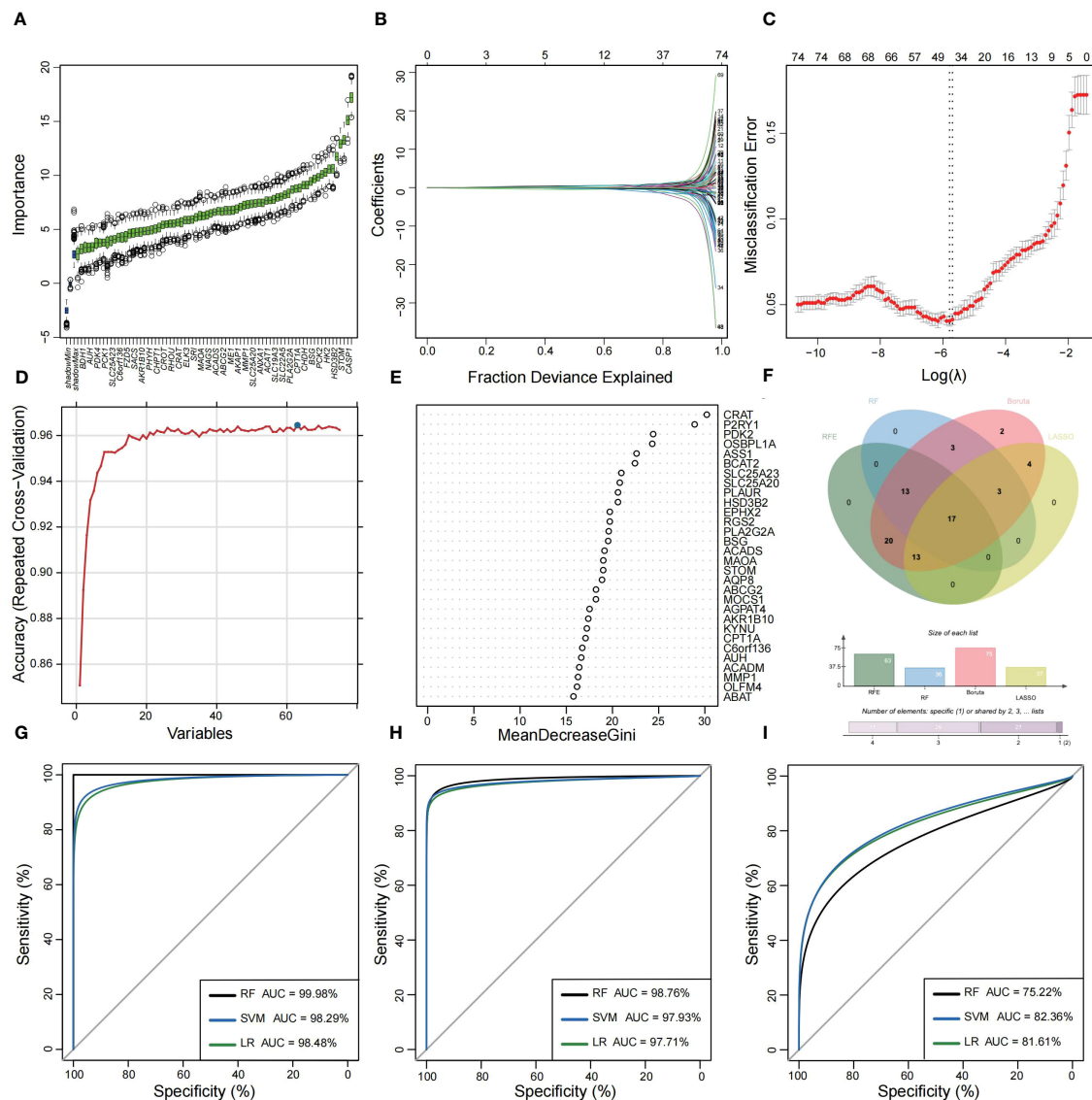


FIGURE 4

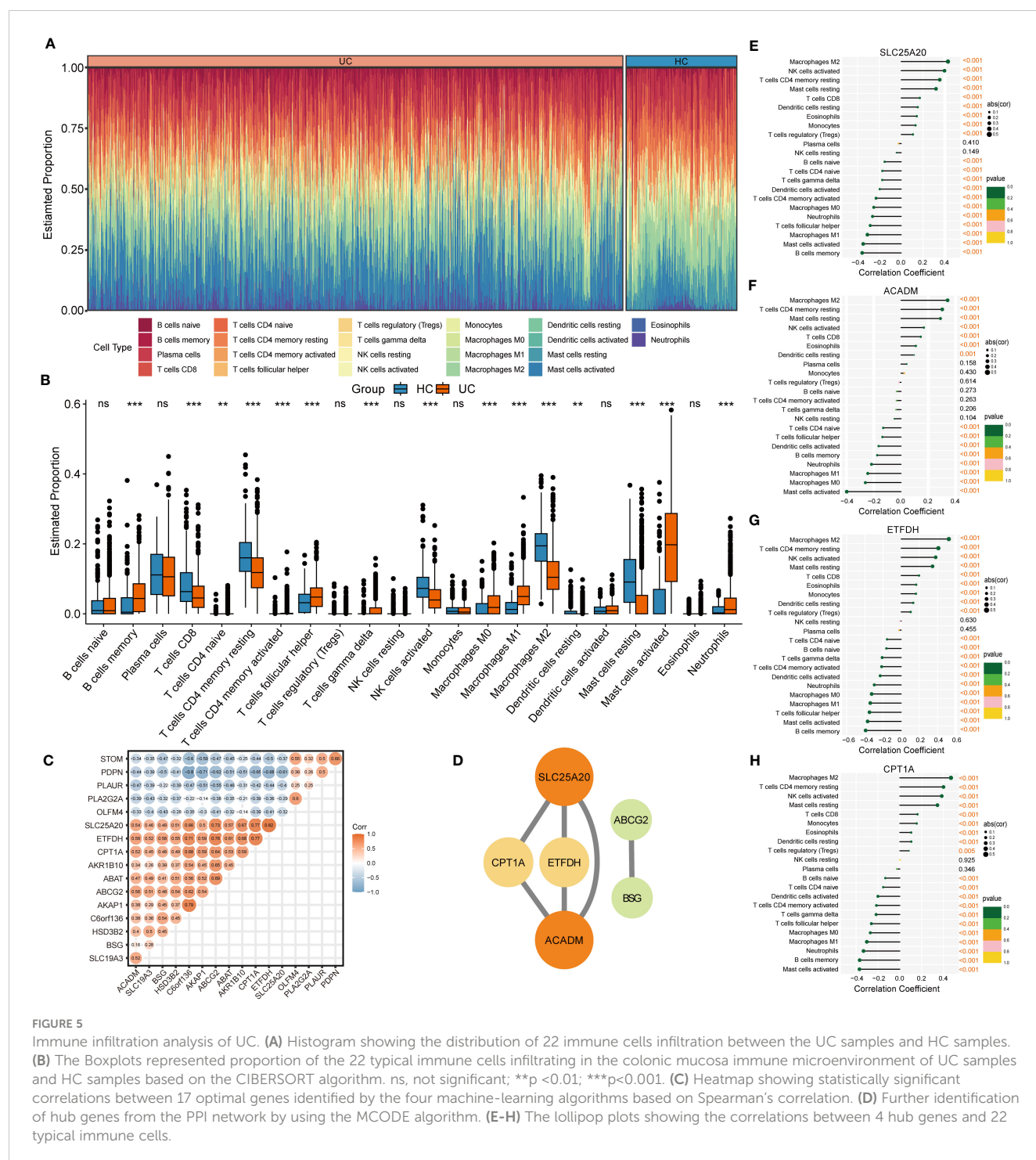
Feature selection and diagnostic model construction via several machine learning algorithms. (A) Candidate optimal genes selection based on the Boruta algorithm. The horizontal axis represents the gene, while the vertical axis represents the Z-value of each gene. (B,C) The LASSO regression used to select candidate optimal gene for UC. LASSO coefficient profiles of the 75 genes. Optimal parameter (lambda) selection in the LASSO model used tenfold cross-validation via minimum criteria. (D) Optimal genes selection using the SVM-RFE algorithm with the highest accuracy and lowest error obtained in the curves. (E) Optimal genes identified using the random forest algorithm. Genes importance in random forest assessed by mean decrease in Gini index. (F) Venn diagram showed the intersection of Candidate optimal genes identified by the four algorithms. ROC curve of the three machine-learning diagnosis model based on 17 optimal genes in the training cohort (G), internal test cohort (H), and external validation cohort (I).

acid metabolism, and taurine and hypotaurine metabolism. In comparison, immune- and inflammation-related pathways, such as CXCR chemokine receptor binding, the NOD-like receptor signaling pathway, Toll-like receptor signaling pathway, and FCγR-mediated phagocytosis, were significantly enriched in UC-I subtype. Most immune cells and immune-related pathways showed modest activation in the UC-T subtype. These results suggest significant differences in immune infiltration, biological function, and pathway activity between different UC subtypes. Thus, utilizing these molecular features, the UC-M subtype was defined as a metabolic subtype enriched in epithelial cells, the UC-T subtype was described

as a transitional subtype, and the UC-I subtype was defined as an immune-inflamed subtype enriched in antigen-presenting cells. In addition, DCs showed particular infiltration in this study, so their role in UC deserves further investigation.

3.5 Validation of classification by external cohort

The robustness of classification results was confirmed by integrating five publicly available UC mucosal biopsy RNA-seq



datasets. Patients were categorized into three subtypes using the gene expression profiles of 108 DE-MiRGs (designated as UC-M (n = 283), UC-T (n = 291), and UC-I (n = 180) subtypes (Supplementary Figure 1). Our findings were consistent with the enrichment scores of UC-related cell subpopulations and pathways (Supplementary Figure 2,3). The UC-M subtype was characterized by epithelial proliferation and mitochondrial metabolism, UC-T was described as showing modest immune activation, and UC-I was identified as an immune-inflamed type.

3.6 The efficacy of biological agents was significantly different between the three UC subtypes

According to the latest IBD Treatment Guidelines 2023, biological agents such as the tumor necrosis factor (TNF)- α inhibitors, infliximab (IFX) and Golimumab (GLM), anti- $\alpha 4\beta 7$ integrin antibodies, vedolizumab (VDZ), and the IL-12/IL-23 inhibitor, ustekinumab (UST), are approved for the treatment of

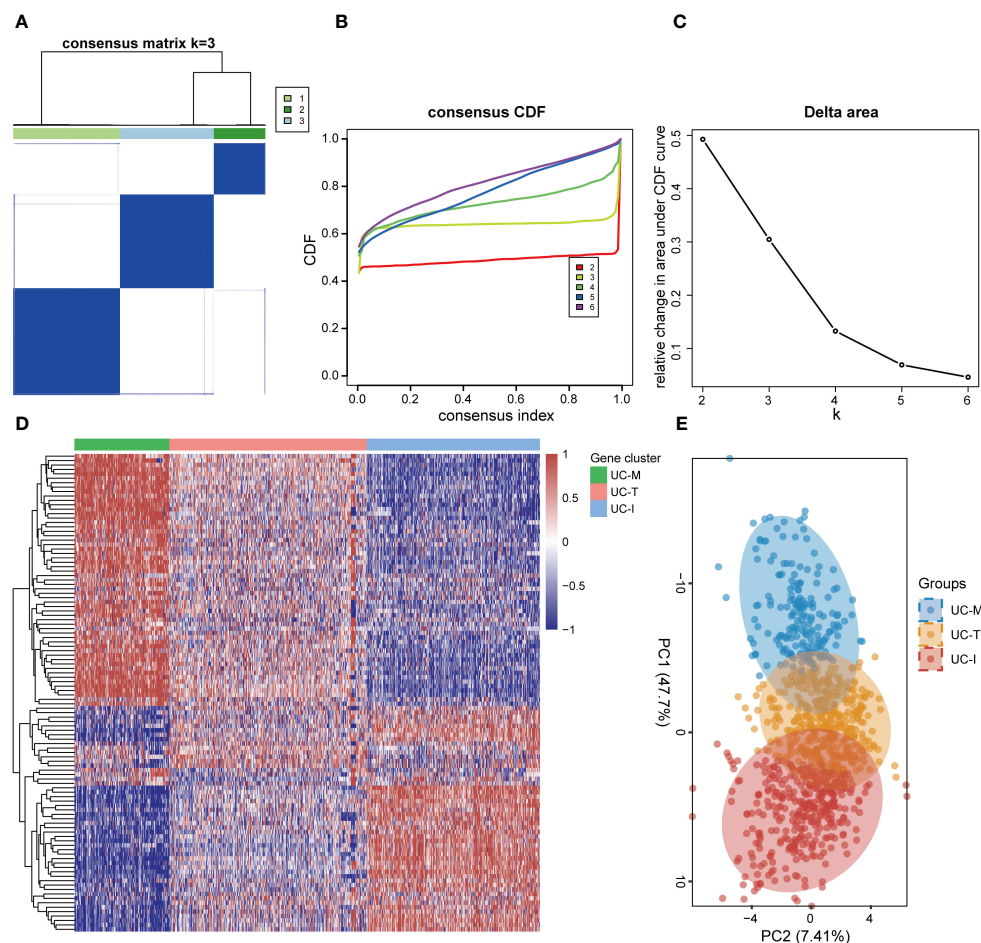


FIGURE 6

Unsupervised consensus clustering identified the subtype of UC driven by MiRGs. **(A)** Consensus matrix heatmap defining three clusters. The bars between the dendrogram and the heatmap represent the molecular clusters. A stable and robust clustering of the samples is evident from the boundary of the consensus matrix. **(B)** The Cumulative distribution function (CDF) curve of different K -values. When $k = 3$, the CDF curve with the slowest downward trend represents the most stable clustering. **(C)** The Delta area plot of different K -values, indicating the relative change in area under the CDF curve between K and $K-1$. **(D)** Gene expression heatmaps of 941 UC samples based on consensus cluster assignment. The colorful scale of heatmap reflects the relative expression levels where blue represents low expression and red represents high expression. **(E)** Principal components analysis (PCA) for the DE-MiRGs expression profiles showing the stability and reliability of the classification.

UC and have been proposed as first-line treatment options for moderate to severe UC. Thus, we evaluated the response to treatment in different subtypes using the above four biologics (Figures 9A–D). The proportion of favorable responses observed for the UC-M and UC-T subtypes consistently exceeded that of the UC-I subtype. All biological agents showed the highest response rates in the UC-M subtype. It is worth mentioning that the UC-M [69.23% and 39.29%] and UC-T [63.46% and 35.71%] subtypes exhibited favorable responses for IFX and GLM, while the UC-I subtype was completely unresponsive to VDZ. Moreover, UST elicited relatively inadequate responses in all three subtypes. However, due to insufficient sample size, these differences may not have reached statistical significance. In conclusion, the efficacy of biological agents in UC patients may be influenced by the unique pathological characteristics of individual colonic mucosal tissues and their molecular activity. Our study indicates that the diverse molecular subtypes of colonic mucosa in ulcerative colitis could impact the response to drug therapy, highlighting the importance of

considering these factors in the clinical application of these medications.

4 Discussion

Emerging evidence indicates that mitochondrial dysfunction plays a role in the progression of UC by affecting the integrity of the intestinal epithelial barrier and mucosal immune tolerance. This study establishes a connection between mitochondrial-related genes and UC, investigates potential pathogenic mechanisms, and presents novel avenues for the diagnosis, categorization, and management of the disease.

By conducting a thorough bioinformatics analysis of the largest cohort of patients with UC to date, we identified 17 signature genes associated with mitochondria that play a significant role in the progression of the UC. We developed genetic diagnostic models for UC using multiple machine learning algorithms that demonstrated

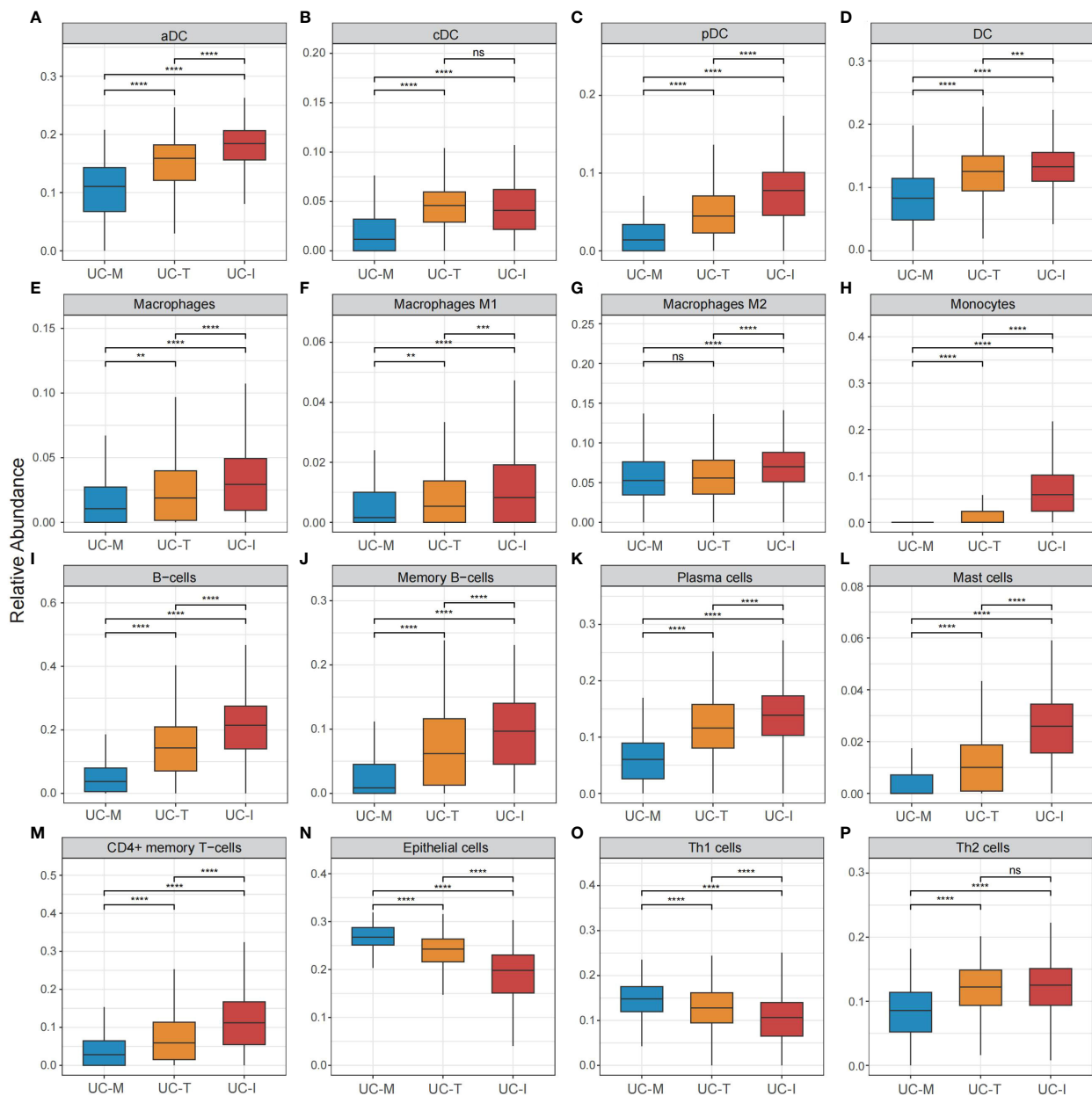


FIGURE 7

Cell subpopulation-driven characterization of UC subtypes. (A–P) Box plots revealed cell subpopulation enrichment scores across the UC colonic mucosa subgroups. Differences across the three subgroups were analyzed using the Wilcoxon test; ns, not significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

strong predictive capabilities and clinical utility. Furthermore, a systematic bioinformatic analysis was performed on the screened genes to investigate the intrinsic relationship between gene-gene and gene-disease. Our study identified a significant correlation, either positive or negative, among the signature genes, indicating potential synergistic or antagonistic interactions that may contribute to the development and advancement of ulcerative colitis. Four hub genes (SLC25A20, ACADM, CPT1A and ETFDH) were subsequently identified by PPI network analysis.

As of now, there has been no comprehensive investigation into the role of UC resulting from the activity of these four genes. CPT1A, identified as a promising target for clinical therapy,

catalyzes the transfer of a long-chain acyl group from an acyl-CoA ester to carnitine, allowing fatty acids to enter the mitochondrial matrix for oxidation (36). The DSS-induced mice model of UC has been reported that downregulation of CPT1A can protect UC partially by inhibiting PPAR α signaling, which suggest that the development of small molecule drugs targeting CPT1A may provide prospective therapeutic options for the UC clinical therapy (37). Additionally, a Mendelian randomization analysis of the specific MiRGs in IBD (38) indicated that genetically predicted levels of ACADM methylation, expression, and the corresponding protein were highly correlated with UC. Nevertheless, there is limited evidence from observational epidemiological and

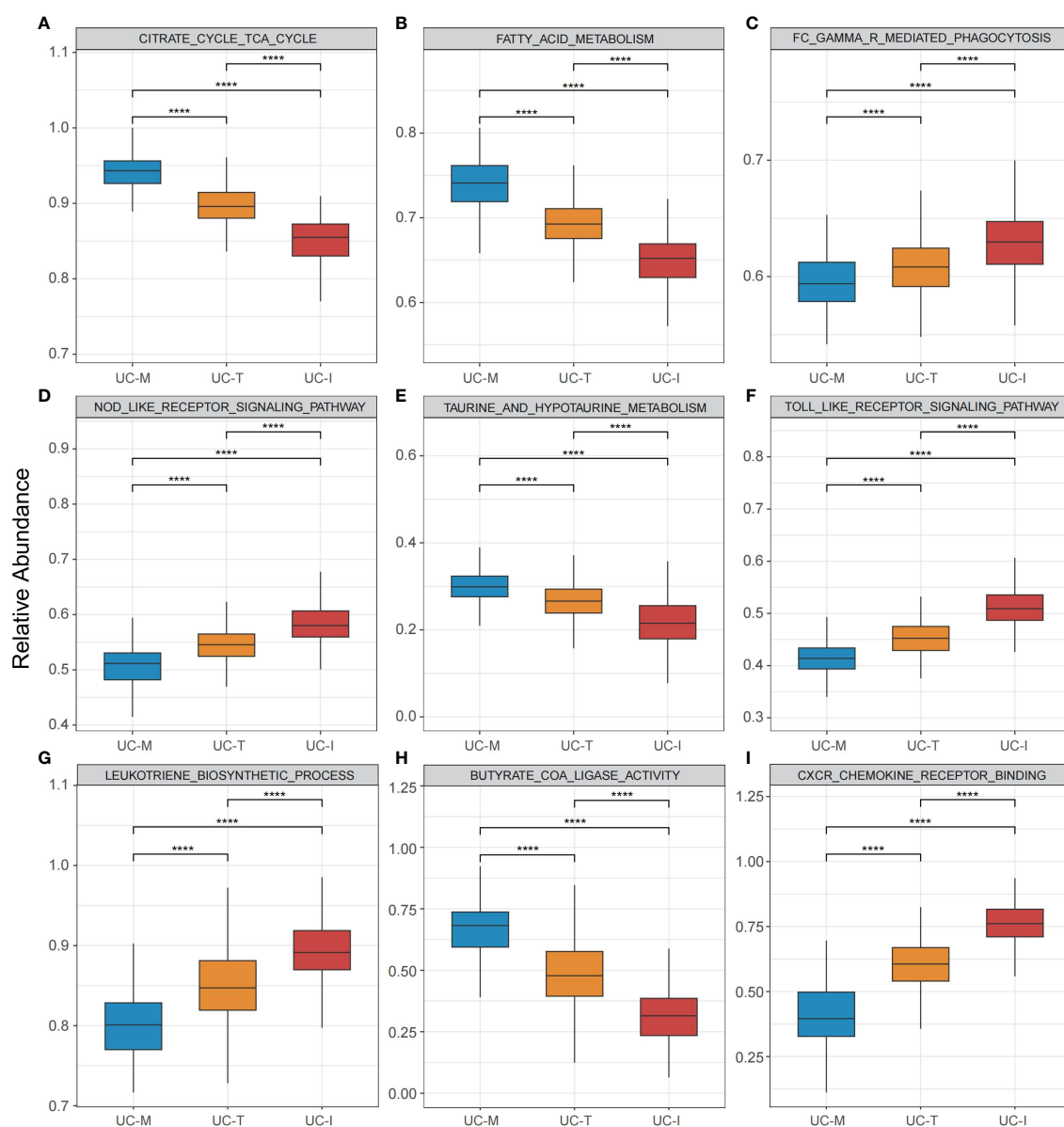


FIGURE 8

Pathway-driven characterization of UC subtypes. (A–I) Box plots revealed pathway activation scores across the UC colonic mucosa subgroups. Differences across the three subgroups were analyzed using the Wilcoxon test; **** $p < 0.0001$.

experimental studies regarding the association between ACADM and UC. Thus, ACADM is prioritized as a potential drug target for UC, and needs to be validated in future trials. For inflammatory UC, the study by Jan Söderman et al. showed that SLC25A20 has been documented downregulation in inflamed UC mucosa but upregulation was detected in non-inflamed UC mucosa (39). Therefore, we suggest that targeting SLC25A20 in pharmacological application of our UC-I subtype has great potential. A significant role for feasible therapy against ETFDH in UC has not been reported. In the future, we will try animal experiments or other measures to explore the role of ETFDH in UC and the mechanisms of these four hub genes in UC treatment. Our research will also focus on elucidating the underlying drug-gene interactions in order to identify potential therapeutic agents.

Currently, anti-TNF- α drugs and other biological agents have been used in UC for many years. It is urgent and essential to develop specific drugs targeting MiRGs for refractory patients who do not show an objective treatment response.

According to reports from the last few years, mitochondrial dysfunction may underlie intestinal mucosal injury (40). Mitochondrial metabolism is key to the regulation of the function of the intestinal epithelial barrier function. For instance, due to defective mitochondrial acetoacetyl CoA thiolase activity in UC, disrupted β -oxidation of butyrate, the preferred energy source of colonic epithelial cells (41), as first shown by Roediger (42), has been implicated in the pathogenesis of UC. However, mitochondrial dysfunction induces inflammatory responses in innate immune cells by promoting the production of pro-inflammatory cytokines

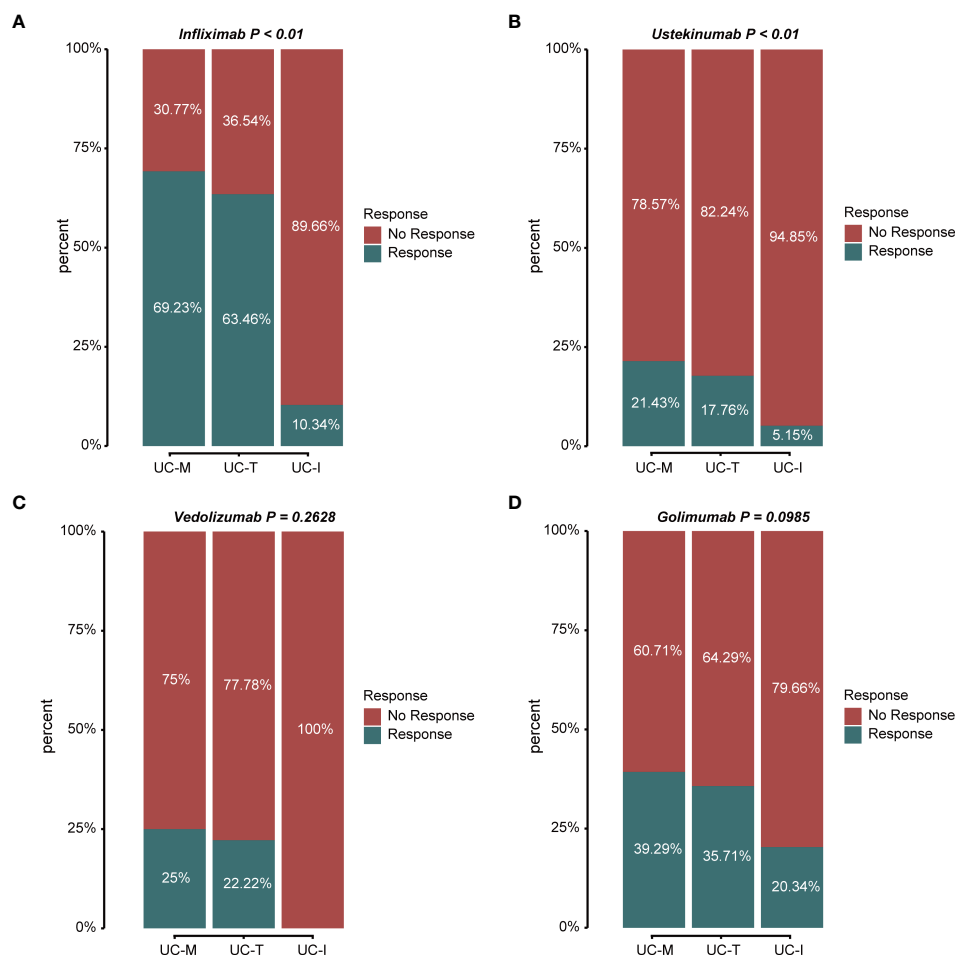


FIGURE 9

Stacked bar graphs showing the response status of different subtypes to multiple biological agents. Response: responded to the biologics; non-response: did not respond to the biologics. (A) Response/non-response to IFX: 69.23%/30.77% in subtype UC-M, 63.46%/36.54% in subtype UC-T, and 10.34%/89.66% in subtype UC-I. (B) Response/non-response to UST: 21.43%/78.57% in subtype UC-M, 17.76%/82.24% in subtype UC-T, and 5.15%/94.85% in subtype UC-I. (C) Response/non-response to VDZ: 25.00%/75.00% in subtype UC-M, 22.22%/77.78% in subtype UC-T, and 0%/100% in subtype UC-I. (D) Response/non-response to GLM: 39.29%/60.71% in subtype UC-M, 35.71%/64.29% in subtype UC-T, and 20.34%/79.66% in subtype UC-I.

(TNF- α , IL-1 β , and interferon [IFN]- γ) through excessive derived reactive oxygen species and an active endogenous damage-associated molecular pattern (43–46). In addition, mitochondrial metabolism is also intimately linked to immune inflammation. Studies have shown that the mitochondrial metabolic state of immune cells can influence their phenotype, pro-inflammatory or anti-inflammatory polarization states, and the efficacy of immune responses (17). In the case of macrophages, M1 macrophage polarization depends on anaerobic glycolysis, whereas M2 macrophage polarization relies on fatty acid metabolism and OXPHOS (47). These are consistent with our subtype classifications driven by mitochondrial gene expression. Specifically, the UC-M subtype has a transcriptomic signature in epithelial cell proliferative-related pathways, while the UC-I subtype exhibits high enrichment in immune cells and proinflammatory activation-related pathways. Expectedly, the UC-T subtype is considered a transitional subtype.

The crucial role of the immune response in the UC pathogenesis has been the focus of much attention. Our systematic evaluation of

immune infiltration revealed that the expression levels of almost all immune cells in UC were significantly higher than those in the HC cohort, which is consistent with the results of many previous studies. Magnusson et al. (48) found that increased numbers of DCs and macrophages, activated upon Toll-like receptors, are characterize the inflamed intestinal lamina propria in UC. This is consistent with our observation of immune cell infiltration in an immune-inflamed subtype. Notably, the metabolism of DC subsets is intrinsically linked to their ability to control Th cell polarization (49). Gut cDC1 produces more IL-12 to drive Th1 polarization than any other DC subsets especially cDC2, which is responsible for Th2 differentiation in a variety of type 2 immune responses (50). Paradoxically, high Th1 expression was observed in the metabolic subtype in our study. This could possibly because the TCA cycle supports Th1 cell proliferation and function through distinct mechanisms. An example is promoting the production of IFN- γ by elevating cytosolic acetyl-CoA pools through mitochondrial citrate export, which cooperated with TNF- α to induce apoptosis of intestinal epithelial cells and damage to the intestinal mucosal

barrier (51, 52). Moreover, IL-12 expression of DCs has been demonstrated to be critically dependent on glycolysis (49). Such prior studies all strongly support our findings. We further speculate that DCs with different Th cell-polarizing properties have distinct mitochondrial metabolic profiles. Additionally, gut DCs have been reported to induce more IL-4 (Th2 cytokine) production but less IFN- γ (Th1 cytokine) and less IL-22 production by T cells. This leads to an enhanced capacity to imprint gut-specific homing properties on effector T cells, skewing gut-specific T cell responses towards a Th2 profile (53, 54). This supports the evidence of UC being a Th2-dominant disease.

In light of our analysis, the metabolic subtype exhibited a higher abundance of epithelial cells and exhibited favorable responses to diverse pharmacological interventions. Whereas the immune-inflamed subtype, characterized by antigen-presenting cell infiltration, presented divergent outcomes and demonstrated inadequate responsiveness to multiple medications. The observed variations in disease activity and drug efficacy across distinct subtypes hold promising clinical implications for the management of UC in patients. The ability of IL-22 to promote stem cell-mediated intestinal epithelial regeneration in mice and humans has been repeatedly demonstrated by Lindemans et al. (55) using different experimental approaches. Others have shown the transfer of IL-22 producing immune cells can enhance recovery from dextran sodium sulfate-induced (56, 57). In addition, further studies revealed that increased IL-22 production after IFX therapy responded well to epithelial cell repair (58). Besides its anti-inflammatory properties, IFX can also enhance the glycerophosphatidylcholine level by regulating lipid metabolism to protect the integrity of intestinal mucosa (59, 60). These findings highlight the superior outcomes of anti-TNF α agents in the UC metabolic subtype.

The immune-inflamed subtype exhibited a less-than-ideal response to various drugs, with a secondary loss of response. The reason for this mechanistic escape remains unclear but is thought to be primarily immunogenicity or an inflammatory burden, resulting in increased drug clearance (61–63). It was deduced that patients exhibiting immune-active characteristics and high levels of immune inflammation are likely to have refractory UC and may warrant therapeutic drug monitoring. For such refractory patients, combining targeted therapies (CTT) may act synergistically to enhance the response to therapy, cover extraintestinal manifestations of disease, or reduce the risk of treatment failure (64). IFX has more potent immunogenicity than other anti-TNF- α agents, because IFX is a chimeric antibody of which 25% has a murine structure (63). In the UC-SUCCESS randomized double-blind trial (65), patients receiving combination therapy with IFX and azathioprine (39.7%) had a significantly higher remission rate at week 16, compared with IFX alone (22.1%). Patients also showed reduced antibody formation against IFX, but had an increased risk of infection and tumor induction. Not enough evidence exists to support the combination of immunosuppressants with other anti-TNF- α agents such as GLM. The gut-selective properties of VDZ would theoretically support its selection as one of the options for combination regimens to reduce the risk of adverse events (63). Nevertheless, the TREAT registry dose not indicated any benefit in

a combination of immunosuppressants with VDZ or UST. Hence, combining small molecule agents targeting specific inflammatory pathways may be one of the most promising emerging therapeutic options for patients with refractory UC who lack effective alternatives. Tofacitinib, a pan-active small molecule pan-active JAK inhibitor, has been demonstrated to inhibit the inflammatory process in UC, mainly by mediating the JAK/STAT inflammatory signaling and pro-inflammatory cytokine signaling, such as by IL-6 and IFN- γ (66, 67). It is also an attractive choice as an oral agent without concerns about being immunogenic. The efficacy and safety of tofacitinib have been confirmed in clinical trials and real-world practice in the maintenance treatment of refractory UC patients (68–70). Recent retrospective studies by Gilmore et al. (71) and Hilley et al. (72) reported that all patients with refractory UC who showed a partial response to a single biologic agent remained on biologics/tofacitinib combination therapy. Of this, 71% vs. 80%, respectively, achieved clinical remission, and 43% vs. 40%, respectively, achieved endoscopic remission; clinical response rates were both higher than with monotherapy. In addition, apremilast, a new oral small-molecule PDE4 inhibitor, can cause an increase in intracellular cAMP levels to reduce those of pro-inflammatory mediators (e.g., TNF- α and IL-23, activating DCs) and increase levels of anti-inflammatory mediators (e.g., IL-10, inhibiting DCs) (73). A recent 12-week phase II clinical trial (No. NCT02289417) conducted by Danese et al. (74) revealed improvements in multiple efficacy indicators in patients with active UC treated with apremilast. Together with our above analysis, we suggest that apremilast functions as a safe and well-tolerated anti-inflammatory agent, targeting DCs specifically, for patients with UC. The therapeutic response in combination with biological agents still needs to be validated in future trials. That is, biologics/small molecule agent combination therapy, to the extent that it reduces both immunogenicity and a high inflammatory burden, has the potential to minimize steroid exposure, induce remission, and avoid colectomy by facilitating a safe transition to maintenance therapy. However, relevant research is still limited. Rigorous randomized trials are needed to obtain efficacy and safety data as well as to assess and carefully discuss risks and benefits for short durations to minimize the risk of adverse events. Hence, further investigation is warranted to assess the efficacy of combining biologics with small molecule inhibitors in the treatment of refractory ulcerative colitis, potentially yielding valuable insights for managing patients with immune-inflammatory subtypes of the disease.

Nonetheless, several limitations in this study bear mentioning. First of all, more metadata would be ideal, but an unavoidable selection bias may exist. Second the complete annotation of clinical information for each UC sample is lacking. Furthermore, we found that the robustness of the diagnostic model in the validation cohort was worse than that in the training and testing cohorts (82.36% vs 97.93%/98.29%). The main reason for this discrepancy may be the different data sources that the clinical samples in the internal training and testing cohorts were from microarray datasets, while RNA-seq datasets was applied in the external validation cohort. However, this bias does not affect our results. Considering the above results, our study was conducted using only clinical samples,

without *in vivo* or *in vitro* validation. Therefore, further studies are required to provide convincing evidence for our results, which will be the focus of our future research.

5 Conclusions

In conclusion, this is the first and largest study published to date on the diagnosis and classification of UC from the perspective of mitochondrial dysfunction. We employed bioinformatics methods to identify signature genes of high value, and developed a predictive model with precise diagnostic capabilities. Furthermore, we categorized ulcerative colitis into three distinct groups using MiRGs. Our findings can be used to better elucidate the heterogeneity and treatment response of patients with UC in order to gain an insight into molecular mechanisms and to design stratified treatment protocols for such patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Author contributions

Y-fZ: Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. M-yF: Writing – original draft, Writing – review & editing. Q-rB: Data curation, Writing – review & editing. RZ: Data curation, Writing – review & editing. SS: Data curation, Writing – review & editing. LW: Data curation, Writing – review & editing. J-hL: Visualization, Writing – review & editing. J-wL: Visualization, Writing – review & editing. QW: Data curation, Writing – review & editing. YL: Data curation, Writing – review & editing. XC: Writing – review & editing.

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Supplementary material

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Exploring the impact of m⁶A modification on immune diseases: mechanisms and therapeutic implication

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N⁶-methyladenosine (m⁶A) is a chemical modification of RNA and has become a widely discussed topic among scientific researchers in recent years. It is distributed in various organisms, including eukaryotes and bacteria. It has been found that m⁶A is composed of writers, erasers and readers and is involved in biological functions such as splicing, transport and translation of RNA. The balance of the human immune microenvironment is important for human health abnormalities. Increasing studies have found that m⁶A affects the development of immune diseases such as inflammatory enteritis and systemic lupus erythematosus (SLE) by participating in the homeostatic regulation of the immune microenvironment *in vivo*. In this manuscript, we introduce the composition, biological function, regulation of m⁶A in the immune microenvironment and its progression in various immune diseases, providing new targets and directions for the treatment of immune diseases in clinical practice.

KEYWORDS

N6-methyladenosine, M6A, autoimmune diseases, immune cells, T cell

Introduction

RNA modifications, as chemical alterations are prevalent across diverse cell types and hold significant relevance in various pathophysiological processes. These modifications include N⁶-methyladenosine (m⁶A), 5-methylcytosine (m⁵C), N¹-methyladenosine (m¹A), N⁷-methylguanosine (m⁷G), and N⁴-acetylcytosine (ac⁴C) (1–4) (Figure 1). Notably, m⁶A, discovered in 1974, stands out as the most extensively studied RNA modification. Advancements, notably methyl-RNA-immunoprecipitation-sequencing (MeRIP-seq) in 2012, have deepened our understanding of m⁶A's role in gene expression (5, 6) (Figure 1).

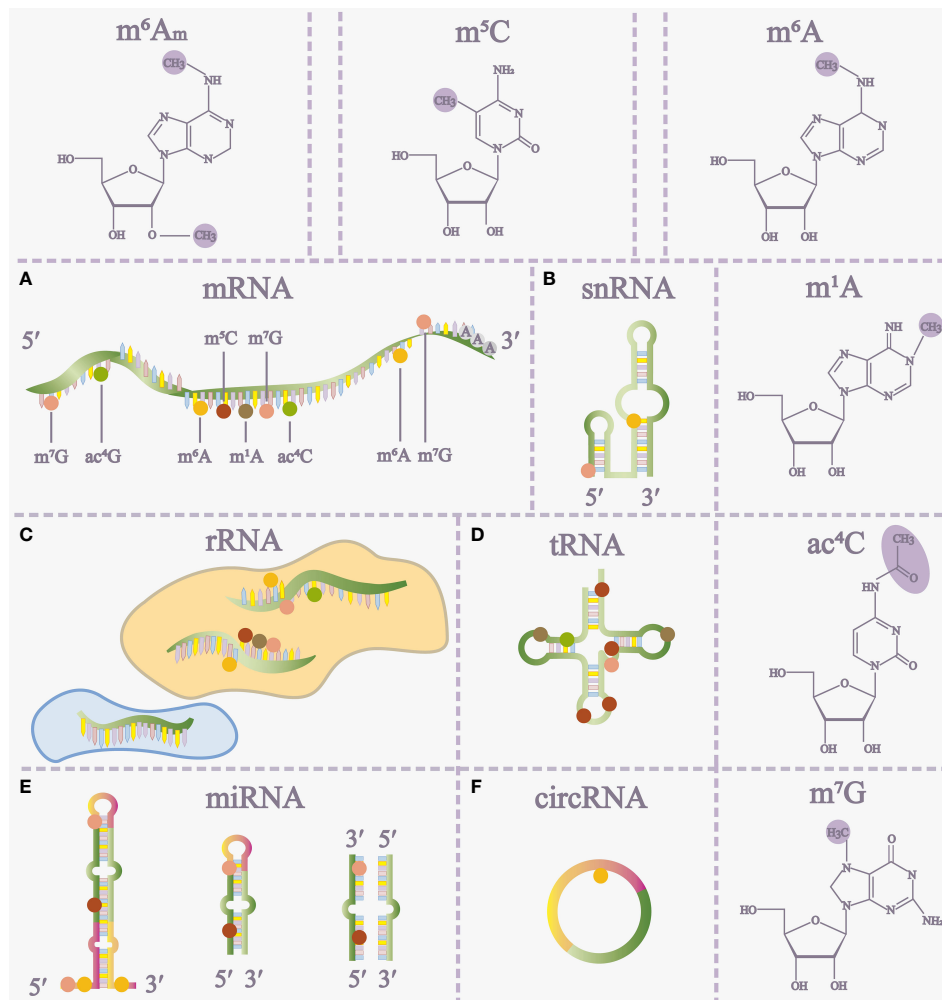


FIGURE 1

The distribution of RNA modifications on different RNAs (A–F) and some RNA modification structures. m⁵C 5-methylcytosine, m⁶A N⁶-methyladenosine, m¹A N¹-methyladenosine, ac⁴C N⁴-acetylcytidine, m⁷G 7-methylguanosine, m⁶Am, N⁶, 2'-O-dimethyladenosine, miRNA microRNA.

m⁶A modifications are pervasive across a wide range of RNA molecules, encompassing both coding and non-coding RNAs. They play pivotal roles in regulating transcription, translation, and RNA decay (7). Growing research links m⁶A methylation to various human diseases. Numerous studies highlight a close connection between m⁶A and the immune microenvironment, emphasizing its role in immune regulation and its impact on autoimmune diseases (ADs).

In this manuscript, we delve into the regulatory role of m⁶A methylation in the immune microenvironment, particularly in immune cells. We focus on the latest research findings regarding the involvement of m⁶A methylation in diverse immune diseases and viral infections. Additionally, we explore the feasibility and challenges of targeting m⁶A as a potential treatment for ADs.

Modification mechanism of m⁶A

m⁶A illustrates methylation of the adenine base at the nitrogen-6-position and consists of three parts: “writers” (methyltransferase),

“erasers” (demethylase), and “readers” (m⁶A-binding protein), in which they are responsible for the addition, removal, and recognition of m⁶A, respectively (8) (Figure 2).

Writers

Writers promote methylation and include methyltransferase-like 3 (METTL3), methyltransferase-like 5 (METTL5), methyltransferase-like 14 (METTL14), Wilms' tumor 1-associating protein (WTAP), RNA binding motif protein (RBM15), Zinc finger CCCH-type containing 13 (ZC3H13), and protein virilizer homolog (VIRMA) and so on.

Among them, METTL3 was the core component of the m⁶A methyltransferase complex (MTC) first discovered in 1997 and plays a role in promoting translation (9). METTL14 is another component that does not catalyze methyl transfer but can form a heterodimer MTTTL3-METTL14 complex with METTL3 to improve the catalytic activity of m⁶A (10–12). METTL5 is a

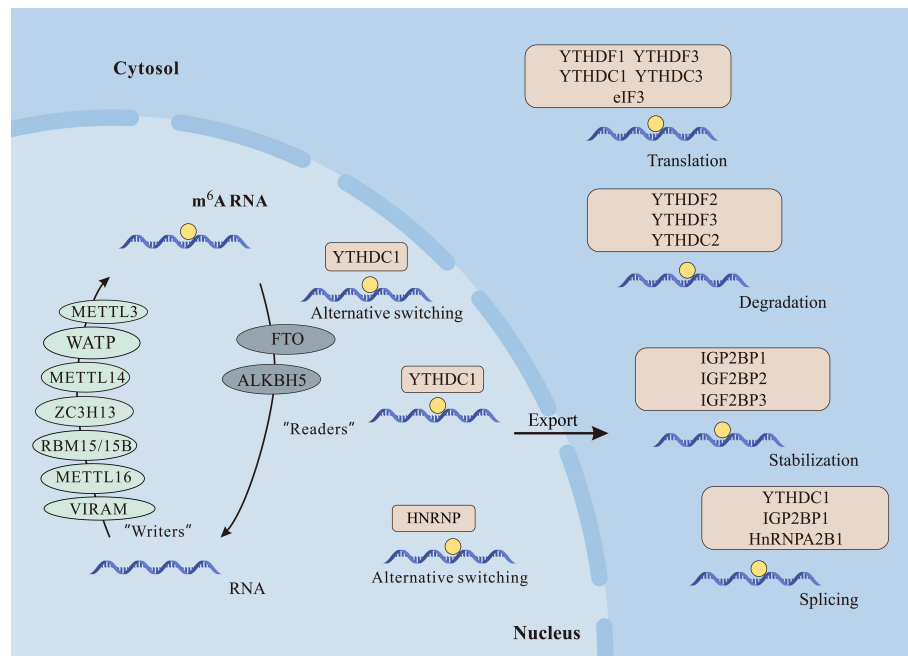


FIGURE 2

The m^6A modification plays crucial roles in mRNA function. Writers like METTL3, METTL14, METTL16, WTAP, VIRMA, RBM15/15B, and ZC3H13 catalyze m^6A methylation. Erasers FTO and ALKBH5 demethylate m^6A , while readers including YTHDF1/2/3, YTHDC1/2, IGF2BP1/2/3, and HNRNPC/A2B1 recognize m^6A , influencing mRNA processes like alternative splicing, nuclear export, translation, degradation, and stabilization.

methyltransferase of 18S ribosomal RNA (rRNA) that forms a heterodimer with transfer RNA (tRNA) methyltransferase subunit 112 (TRMT112), thereby facilitating modification of N6-methylation of m^6A_{1832} (13). WATP is another core part of MTC, which acts as a regulatory and recruits METTL3-METTL14 complex to messenger RNA (mRNA) (14). RBM15 and its analog RBM15B bind the m^6A methyltransferase complexes to specific mRNAs because of interaction with WATP. They also affect the long non-coding RNA X-inactive specific transcript (XIST) m^6A methylation (15). ZC3H13 promotes m^6A modification and it has been shown that reduction of ZC3H13 decreases methylation level of the 3' untranslated regions (3'UTRs) (16). VIRMA mediates preferential mRNA methylation in the 3' UTR and nearby stop codons, which selectively methylates primarily by recruiting the catalytic m^6A core assembly METTL3/METTL14/WTAP guide region (17).

Erasers

m^6A demethylases, functioning akin to erasers, eliminate m^6A modifications in RNA. The initial discovery in 2011 identified the fat mass and obesity-associated protein (FTO) as the pioneer m^6A demethylase, positioned in both the cytoplasm and nucleus. FTO plays a crucial role in glycolysis and adipogenesis by eradicating m^6A methylation (18, 19). Another demethylase, alkylated DNA repair protein alkB homologue 5 (ALKBH5), uncovered in 2013, is predominantly localized in the nucleus. ALKBH5 induces conformational changes in Flip 3, expediting nuclear export and

influencing mRNA export and metabolism (20, 21). Recent findings indicate that the methyltransferase ALKBH3 predominantly mediates m^6A modification in tRNA (22).

Readers

Multiple readers of m^6A serve as recognition functions during m^6A methylation and affect the fate of RNA (23). Among them, YTH21-B homology domain-containing family (YTHDF) is one of the main players (24). The YTHDF family consists of YTHDF1-3 and YTHDC-2. Within this family, YTHDF1-3 serve distinct roles in m^6A modification. YTHDF1 interacts with translation initiation factors, enhancing mRNA translation. YTHDF2 hinders the binding of mRNA stability proteins, facilitating the degradation of 3' UTR m^6A -modified mRNA. Meanwhile, YTHDF3 both promotes mRNA translation alongside YTHDF1 and mediates mRNA degradation in collaboration with YTHDF2 (25–27). YTHDC2 is an RNA helicase whose structure can aid RNA binding, is involved in mRNA regulation, and is particularly highly expressed in germ cells (28). YTHDC1 is able to interact with transcripts containing m^6A and regulate downstream targeted gene expression (29).

Biological function of m^6A

Certainly, m^6A assumes a pivotal role in diverse biological functions, participating in various facets of RNA metabolism. This

comprehensive involvement influences the entirety of the RNA life cycle, regulating a myriad of cellular processes (30). Among them, m⁶A is involved in multiple aspects of mRNA, including splicing, nuclear export, translation, and RNA stability (14, 31, 32) (Figure 2).

YTHDC1 regulates mRNA splicing by directly recruiting and regulating pre-mRNA splicing factors, in addition to the abundant nuclear protein HNRNPs that regulate pre-mRNA processing that can also indirectly affect alternative splicing of mRNA (33). Additionally, the m⁶A-binding protein YTHDC1 has been shown to interact with export proteins serine/arginine-rich splicing factor 3 (SRSF3) and nuclear RNA export factor 1 (NXF1), facilitating the transport of methylated mRNA from the nucleus to the cytoplasm in HeLa cells and playing a role in mRNA nuclear export. In mouse embryonic stem cells, m⁶A-modified mRNAs exhibit reduced stability compared to unmodified transcripts, leading to decreased abundance and translational potential (34).

YTHDF2 has been implicated in influencing the efficiency and stability of RNA translation of m⁶A. Mechanistically, YTHDF2 recruits the CCR4-NOT deadenylase complex, promoting mRNA degradation through a direct interaction between the N-terminal region of YTHDF2 and the SH domain of the CCR4-NOT transcription complex, subunit 1 (CNOT1) subunit. In contrast, in *T. brucei*, mRNA stability increases when the poly A tail of transcripts is methylated in association with variant surface glycoproteins (35). Moreover, m⁶A plays a crucial role in regulating mRNA translation. On the one hand, m⁶A at the 5' UTR position enhances translation independently of the cap structure. Mechanistically, m⁶A directly binds to eIF3, initiating translation. On the other hand, at the 3' UTR, m⁶A interacts with YTHDF1 or YTHDF3, promoting cap-related translation (19, 36, 37).

In rRNAs, novel m⁶A methyltransferases, such as ZCCHC, have been experimentally identified for methylating human 28S rRNA. Knockdown of ZCCHC4 in mammalian cell lines resulted in the elimination of the m⁶A4220 modification in 28S rRNA, leading to reduced global translation in hepatoma cell lines and inhibiting cell proliferation (38, 39).

Roles of m⁶A modification in immune cell biology

The immune system includes innate and adaptive immunity, in which various immune cells perform their duties. Macrophages, natural killer (NK) cells, Dendritic cells (DCs), neutrophils, are involved in immune responses in innate immunity. T cells and B cells are able to specifically clear pathogens and participate in adaptive immunity (40). An increasing number of experiments have demonstrated that m⁶A is involved in regulating the immune microenvironment by mediating various immune cells (Figure 3).

m⁶A and macrophages

Macrophages serve as the frontline of host defense, playing a crucial role in both innate and adaptive immunity. When activated,

they not only engulf pathogens but also release cytokines, actively participating in immune responses. Activated macrophages are categorized into M1 and M2. While M1 primarily assumes a pro-inflammatory role, M2 enhances anti-inflammatory factor production, mitigating the inflammatory response (41, 42).

It has been shown that m⁶A is involved in macrophage polarization. Macrophage polarization impairment is a major cause of RA disease progression. Signal transducer and activator of transcription 1 (STAT1) mRNA plays an important role in M1 macrophage polarization. The “writers” protein METTL3 in m⁶A was found to methylate STAT1 mRNA thereby increasing STAT1 levels and promoting M1 polarization, and conversely, knockdown of METTL3 decreased M1 and increased M2 in macrophages (43). METTL3 levels were upregulated in peripheral blood mononuclear cells from RA patients and positively correlated with C-reactive protein (CRP) and erythrocyte sedimentation rate, two markers of RA disease activity. Increased METTL3 may ameliorate inflammation in RA patients by inhibiting nuclear factor kappa-B (NF-κB) signaling to suppress lipopolysaccharide (LPS)-induced expression of inflammatory cytokines Interleukin-6 (IL-6) and tumor necrosis factor alpha-α (TNF-α) in macrophages (44).

Macrophage toll-like receptors (TLRs) have an important role in the immune microenvironment. Once pathogens invade, they trigger a series of intracellular signaling pathways that produce various pro-inflammatory factors and clear pathogens. Upon LPS stimulation *in vitro*, macrophages lacking METTL3 showed loss of m⁶A modification of recombinant interleukin 1 receptor associated kinase 3 (IRAKM) mRNA, decreased TNF-α levels, and ultimately suppressed TLR signaling-mediated macrophage activation (45).

Pulpitis is a bacterial infection in the oral cavity that causes an inflammatory response that can further deteriorate into pulp necrosis and periodontal disease. METTL3-induced m⁶A levels have been found to be elevated in lipopolysaccharide LPS-stimulated human dental pulp cells (HDPCs). Whereas knockdown of METTL3 promotes MyD88S, which is a splice variant of myeloid differential protein-88 (MyD88) and inhibits various inflammatory factors, it alleviates the inflammatory response of HDPCs (46). Furthermore, METTL3 overexpression induces heightened secretion of IL-6 and Inducible Nitric Oxide Synthase (iNOS) in M1 macrophages, while also boosting the osteogenic and migratory capacities of bone marrow stromal cell (BMSC) (47).

Alternatively, METTL14-mediated m⁶A modification is implicated in the regulation of macrophages. Depleting the m⁶A methyltransferase subunit METTL14 in myeloid cells heightened the macrophage response to acute bacterial infection in mice. METTL14 depletion weakened suppressor of cytokine signaling 1 (SOCS1) m⁶A methylation, reducing YTHDF1 binding to m⁶A sites, subsequently diminishing SOCS1 induction and leading to overactivation of the TLR4/NF-κB signaling pathway (48).

In addition, m⁶A can regulate C1q⁺ tumor-associated macrophages (TAM), but also CD8⁺ T cells through some ligands. Knockdown of METTL14 was found to promote CD8⁺ T cell differentiation and impair their function thereby eliminating tumors (49). In a separate study, the knockdown of YTHDF2 significantly increased the expression of inflammatory factors

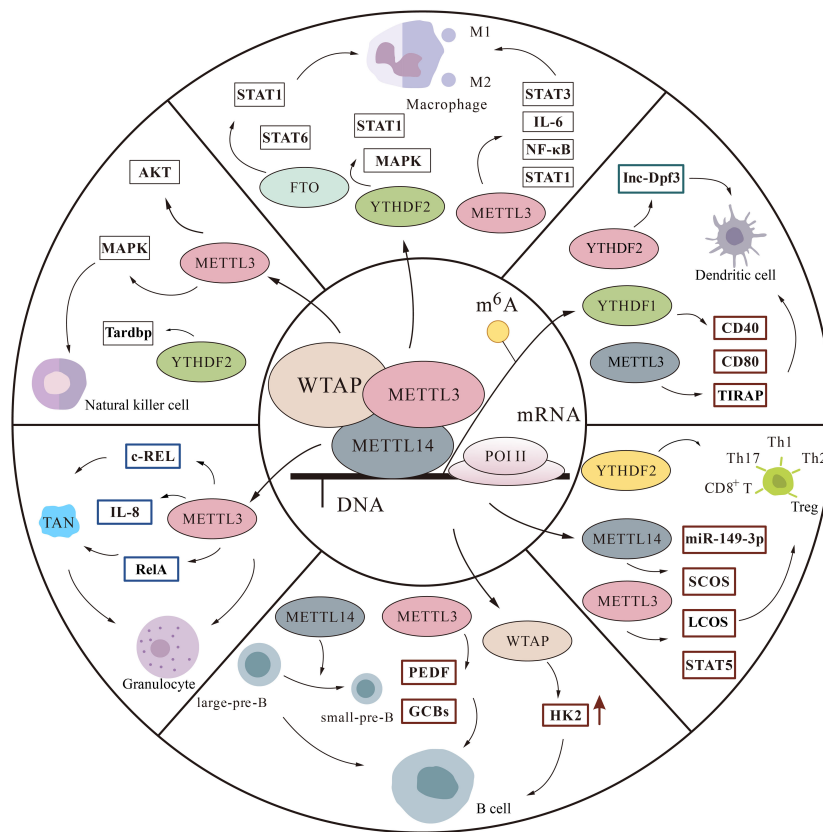


FIGURE 3

The m⁶A modification is involved in regulatory pathways in immune cells. We mainly describe some protein factor changes of m⁶A in macrophages, monocytes, natural killer cells, T cells, B cells, and granulocytes thereby affecting the immune microenvironment.

such as IL-6, TNF- α , IL-1 β , and IL-12 in LPS-induced macrophages. This resulted in the activation of the mitogen-activated protein kinase (MAPK) and NF- κ B signaling pathways, thereby intensifying LPS-induced inflammation in RAW 264.7 cells (50).

These findings imply that m⁶A plays a role in various aspects of macrophage activation and function, suggesting it could serve as a target for improving a range of inflammatory diseases through the regulation of m⁶A modification.

m⁶A and NK cells

NK cells are critical cells in innate immunity and can non-specifically and directly remove cancer cells and infected cells (51, 52). METTL3 expression was found to be decreased in tumor-infiltrating NK cells, and its protein expression level was positively correlated with NK cell effector molecules. Absence of METTL3 protein imbalances NK cells, thereby inhibiting NK cells from playing a role in immunity, leading to rapid tumor cell growth. This was associated with decreased activity of the m⁶A-modified gene encoding Tyrosine Phosphatase Shp-2 (SHP-2), inhibition of KT-mTOR and MAPK-ERK signaling pathways as well as decreased response to IL-15 and suggests that METTL3-mediated m⁶A promotes NK cells and can alleviate tumor progression,

providing new possibilities for cancer immunotherapy in the future (53).

In Testicular germ cell tumors (TGCTs), METTL3 protein levels decreased, tumor growth was evident, and patient survival decreased. The expression of METTL3 was positively correlated with molecular markers and infiltration levels of CD8⁺ and CD4⁺ T cells as well as natural killer cells, as illustrated by this study (54).

Research has highlighted the significance of the “readers” protein YTHDF2 in recognizing and interpreting m⁶A modifications within NK cells, which is critical for maintaining NK cell homeostasis, as well as for their survival and proliferation mediated by IL-15. In response to various stimuli such as tumor cells and infections, the expression of YTHDF2 increases in NK cells. Its absence leads to decreased antitumor and antiviral ability of NK cells. The role of YTHDF2 in NK cell function is associated with its interaction with TAR DNA-binding protein (Tardbp). The specific binding of YTHDF2 to Tardbp likely plays a critical role in the regulation of NK cell responses (55).

m⁶A and DCs

DCs have the ability to phagocytose and present antigens. They are responsible for initiating adaptive immune responses, particularly the antigen-specific activation of naive T cells, and

serve as a bridge between innate and adaptive immunity (56). Recently, many studies have found that m⁶A is involved in regulating DCs (57).

It has been shown that METTL3-modified m⁶A positively regulates maturation and activation of DCs. CD40, CD80, and TIR domain-containing adaptor protein (TIRAP) are important transcripts in DCs. METTL3-mediated m⁶A methylation of these transcripts enhances translation of DCs and stimulates T cell activation, promoting secretion of relevant cytokines by activating the TLR4/NF- κ B signaling pathway (58). In another study, reduced release of major histocompatibility complex II (MHCII), costimulatory molecules (CD80, CD86), and cytokines [interferons- γ (IFN- γ), IL-12] was observed, along with a decreased ability to mediate T-cell activation in DCs that were knocked down for METTL3, exhibiting immature properties (59).

Furthermore, CCR7-induced lncRNA lnc-Dpf3 inhibited CCR7-mediated migration of DCs, and YTHDF2 aggravated the inflammatory response by mediating m⁶A modification of lnc-Dpf3 (60). Studies have shown that m⁶A negatively regulates tumor neoantigen-specific immunity through YTHDF1, and YTHDF1 cross-presents tumor antigens in DCs, thereby stimulating T cell activation (61).

Pathogenic autoantibodies in SLE patients bind to antigens and form immune complexes (ICs) deposited in target organs, which can activate TLR on DCs and produce large amounts of type I IFNs, especially IFN- α and IFN receptors to promote a series of autoimmune responses in CD4⁺ T lymphocytes, CD8⁺ T lymphocytes and B cells, causing abnormal immune regulation. It has been shown that loss of YTHDF1 leads to recruitment of DCs, increased MHCII expression and IL-12 secretion, promotes penetration of CD4⁺ and CD8⁺ T cells, and increases IFN- γ secretion, playing a role in disease remission (62).

m⁶A and T lymphocytes

T lymphocytes play a very important role in adaptive immunity. T cells are mainly divided into CD4⁺ T cells and CD8⁺ T cells, and the imbalance of T cells can cause impaired immune function and cause ADs (63, 64). It has been shown that m⁶A plays a role in maintaining homeostasis of T cells.

Naïve CD4⁺ T cells exhibit the ability to differentiate into distinct cell subsets in response to various stimuli. These subsets encompass CD4⁺ helper T cells, specifically Th1, Th2, Th17 cells, CD4⁺ regulatory T cells (Treg cells), and follicular helper T cells (Tfh cells) (65–67).

The deletion of METTL3 in mouse T cells has been observed to have a significant impact on T cell homeostasis and differentiation. In METTL3-deficient naive T cells, there is a notable increase in the expression levels of mRNA and protein of the SOCS family. This upregulation of SOCS proteins inhibits the IL-7-mediated activation of STAT5, leading to impaired T cell homeostatic proliferation and differentiation (68).

The relationship between enterotoxigenic *Bacteroides fragilis* (ETBF), inflammatory bowel disease (IBD) and rectal cancer (CRC) has been investigated. They found that METTL14-mediated m⁶A

regulated Th17 cell differentiation by mediating exosome miR-149-3p of cells in ETBF-treated cells (69). Similarly, METTL14 deficiency leads to increased Th1 and Th17 cytokines and dysfunctional Treg cells, inducing IBD (70).

Th2-dominant response caused by Th1/Th2 imbalance is one of the important causes of allergic asthma. Five candidate m⁶A regulators [fragile X mental retardation 1 (FMR1), KIAA1429 (VIRMA), WTAP, YTHDF2, ZC3H13] were screened in the Gene Expression Omnibus GSE40888 dataset by a random forest model, and asthmatic children were distinguished into two m⁶A patterns (cluster A and cluster B) using consensus clustering to quantify m⁶A patterns. The results showed that patients in group B had higher m⁶A scores than those in group A. In addition, it was found that patients with group A were associated with Th1 dominant immunity, while patients with group B were associated with Th2 dominant immunity. In summary, m⁶A regulators may be involved in the development of childhood asthma, but the specific mechanism of involvement needs to be further experimentally demonstrated (71).

In addition, m⁶A also maintained the inhibitory effect of Treg cells, and METTL3 deletion resulted in increased SOCS mRNA in Treg cells, which targeted the inhibition of IL-2-STAT5 signaling pathway and finally lost the inhibitory function of Treg cells (72). Tfh cells are T cell subsets that assist B cells in humoral immunity to produce antibodies and play an important role in humoral immunity. There is evidence that m⁶A modification has an inhibitory effect on Tfh cells, mainly by reducing ICOS secretion, a key molecule for Tfh development (73).

CD8⁺ T cells, vital for immune defense, play a crucial role by secreting various cytokines to exert their immune effects. The regulation of CD8⁺ T cell function is closely linked to m⁶A regulation. In a mouse tumor model, mice lacking YTHDF1 exhibited heightened antigen-specific CD8⁺ T cell antitumor capacity (61). Moreover, FTO in tumor cells can suppress the activity of CD8⁺ T cells, enabling tumor cells to evade immune surveillance. Knocking down FTO reduces the glycolytic activity of tumor cells, restoring the functional capabilities of CD8⁺ T cells and effectively blocking immune escape mechanisms, thereby inhibiting tumor growth (74). In tumor-associated macrophages, the absence of METTL14 induces aberrant differentiation in CD8⁺ T cells, compromising functionality and inhibiting activation in CD8⁺ T effector cells (49).

It has been found that the expression of ALKBH5 is down-regulated in both PBMCs and T cells of SLE patients, and ALKBH5 mRNA levels are negatively correlated with SLE disease activity index score, erythrocyte sedimentation rate and anti-dsDNA levels, and positively correlated with complement C3 and C4 levels. Mechanistically, functionally, overexpression of ALKBH5 promoted apoptosis and inhibited T cell proliferation (75).

m⁶A and B lymphocytes

B lymphocytes are one of the main immune cells in the body and play an important role in humoral immunity. Activated mature

B lymphocytes can differentiate memory B cells and plasma cells and produce corresponding antibodies to kill pathogens (76).

According to studies, the lack of the m⁶A “writers” METTL14 significantly interferes with B cell development, impairs IL-7-induced proliferation, and affects the transition from large pre-B to small pre-B cells. The reduced binding of YTHDF2 to its target genes due to the absence of METTL14 is pivotal for the progression from the pro-B stage to the large pre-B stage, which is the first time that m⁶A has been found to be associated with B cell development (77).

The deletion of m⁶A using Mb1-Cre severely impairs B cell development. Interestingly, specifically knocking down METTL3 at the pro-B cell stage shows only minor effects on B cell development and function. Furthermore, this targeted reduction of METTL3 does not influence the fibrotropic activity of B cells in the context of liver fibrosis. This suggests that while m⁶A modifications are crucial for B cell development, the specific role of METTL3 knockdown at the pro-B stage might not significantly affect B cell development or function in certain contexts, such as liver fibrosis (78).

Research has demonstrated the crucial role of METTL14-mediated m⁶A modification in the germinative center (GC) B cell response. Elimination of METTL14 in B cells leads to compromised GC B cell proliferation and flawed antibody responses (79). Recent studies have revealed a significant decrease in m⁶A methylation levels in plasma cells taken from patients diagnosed with multiple myeloma. This decrease is associated with the upregulation of FTO, which targets the heat shock factor 1-heat shock proteins (HSF1-HSPs) pathway, promoting the proliferation, migration, and invasion of multiple myeloma cells. These effects occur through YTHDF2-dependent mechanisms. This demonstrates that the role of m⁶A methylation dysregulation in the pathogenesis and progression of multiple myeloma (80).

In addition, m⁶A was demonstrated to be involved in the progression of diffuse large B-cell lymphoma (DLBCL). In DLBCL patients, m⁶A and METTL3 expression levels were elevated. However, high expression of METTL3 can promote cell proliferation. METTL3 can promote cell proliferation and accelerate DLBCL progression by regulating m⁶A methylation and total mRNA levels of pigment epithelium-derived factor (PEDF) (81).

m⁶A and granulocytes

Granulocytes are white blood cells with special granules in the cytoplasm, which can be mainly divided into three types: neutrophils, eosinophils and basophils (82). In papillary thyroid carcinoma (PTC), METTL3 is able to cooperate with YTHDF2 to inhibit IL-8 secretion, reduce tumor-associated neutrophil (TAN) recruitment, and inhibit tumor growth using c-Rel and RelA as downstream modification targets (83). Currently, our understanding of the effect of m⁶A on granulocytes is still incomplete, and its specific mechanism of action awaits further investigation.

m⁶A and antiviral immunity

Viral infection is one of the important killers endangering human health. It has been shown that mRNA levels in the virus and host change markedly during viral infection replication (84). In antiviral immunity, m⁶A methylation can regulate host response to viral infection by affecting cytokine levels, regulating immune cell function, and affecting stress response. Host cells recognize associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), which initiate innate immune responses, which in turn activate signaling pathways, stimulate cytokine release, and further activate adaptive immune responses (85).

It has been found that m⁶A plays an important role in this process. DEAD-box (DDX) helicase is able to initiate antiviral innate immunity. Following viral infection, DDX46, a nuclear DDX member, was able to recruit ALKBH5 to demethylate some m⁶A-modified antiviral transcripts in macrophages from mice, inhibiting signaling and type I interferon production (86).

m⁶A methylation modification can affect immune cell maturation and activation. In monocytes and macrophages, m⁶A of HIV-1 RNA avoids innate immune sensing in bone marrow cells and weakens wolf virus innate immune responses (87). In NK cells, the m⁶A reader YTHDF2 was upregulated at levels following activation of cytomegalovirus infection. YTHDF2 knockdown disrupted the antitumor and antiviral activity of NK cells, with TAR DNA binding protein (TARDBP) being the YTHDF2 binding target in NK cells (55). In DCs, METTL3-mediated methylation of m⁶A is able to promote maturation of DCs. Mechanistically, METTL3 activates DCs function by promoting m⁶A-modified CD40 and CD80 transcripts and translation of TIRAP, a signaling protein in the TLR4/MyD88 pathway that is important for TLR4 signaling and downstream DCs activation.

Besides, loss of METTL3 renders DCs deficient in their ability to promote T cell proliferation (58). In METTL3 knockout mice, T cells exhibit impaired proliferation and differentiation into effector T cells (68). CD4⁺ T cells, susceptible to HIV infection, demonstrate increased HIV-1 protein and RNA expression, along with enhanced viral replication when YTHDF is overexpressed (88).

Viral infections can trigger cellular stress responses, influencing cellular metabolism. m⁶A methylation plays a role in regulating viral infections by modulating the host cell stress response. Flaviviridae RNA virus infection can alter m⁶A modification of some specific cellular transcripts, including RIO kinase 3 (RIOK3) and Cold-inducible RNA-binding protein (CIRBP), by regulating innate immune sensing and endoplasmic reticulum (ER) stress pathways. During viral infection, m⁶A modification of RIOK3 promotes its translation, while reduced m⁶A modification of CIRBP promotes alternative splicing (89). Research has demonstrated that ER stress responses activated by viral infection are involved in m⁶A changes in RIOK3 or CIRBP. These results suggest that viral infection-induced innate immune responses and ER stress contribute to changes in host mRNA methylation modification levels (90).

Role of m⁶A in immune diseases

Excessive inflammation and activation of immune cells can cause tissue damage and organ dysfunction, which leads to ADs (91). The etiology of ADs is not clear, but the role of m⁶A modification in ADs has gradually been discovered (Figure 4).

Rheumatoid arthritis

RA is an autoimmune disease characterized by abnormal proliferation of synovium, which leads to a series of pathological changes such as synovial inflammation and joint injury, and seriously reduces the quality of life of patients (92). Fibroblast-like slippery fetal cells (FLSs) play a crucial role in this (93). It has been found that m⁶A regulates the progression of RA.

SNPs can affect m⁶A by altering RNA sequences or key flanking nucleotides at target sites, and thus m⁶A-associated SNPs (m⁶A-SNPs) may affect RA by regulating genes. A large scale genome-wide association study (GWAS) was performed to screen 37 SNPs associated with m⁶A. Further integration of RA associated m⁶A-SNPs and gene expression data resulted in 23 SNP-Gene-RA trios. However, how m⁶A-SNP impacts RA needs further experimental validation (94). In the clinical cohort, IGF2BP2 has been found to be elevated in RA Synovial fibroblasts (SFs). Mechanistically, IGF2BP2-

regulated neuropeptides growth hormone receptor (GHR) and recombinant natriuretic peptide receptor 2 (NPR2) affect SFs, thereby promoting RA progression (95). In addition, similar studies have demonstrated that IGF2BP3 expression is increased in RA-FLS and regulates G2/M transition to promote RA-FLS proliferation and affect M1 macrophage polarization (96).

Furthermore, the m⁶A regulator ALKBH5 has been shown to be associated with synovial hyperplasia and infiltration. It has been reported that knockdown of ALKBH5 can inhibit the proliferation, migration and invasion of RA FLSs, and mechanistically, ALKBH5-mediated m⁶A can synergistically modify JARID2 mRNA and enhance its mRNA stability with IGF2BP3. The severity of arthritis was also found to be reduced in ALKBH5 knockout delayed-type hypersensitivity arthritis (DTHA) model mice or collagen-induced arthritis (CIA) and rats injected intra-articularly with ALKBH5-shRNA RNA (97). Another study is demonstrating that ALKBH5 may play a role in the activation of RA FLSs regulated by secreted modular calbindin 2 (SMOC2). SMOC2 was significantly increased in RA FLSs and synovial tissue, and knockdown of SMOC2 specifically regulated cytoskeletal remodeling and reduced migration and invasion of RA FLSs. In addition, intra-articular injection of Ad-shRNA-SMOC2 treatment reduced synovial inflammation and reduced erosion of bone and cartilage in CIA rats (98).

In RA patients, expression of METTL3 was significantly increased, as was m⁶A content. Overexpression of METTL3

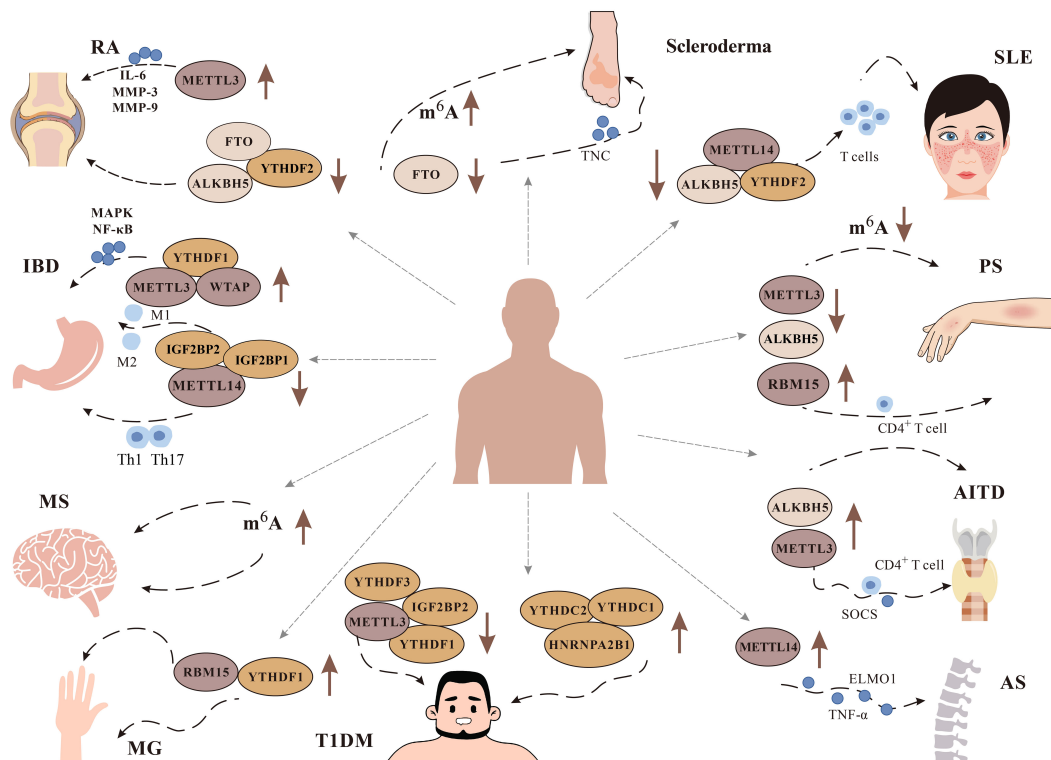


FIGURE 4

Role of m⁶A modification in immune diseases. It has been found that m⁶A plays a role in a variety of immune diseases, including Rheumatoid arthritis (RA), Systemic lupus erythematosus (SLE), Multiple Sclerosis (MS), Psoriasis (PS), Inflammatory bowel disease (IBD), Type 1 diabetes mellitus (T1DM), Autoimmune thyroid disease (AITD), Ankylosing spondylitis (AS), and Scleroderma. We focus on the changes of related m⁶A methylation-related enzymes METTL3, METTL14, WTAP, YTHDF1, YTHDF2, FTO And ALKBH5 in related diseases.

significantly attenuated LPS-induced macrophage inflammatory responses. Downregulation of METTL3 decreased IL-6, matrix metalloproteinase (MMP) -3, and MMP-9 levels in human RA FLSs and rat AIA FLSs. This process is mediated through modulation of the NF- κ B pathway (44, 99).

It has been shown that the content of METTL14 in synovial tissue of RA rats is significantly increased and promotes FLS activation and inflammation in RA rat models. Knockdown of METTL14 significantly increased apoptosis, inhibited cell migration and invasion, and reduced the production of cytokines such as IL-6, IL-18, and CXCL10 in RA FLSs. Mechanistically, METTL14 silencing suppressed LASP1 expression and TNF- α -induced activation of the SRC/AKT axis in FLS, mediating m⁶A modification to improve mRNA stability of LIM and SH3 domain protein 1(LASP1) (100).

Recent studies have found that circ.0066715 is less expressed in RA. Circ.0066715 regulates the methylation process of WTAP and the expression of the downstream transcriptional gene avian erythroblastosis virus E26 oncogene homolog-1 (ETS-1), affecting macrophage polarization in RA. When circ.0066715 was overexpressed, the content of WTAP decreased, increasing ETS1 levels in RA-FLS cells, reducing cytokine secretion on in M1 macrophages, increasing M2 cytokine secretion, and inhibiting FLS proliferation (101).

SLE

SLE is a systemic inflammatory disease typically caused by immune system abnormalities. Its occurrence is related to genetic factors, environmental factors, estrogen levels and other factors, mainly deposited in the skin, joints, small blood vessels, glomeruli and other parts (102, 103). Recently the role of RNA modification in SLE has gained increasing attention (104).

Decreased mRNA expression of MTEEL14, ALKBH5, and YTHDF2 has been found in peripheral blood mononuclear cells (PBMCs) of SLE patients, and decreased mRNA expression of YTHDF2 has additionally been shown to be a risk factor for SLE according to logistic regression analysis (105).

It has been found by bioinformatics analysis that m⁶A “readers” IGFBP3 and two key immune genes (CD14 and IDO1) of m⁶A may be useful in the diagnosis and treatment of SLE (106). The imbalance of CD4⁺ T cells *in vivo* is one of the important causes of SLE.

In a mouse model immunized with sheep red blood cells (SRBC) and a mouse model of chronic graft-versus-host disease (cGVHD), silencing METTL3 promoted the activation of CD4⁺ T cells and the differentiation of effector T cells (mainly Treg cells), increased antibody production and aggravated the lupus-like phenotype in cGVHD mice. Mechanistically, inhibition of METTL3-mediated m⁶A modification stabilizes Forkhead box protein P3 (Foxp3) mRNA, thereby attenuating Treg differentiation (107).

Type I IFN is the most important cytokine involved in the pathogenesis of SLE, and IFN stimulated gene (ISG) imbalance is an important manifestation of SLE. It has been shown that YTHDF3

inhibits ISG expression by promoting translation of the transcriptional co-repressor forkhead box protein O3 (FOXO3) mRNA, and thus YTHDF3 may play an important role in SLE pathogenesis (108).

At present, there is still a lack of understanding of RNA modification in SLE, but it provides new ideas for the treatment of SLE.

Multiple sclerosis

MS is a chronic autoimmune disease with inflammatory demyelination of the central nervous system as the main pathological change (109). MS mainly includes progressive multiple sclerosis (PMS) and relapsing-remitting MS (RRMS) (110). Increasing evidence suggests that epigenetic modifications including RNA methylation are involved in MS initiation and progression.

It has been found that 13 central m⁶A RNA methylation modulators are upregulated in MS patients compared with non-MS patients. However, compared with cerebrospinal fluid from RRMS samples and PMS samples, the researchers found that RRMS samples showed significantly higher gene expression levels than m⁶A RNA methylation and m⁶A-related genes. It can be seen that m⁶A methylation is associated with the progression of MS, and can diagnose and distinguish PMS from RRMS (111).

DNA methylation and gene expression are significant risk factors for MS. Experiments integrating data from large-scale genome-wide association studies (GWAS) and quantitative trait loci (QTL) studies through Mendelian randomization have identified mRNA expression for 178 DNA methylation loci and 29 MS-related genes. Among the non-MHC genes identified, METTL21B, METTL1, and the translation elongation factor, mitochondrial (TSFM) showed strong associations. Additionally, MS-associated SNPs in DDR1 were significantly linked to plasma levels of MHC class I peptide-associated sequence B (MICB) and granzyme A. There is a causal relationship between plasma MICB and granzyme A levels and MS. Moreover, rs2288481 in DKKL1 and rs923829 in METTL21B are m⁶A SNPs associated with MS. These SNPs, rs2288481 and rs923829, may influence the regulation of gene expression in DKKL1 and METTL21B, respectively (112).

Psoriasis

PS is an immune-mediated inflammatory skin disease whose pathogenesis is associated with inflammatory cell infiltration and inflammatory factors. Its etiology is not clear and may be related to genetics, infection, and immune abnormalities (113–115).

RNA methylation has been poorly investigated in psoriasis so far. m⁶A methylation and METTL3 levels show a decrease in psoriatic skin lesions, displaying a negative correlation with psoriasis sarea and severity index (PASI) scores. In experimental models, reducing METTL3 inhibits m⁶A methylation, consequently promoting the development and exacerbating the severity of PS in imiquimod-induced psoriasis-like mice (116). Methyltransferase

RNA-binding motif protein 15 (RBM15) has the capacity to modify keratin 17 (K17). Skin samples from PS patients exhibited heightened levels of both RBM15 and K17. When RBM15 was silenced, it hindered proliferation and inflammation by mediating m⁶A modification of K17, thereby reducing K17 stability in IL-17A-induced keratinocytes (117).

m⁶A modification plays a role in PS development through ALKBH5 and METTL3-mediated IL-17a modification in CD4⁺ T cells. In a mouse model of PS, knockdown of ALKBH5 within CD4⁺ T cells aggravated PS-like phenotype and inflammation, and knockdown of METTL3 alleviated this symptom (118).

m⁶A modification is able to play a role in PS by regulating different classes of RNAs. CircRNA is associated with psoriasis. Some research found that a circRNA expressed by macrophages in the inflammatory environment, hsa.circ.0004287, was upregulated in PBMCs from AD and PS patients. Hsacirc0004287 reduced the stability of its host gene metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) by competitively binding IGF2BP3 to MALAT1 in an m⁶A-dependent manner. MALAT1 levels were low and promoted ubiquitinated degradation of S100A8/S100A9, thereby preventing p38/mitogen activated protein kinase phosphorylation and macrophage-mediated inflammation (119).

lncRNAs have also been found to play a role in PS. Urothelial carcinoma associated 1 (UCA1) was identified as a lncRNA associated with PS and highly expressed in psoriatic lesions. In psoriatic lesions, UCA1 is able to attenuate keratinocyte-driven inflammation and PS development by binding to METTL14 and activating hypoxia-inducible factor 1 α (HIF-1 α) and NF- κ B signaling pathways (120). The expression of another lncRNA AGAP2-AS1 is also increased in PS patients and plays a role in promoting the proliferation of keratinocytes and inhibiting apoptosis. m⁶A methylation is involved in its upregulation. METTL3-mediated m⁶A modification suppresses the expression of AGAP2-AS1 via YTHDF2-dependent AGAP2-AS1. In addition, AGAP2-AS1 promotes keratinocyte proliferation by activating the miR-424-5p/AKT/mTOR axis (121). All of the above suggest that m⁶A modification may provide new ideas and directions for the treatment of PS in the future.

IBD

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic nonspecific intestinal inflammatory disease (122). So far, the etiology of IBD is not clear, but genetic susceptibility, environmental factors and complex interactions between gut microbes play a very important role in it (123).

In recent years, increasing evidence has shown that multiple m⁶A regulators regulate the development of IBD (124). Among them, METTL3 plays different roles in different stages of IBD development. METTL3 overexpression has been found to increase viability, induce apoptosis, increase inflammatory factor release, and aggravate inflammation in epithelial cells treated with LPS (125). Silencing METTL3 reduced the level of TNF receptor-associated factor 6 (Traf6) and inhibited the MAPK and NF- κ B

signaling pathways, thereby relieving dextran sodium sulfate (DSS)-induced IBD (126).

Dysfunction of T cells is an important cause of IBD. It has been found that METTL14 levels are significantly decreased in IBD patients and promote Th1 and Th17 cytokine secretion, thereby inducing IBD (69).

IGF2BP1 assumes a dual regulatory role in IBD. On the one hand, its absence can foster autophagy in intestinal epithelial cells, nurturing the healing of intestinal inflammation. On the other hand, the absence of IGF2BP1 can diminish the level of occludin, disrupting the delicate balance of the intestinal barrier, hence intensifying the inflammation within the intestine (127, 128).

Furthermore, IGF2BP2 can participate in IBD by mediating macrophage polarization. IGF2BP2-deficient mice have been found to exhibit a more severe inflammatory response in the DSS-induced acute colitis in mice model. This is mainly caused by IGF2BP2 depletion promoting pro-inflammatory M1 macrophage polarization and inhibiting anti-inflammatory M2 macrophage polarization (129, 130).

ALKBH5 acts as an eraser of m⁶A, and its loss can hinder T cell infiltration into colonic tissue and then relieve colitis (131). YTHDF1, as a reader of m⁶A, was significantly increased in intestinal epithelial cells induced by IBD patients and IFN- γ stimulation. Silencing YTHDF1 resulted in decreased pro-inflammatory cytokine release in LPS-stimulated intestinal epithelial cells. Mechanistically, YTHDF1 governs Traf6 mRNA translation. Its loss hinders Traf6 translation, activating NF- κ B signaling and the NLRP3 inflammasome, ultimately reducing intestinal inflammation (132, 133).

Type 1 diabetes mellitus

T1DM is a condition affecting glucose metabolism due to a substantial decrease in insulin secretion, primarily stemming from the extensive destruction of pancreatic β -cells driven by autoimmunity (134). Genetic and environmental factors are the main causes of morbidity (135). In patients with T1DM, there is a notable decrease in the expression of METTL3 and IGF2BP2, while the expression of YTHDC1 and HNRNPA2B1 is significantly increased. Further studies have found that differentially methylated transcripts are involved in some immune pathways closely related to T1DM (136).

Three single nucleotide polymorphisms (SNPs) in non-coding regions of PRRC2A (rs2260051, rs3130623) and YTHDC2 (rs1862315) genes have been found to be closely associated with T1DM. Among them, PRRC2A is increased in T1DM patients, and plays a role in cytokine-cytokine receptor interaction, cell adhesion and chemotaxis, and neurotransmitter regulatory pathways. This process is associated with the PI3K/AKT pathway (137).

Cognitive impairment is a recognized and severe complication of diabetes, particularly in cases of T1DM. Studies in mouse models of T1DM have revealed significant cognitive impairment, with observed alterations in m⁶A-related protein levels within their hippocampus. Specifically, there was notable upregulation in the

protein levels of YTHDC2 and ALKBH5, while the expression of YTHDF1, YTHDF3, and WTAP was significantly downregulated. Remarkably, the overexpression of YTHDF1 notably alleviated STZ-induced cognitive dysfunction in diabetic conditions (138).

Islet transplantation is an ideal treatment for T1DM, but hypoxia-induced pancreatic β -cell death after islet transplantation is a huge obstacle leading to this treatment failure. The researchers designed extracellular vesicles (EVs) (HIF-1 α -EVs) derived from mesenchymal stem cells (MSCs) with overexpression of HIF-1 α . They found that HIF-1 α -EV activated autophagy in a YTHDF1-dependent manner, thereby promoting pancreatic β -cells to resist the hypoxic environment and reversing pancreatic β -cell apoptosis and aging (139).

Autoimmune thyroid disease

AITD, including Graves' disease (GD) and Hashimoto's thyroiditis (HT), is caused by autoimmune disorders (140). ALKBH5 gene polymorphisms have been found to be associated with AITD, GD, and HT patients, and it is speculated that ALKBH5 may be a candidate gene for AITD susceptibility (141).

Other studies have also found that m⁶A plays a role in AITD. In HT patients, there were significantly more cells in the recombinant activating transcription factor 4 (ATF4)-positive thyroid follicular epithelium (ThyFoEp), which triggered ER stress and led to cell death including cell necrosis and apoptosis. Further studies revealed that HNRNPC as a reader of m⁶A was able to mediate m⁶A modification of ATF4, thereby promoting endothelial cell apoptosis and necrosis (142). Additional experiments have also demonstrated that METTL3 participates in GD by mediating m⁶A modification of SOCS. In GD patients, METTL3 and SOCS molecules are aberrantly expressed in CD4⁺ T cells. Silencing METTL3 increased SOCS family expression (143).

Ankylosing spondylitis

AS is a chronic inflammatory disease that causes inflammation of the axial joints, leading to tendon ligament calcification and synovial tissue proliferation (144). Evidence suggests that m⁶A modification is reduced in T cells from AS because METTL14 levels are decreased in AS patients, leading to reduced FOXO3a expression, diminished autophagy, and exacerbated inflammation (145).

Recently, it has been found that TNF- α induces enhanced directional migration of AS-mesenchymal stem cells (AS-MSCs) *in vitro* and *in vivo* and promotes AS pathogenesis. In AS-MSC, TNF- α triggers elevated engulfment and cell motility 1 (ELMO1) expression, while METTL14 governs MSC migration by influencing specific m⁶A modification sites in ELMO1's 30 UTR (146).

Scleroderma

Scleroderma, a connective tissue disease primarily impacting the skin and mucosal tissues, is marked by fibrosis and sclerosis. In a scleroderma mouse model, there was a notable increase in methylation of Hras, Lama3, and Tnc, while methylation of Ccl3, Ccl9, Saa1, and Il1b exhibited a significant decrease. This suggests an association between m⁶A methylation and scleroderma, although the precise underlying mechanism remains elusive (147).

The aggregation of Tenascin C (TNC) is widely recognized as a significant contributor to collagen deposition in the development of scleroderma. Several studies have identified a connection between TNC and m⁶A methylation. In a bleomycin (BLM)-induced scleroderma mouse model, overexpression of FTO led to reduced m⁶A and mRNA levels of TNC, resulting in the alleviation of mouse skin fibrosis. FTO/TNC may become a new target for the treatment of Scleroderma in the future (148).

Myasthenia gravis

MG is one of the ADs in which lesions occur at the neuromuscular junction (NMJ) of skeletal muscle (149). The m⁶A-related modulators CBLL1, RBM15, and YTHDF1 have been found to be upregulated in MG patients, and primarily target histone-rich modifications and Wnt signaling pathways. Among these, RBM15 showed the strongest association with immune profiles (150).

Autoimmune hepatitis

AIH is an inflammatory disease of the liver caused by unexplained immune abnormalities, severe cases may develop cirrhosis, liver failure, and even lead to death (151). YTHDF2 expression has been found to be significantly upregulated in AIH patients. Deletion of YTHDF2 in mice led to a progressive rise in bone marrow-derived suppressor cells (MDSC) within the liver. This effect mitigated concanavalin-induced liver injury, enhanced liver expansion, chemotaxis, and ultimately alleviated AIH (152).

Experimental autoimmune encephalomyelitis

EAE is one of the ADs mainly mediated by specifically primed CD4⁺ T cells, and the cause of the disease is not yet clear (153). Reducing ALKBH5 resulted in heightened m⁶A modification on IFNG and recombinant human C-X-C motif chemokine 2 (CXCL2) mRNA, impairing CD4⁺ T cell responses and contributing to the suppression of autoimmunity (131).

Celiac disease

CD is a persistent, systemic autoimmune condition affecting the small intestine. It arises from the consumption of gluten-containing cereals by individuals genetically predisposed to the disease (154). The 5' UTR of XPO1 RNA in CD patients has higher YTHDF1 protein-mediated m⁶A methylation and activates downstream NF- κ B activity, forming a novel m⁶A-XPO1-NF- κ B pathway that causes inflammation (155).

Potential treatment and application of m⁶A

In recent years, the incidence of ADs has become one of the important causes of damage to human health, and seeking effective therapies for the treatment of ADs has attracted increasing attention. In the above, we focus on the important role-played by m⁶A RNA methylation in the immune microenvironment, which has also been found to be a potential direction and idea for targeted therapy of ADs.

For RA, the m⁶A reader IGF2BP3 promotes RA-FLS by affecting G2/M transition (96). Tripterygium wilfordii Hook F (TwHF) is one of the drugs for the treatment of RA, and triptolide (TP) is its main active component. TP has been found to have a high binding affinity to IGF2BP3 and TP can reduce mRNA expression of IGF2BP3 in PBMC and rheumatoid arthritis fibroblast-like synoviocyte (MH7A) (156, 157). Therefore, IGF2BP3 may be a potential therapeutic target for TP during RA treatment, but this needs further experimental validation. In addition, it has been shown that miR-223p and miR-149-3p regulate osteoblast differentiation of BMSCs by targeting FTO, so FTO may become a new target for the treatment of RA (158).

The m⁶A-related proteins ALKBH5, METTL14, and YTHDF2 are involved in the regulation of SLE. Moreover, m⁶A-related lncRNAs (Xist, PSMB8-AS1, linc02446) are closely related to SLE, as shown by the downregulation of Xist and PSMB8-AS1 expression in SLE patients and the rise of linc02446 expression. Therefore, investigation of m⁶A modification targeting lncRNA could help to innovate the treatment of SLE (159).

Currently, m⁶A inhibitors mostly target FTO and METTL3, whereas eraser of m⁶A ALKBH5 acts on a variety of ADs, and five ALKBH5 gene variants have been found to be detected in AITD patients; deletion of ALKBH5 reduces T cell infiltration into colon tissue and alleviates IBD; ALKBH5 acts on CD4⁺ T cells, and knockdown of ALKBH5 aggravates PS, while inhibition of ALKBH5 may alleviate EAE. Therefore, studies targeting ALKBH5 will provide new therapeutic directions and ideas for the treatment of a variety of ADs (118, 131, 141).

Conclusion and future perspectives

The coordination among the “writers” (methyltransferase), “erasers” (demethylase), and “readers” (m⁶A-binding protein)

intricately governs m⁶A methylation, shaping the destiny of RNA. Beyond influencing transcription, maturation, localization, translation, and degradation of mRNAs, m⁶A methylation extends its impact to various facets of other RNAs, including snRNAs, snoRNAs, miRNAs, and lncRNAs.

Notably, ADs are due to disruption of immune system balance in the body, while m⁶A can influence disease progression by regulating the immune system. In this article, we focus on summarizing the effects of m⁶A on various immune cells, but the role of m⁶A in them remains controversial, especially m⁶A is a dynamically reversible process. In addition, the immune system is an extremely complex mechanism. At present, the regulation of m⁶A on the immune system is mainly concentrated in several major immune cells, but the related inflammatory factors and pathways need to be further studied.

Currently, our understanding of the relationship between m⁶A and ADs is superficial, and the specific regulatory mechanisms and effects on other ADs need to be further experimentally demonstrated. Additionally, m⁶A methylation is a dynamically reversible process, and its precise modification sites in ADs and complex contradictory action relationships need to be clarified. Our research on m⁶A-targeted ADs is still in the basic research stage. The focus of attention in the future will be on accurately providing clinical treatment and developing targeted drugs.

Author contributions

YC: Writing – original draft. MLi: Writing – original draft. MLu: Writing – original draft. LL: Writing – original draft. HZ: Writing – review & editing. XL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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