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Study on immune mechanism and immune intervention in connective tissue diseases

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Study on immune mechanism and immune intervention in connective tissue diseases

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Editorial: Study on immune mechanism and immune intervention in connective tissue diseases

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Editorial on the Research Topic
Study on immune mechanism and immune intervention in connective tissue diseases

Connective tissue diseases (CTDs) are a group of autoimmune disorders that primarily affect the connective tissues, including the skin, joints, blood vessels, and internal organs. This group of diseases include conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SS), and mixed connective tissue disease (MCTD). They occur when the body's immune system mistakenly targets and attacks its own tissues. The immune mechanisms involved in CTDs are complex and vary depending on the specific disease, but generally, several common immune processes are implicated.

Among the shared immunological mechanisms in CTDs, the production of autoantibodies specific to each disease plays a central role in diagnosis. For example, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are strongly related to RA, while antinuclear antibodies (ANA) and anti-double-stranded DNA (dsDNA) are associated with SLE. Anti-topoisomerase I and anti-centromere antibodies are markers for SS, and Anti-U1 RNP antibodies are found in MCTD (1). Recently, anti-Ro60 and anti-Ro52 antibodies have been identified as clinically relevant to the severity of diseases such as SS and SLE (2). In the case of anti-U1RNP+ MCTDs, autoantibodies against the motor neuron complex (anti-SMN antibodies) may help define not only the clinical severity in terms of multi-organ involvement but also the disease's phenotypic characteristics, thus offering a useful diagnostic and prognostic tool (3).

T-cell involvement, particularly CD4+ T-helper cells, is also crucial in the pathogenesis of CTDs. These cells initiate and sustain autoimmune responses by producing and releasing specific inflammatory mediators such as interleukins (IL-1, IL-6) and tumor necrosis factor (TNF), which recruit additional immune cells (macrophages, neutrophils) to the site of tissue damage. Yixian et al. demosntrated that elevated CD28 level on CD4-secreting CD39+ regulatory T cells increases the risk of SS, while elevated CD3 level on CD39+ CD8+ T cells decreases it. Furthermore, increased expression of CD24 on memory B cells and CD27 on IgD+ CD24+ B cells promotes the development of SS, while increased CD38 on IgD+ CD38+ B cells reduces it. Notably, efferocytosis is the process by which apoptotic cells are removed by phagocytic cells. It can be thought of as the "burial of dead cells." Defective efferocytosis has been demonstrated in several inflammatory diseases including RA and SLE. The resulting inflammation and cellular necrosis releases the cell contents sustaining the chronic inflammation. Lofaro et al. developed a systematic review that includes 1,003 papers confirming the ever-increasing scientific attention on this Research Topic supported by the constant increase in the number of publications.

Mo et al. emphasized the regulatory role of competing endogenous RNAs (ceRNAs) in the pathogenesis and treatment of SLE. However, many CTDs have a genetic component, with specific genetic variants (e.g., HLA genes) linked to an increased risk of developing these diseases. In particular, Ivanova et al. confirmed that HLA-B*08:01 allele was the primary risk factor for early-onset myastenia gravis (MG) and HLA-DRB1*15:01 allele for late-onset MG. Furthermore, the expression of HLA-A*25, HLA-B*40:01 and HLA-DRB1*16 predisposes to a higher risk of developing thymoma-associated MG.

Vitamin D receptor (VDR) polymorphisms have also been associated with susceptibility to diseases such as SLE, primary Sjögren's syndrome (pSS), and RA (4).

Immunotherapy for CTDs aims to modulate the immune system to reduce inflammation, prevent tissue damage, and improve symptoms. The goal is to suppress the overactive immune response or target specific molecules involved in the inflammatory process.

The first line of treatment often involves corticosteroids, which are powerful anti-inflammatory drugs that suppress the immune system. While effective, corticosteroids have significant side effects, especially with long-term use, such as weight gain, osteoporosis, diabetes, and an increased risk of infection. In addition to corticosteroids, immunosuppressive drugs are commonly used. These include methotrexate (for RA), azathioprine (for SLE and RA), cyclophosphamide, and mycophenolate mofetil (for lupus nephritis).

TNF-α inhibitors, such as infliximab, adalimumab, and etanercept, are biologic therapies used to treat various autoimmune diseases, including several CTDs. By inhibiting TNF, these drugs reduce inflammation and prevent further tissue and organ damage.

Janus Kinase inhibitors (JAK inhibitors) are another class of medications that target specific enzymes involved in immune response and inflammation. These inhibitors, which include tofacitinib, baricitinib, upadacitinib, and filgotinib, have become an important treatment option for patients with diseases difficult to manage with traditional therapies. They help control inflammation, reduce disease activity, and prevent further organ damage.

B-cell depletion therapy is an advanced treatment used in several autoimmune and connective tissue diseases. The most widely used B-cell depletion therapy involves monoclonal antibodies that target CD20, a protein on the surface of most B cells. Rituximab and ofatumumab are the main monoclonal antibodies used for this purpose (5). These therapies reduce the number of active B cells in the body, thereby reducing autoimmune activity. In the context of advanced cellular therapies, mesenchymal stem cells (MSCs) and chimeric antigen receptor T cells (CAR-T cells) represent two innovative and promising approaches for the management of scleroderma (Chen et al.).

Monoclonal antibodies are also a key part of modern treatments for connective tissue diseases. They offer targeted therapies that provide better disease control with fewer side effects compared to traditional treatments. Examples include rituximab, abatacept, belimumab, tocilizumab, and secukinumab, which are used in conditions like lupus, rheumatoid arthritis, scleroderma, and vasculitis (6).

Gene therapy holds great promise for CTDs by targeting the underlying genetic causes of these diseases, correcting immune dysfunction, and promoting tissue repair. Although gene therapy for CTDs is still in its early stages, ongoing clinical trials and preclinical studies are investigating approaches such as CRISPR-Cas9 gene editing to modify immune cell behavior in diseases like lupus and rheumatoid arthritis (7).

Author contributions

GM: Conceptualization, Supervision, Writing – review & editing. WS: Writing – review & editing. ZL: Writing – review & editing. CA: Writing – review & editing. PL: Writing – review & editing. MS: Writing – review & editing. FP: Writing – original draft.

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Construction of competing endogenous RNA networks in systemic lupus erythematosus by integrated analysis

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Objective: Systemic lupus erythematosus (SLE) is a disease characterised by immune inflammation and damage to multiple organs. Recent investigations have linked competing endogenous RNAs (ceRNAs) to lupus. However, the exact mechanism through which the ceRNAs network affects SLE is still unclear. This study aims to investigate the regulatory functions of the ceRNAs network, which are important pathways that control the pathophysiological processes of SLE.

Methods: CircRNA microarray for our tested assays were derived from bone marrow samples from three healthy individuals and three SLE patients in our hospital. The other sequencing data of circRNA, miRNA and mRNA were obtained from Gene Expression Omnibus (GEO) datasets. Using the limma package of R program, the differential expression of mRNA and miRNA in the GEO database was discovered. Then predicted miRNA-mRNA and circRNA-miRNA were established using miRMap, miRanda, miRDB, TargetScan, and miTarBase. CircRNA-miRNA-mRNA ceRNA network was constructed using Cytoscape, and hub genes were screened using a protein-protein interaction network. Immune infiltration analysis of the hub gene was also performed by CIBERSORT and GSEA.

Results: 230 overlapped circRNAs, 86 DEmiRNAs and 2083 DEmRNAs were identified in SLE patients as compared to healthy controls. We constructed a circRNA–miRNA–mRNA ceRNAs network contained 11 overlapped circRNAs, 9 miRNAs and 51 mRNAs. ESR1 and SIRT1 were the most frequently associated protein-protein interactions in the PPI network. KEGG analysis showed that DEGs was enriched in FoxO signaling pathway as well as lipids and atherosclerosis. We constructed a novel circRNA-miRNA-mRNA ceRNA network (HSA circ 0000345- HSA miR-22-3-P-ESR1/SIRT1) that may have a major impact on SLE.

Conclusion: Through this bioinformatics and integrated analysis, we suggest a regulatory role for ceRNA network in the pathogenesis and treatment of SLE.

KEYWORDS

systemic lupus erythematosus, differentially expressed genes, competing endogenous RNAs, bioinformatics analysis, enrichment analysis

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune connective tissue disease that can lead to functional impairment of multiple organs and increased morbidity and mortality (1, 2). Many studies have confirmed the involvement of non-coding RNAs in the pathogenesis of lupus (3), while the exact pathogenesis is not fully understood. Thus, exploring the molecular features and mechanisms of non-coding RNA in SLE is crucial for better insight and treatment of the disease.

Competing endogenous RNAs (ceRNAs) are molecules of both coding and non-coding RNAs that can be targeted by the same miRNAs in the right context and can indirectly regulate each other by competing for them, leading to additional post-transcriptional regulatory layers where non-coding RNAs can find new meaning. miRNA-mediated interactions between different types of RNA molecules have been observed in many different contexts (4). Circular RNAs (circRNAs) lack 5′- and 3′-terminal poly (A) tails, making them less susceptible to nucleic acid exonuclease-driven degradation, resulting in a longer median half-life (5). Therefore, circRNAs are more likely to function as sponges and participate in the competing endogenous RNA (ceRNA) network (6). Researchers have experimentally tested instances of ceRNA crosstalk in a large number of situations and observed in normal and pathological contexts, demonstrating the widespread involvement of this mechanism in brain regenerative mechanisms (7), neuronal and muscle developmental processes (8) and cellular differentiation (9), as well as in diseases which highly complex gene regulatory circuits are most affected by perturbations. Researchers strive to model these regulatory networks to predict and understand how modifications alter the dynamic balance between molecules, leading to the onset of cancer and its progression, cardiovascular problems and neurodegenerative diseases, as well as other pathologies, such as those related to immune and autoimmune responses and degenerative physical conditions. Recently, several studies reported an association between ceRNA network and lupus (10, 11). The development of new computational tools that provide hypothetical predictions of ceRNA interactions facilitates insights into lupus pathogenesis and the discovery of novel therapeutic targets.

CircRNA, miRNA, and mRNA data can be obtained from the Gene Expression Omnibus (GEO) database (12), making it a valuable tool for biological discovery and data mining. Integrative ceRNA regulation circuits can be built to investigate more precise prognostic markers using these publicly available databases. In our study, we constructed ceRNAs networks of lupus by taking the intersection of our self tested circRNA microarray data with datasets from the GEO database. So far, such a study is unique.

In this research, we performed differential expression analysis using circRNA microarray of bone marrow samples from three healthy participants and three patients in our department and circRNA datasets in the GEO dataset. We then used online tools to identify the relationship between miRNA-circRNA and miRNA-mRNA to construct ceRNA network. By using enrichment function analysis, the biological significance of mRNA differential expression was determined. The protein-protein interaction (PPI) network (13) was used to search hub genes. Immune infiltration analysis of the hub gene was also performed by CIBERSORT and GSEA.

Methods

Bone marrow samples collection

Bone marrow samples were obtained from 3 healthy participants and 3 patients with systemic lupus erythematosus (SLE) at Fujian Provincial Hospital. All participants, both healthy and those with SLE, were of Han Chinese origin. The inclusion criteria required patients with untreated first-onset SLE and healthy subjects. SLE diagnosed according to the 1997 updated American College of Rheumatology (ACR) classification criteria (14). This research received approval from the Ethics Committee of the Fujian Provincial Committee. Informed consent was obtained from all participants.

Data collection

We searched for SLE-associated aberrantly expressed RNA microarray data from the GEO database.[1] The selection criteria were as follows: (a) The dataset included lupus patients and healthy controls. (b) Arrays contained at least 3 samples from patients and 3 samples from the normal group; (c) Uploaded data were available for analysis. Finally, GSE84655 (circRNA, 6 SLE vs. 3 control), GSE175840 (miRNA, 5 SLE vs. 5 control) and GSE175839 (mRNA, 5 SLE vs. 5 control) were included in the study. The downloaded files were calibrated, standardized, and log2 transformed using R software (15).

Identification of the differentially expressed circRNAs, miRNAs and mRNAs

Batch effects were normalised using the sva package. Differential analysis was performed using the limma package. DEcircRNAs were identified using a threshold of |log2FC| over 1.2 and an adjusted *p*-value less than 0.05. Also, the ballgown package was employed to identify DEmiRNAs and DEmRNAs. The thresholds set were an adjusted *p*-value less than 0.05 and |log2FC| above 1.2 for DEmiRNAs and DEmRNAs.

ceRNA network

The miRNAs corresponding to circRNAs were predicted by StarBase (16). To obtain the paired miRNA_mRNAs, miRMap (17), miRanda (18), miRDB (19), TargetScan (20), and miTarBase (21) were used to find possible targeted mRNAs of miRNA. Subsequently, the overlapping predictions between the two programs were considered effective target pairs. ceRNA networks were constructed in Cytoscape (version 3.7) (22).

Gene function enrichment analysis

The database for annotation, visualization, and integrated discovery (DAVID) (23) was employed to determine the biological

1 http://www.ncbi.nlm.nih.gov/geo

functions of DEGs. The biological process (BP), molecular function (MF), and cellular component (CC) were applied in GO enrichment analyses.[2] The Kyoto Encyclopedia of Genes and Genomes (KEGG) serves as a database resource for comprehending biological systems and overarching functions (24). The cut-off criteria were the false discovery rate < 0.1 and adjusted p-value < 0.05.

Formation of protein-protein interaction network and identification of hub genes

A PPI network of the DEmRNAs was established using the Search Tool for the Retrieval of Interacting Genes (STRING).[3] Required Confidence (combined score) > 0.4 was selected as the threshold for PPI. The PPI network was visualized using Cytoscape. Molecular Complex Detection (MCODE) app of Cytoscape was used to determine hub genes.

Immune infiltration analysis of hub-gene

CIBERSORT, an universal deconvolution algorithm, was employed to examine the immune cell subset proportions using RNA expression profiles (25). Herein, we obtained a matrix of 22 immune cell subsets in GSE50772 via the R package of CIBERSORT. A bar plot displayed the percent of each immune cell and compare infiltrating levels between SLE patients and controls using the "ggplot2 (v3.3.0)" package, and the "corrplot (v0.90)" package was utilized to depict the relationship of immune cell subsets. $p < 0.05$ was determined to be statistically significant.

Gene set enrichment analysis

GSEA software (version 3.0) was obtained from the GSEA website.[4] The Molecular Signatures Database[5] was used to assess the pathways and molecular mechanisms involved in the hub genes. $p < 0.05$ was determined to be statistically significant.

Result

Identification of DEcircRNAs in SLE

First, we analyzed 2 profiles of circRNAs including our self data and GSE84655, basic information was shown in Figure 1A. We detected 2,668 different expressed circRNAs (1,602 up-regulated, 1,066 down-regulated) in lupus samples using our tested bone marrow circRNA microarray (Figure 1B). Figure 1C showed the heatmap of top 20 differentially expressed circRNAs. To refine the identification further, a search for DEcircRNAs was conducted in GSE84655 and 1739 DEcircRNAs (847 up-regulated, 892 down-regulated) were found (Figure 1D). Subsequently, the top 20 circRNAs displaying the most significant variations were selected and are exhibited in Figure 1E. Ultimately, we integrated 230 overlapped circRNAs in SLE patients as compared to healthy controls (66 down regulated circRNAs Figure 1F and 164 up regulated Figure 1G).

Determination of lupus-associated DEmRNAs and DEmiRNA

To explore the potential functions of mRNAs/miRNAs/circRNAs in SLE, The mRNA (GSE175839) and miRNA (GSE175840) datasets were further analyzed. In SLE group, 86 DEmiRNAs (15 up-regulated, 71 down-regulated), and 2083 DEmRNAs (167 up-regulated, 1916 down-regulated) were discovered based on predetermined thresholds (Figure 2A). The top 20 DEmiRNA and DEmRNAs revealed in Figures 2B,C.

In order to find out the targeted miRNAs corresponding to GSE175839, DEmRNAs were searched in the miRMap, miRanda, miRDB, TargetScan and miTarBase. Based on the principle that paired miRNA-mRNA presented at least four databases, we confirmed 34 pairs of down-regulated miRNA and up-regulated interacted mRNA (Figure 2D) and 115 pairs of up-regulated miRNA and down-interacted mRNA (Figure 2E).

ceRNA network in SLE

Using the overlapped DEcircRNAs discovered in Figure 1, the 156 pairs of miRNAs that were down-regulated and the 1,180 pairs of miRNAs that were up-regulated were extracted from Starbase.

As shown in the flowchart (Figure 3), overlapped DEcircRNAs of (Figure 1) predicted cirRNA-miRNA, mRNA-miRNAs predicted by DEmRNAs of GSE175839 and the DEmiRNAs of GSE175840 were taken interacted, and identified the experimentally strongly supported SLE specific differentially expressed of the circRNA-miRNA-mRNA network. This network contained 11 circRNAs, 9 miRNAs and 51 mRNAs (Table 1). Then, we visualized these candidate genes and constructed the ceRNA network using cytoscapeV3.7 (Figure 4).

Functional and pathway enrichment analysis for the targeted genes in the ceRNA network

We used DAVID to conduct functional and pathway enrichment analyses to gain more insight into the biological roles of the 51 DEmRNAs implicated in SLE. The top 15 KEGG pathways and enriched GO terms were exhibited in Figure 5. The GO analysis revealed that DEGs were primarily enriched in the following categories: "chromatin organization," "chromosomal organization," and "the negative regulation of fat cells" (BPs); "intracellular membrane-bounding organelle," "nuclear lumen," and "nucleotic" (CCs); and "kinase binding," "protein kinase binding," "RNA polymerase II-specific DNA binding transcription activator activity" (MFs). KEGG pathway analysis

2 http://geneontology.org/page/download-ontology
3 http://string-db.org/
4 http://software.broadinstitute.org/gsea/index.jsp
5 http://www.gsea-msigdb.org/gsea/downloads.jsp

A

Basic information of circRNA in 2 microarray datasets

Data source	Platform	log2Fold change	P_value	Up-DEGs	Down-DEGs	Sample size (SLE/Control)	Detected RNA type
GSE84655	GPL21825	1.2	<0.05	847	892	6/3	circRNA
Self-test dada	/	1.2	<0.05	1602	1066	3/3	circRNA

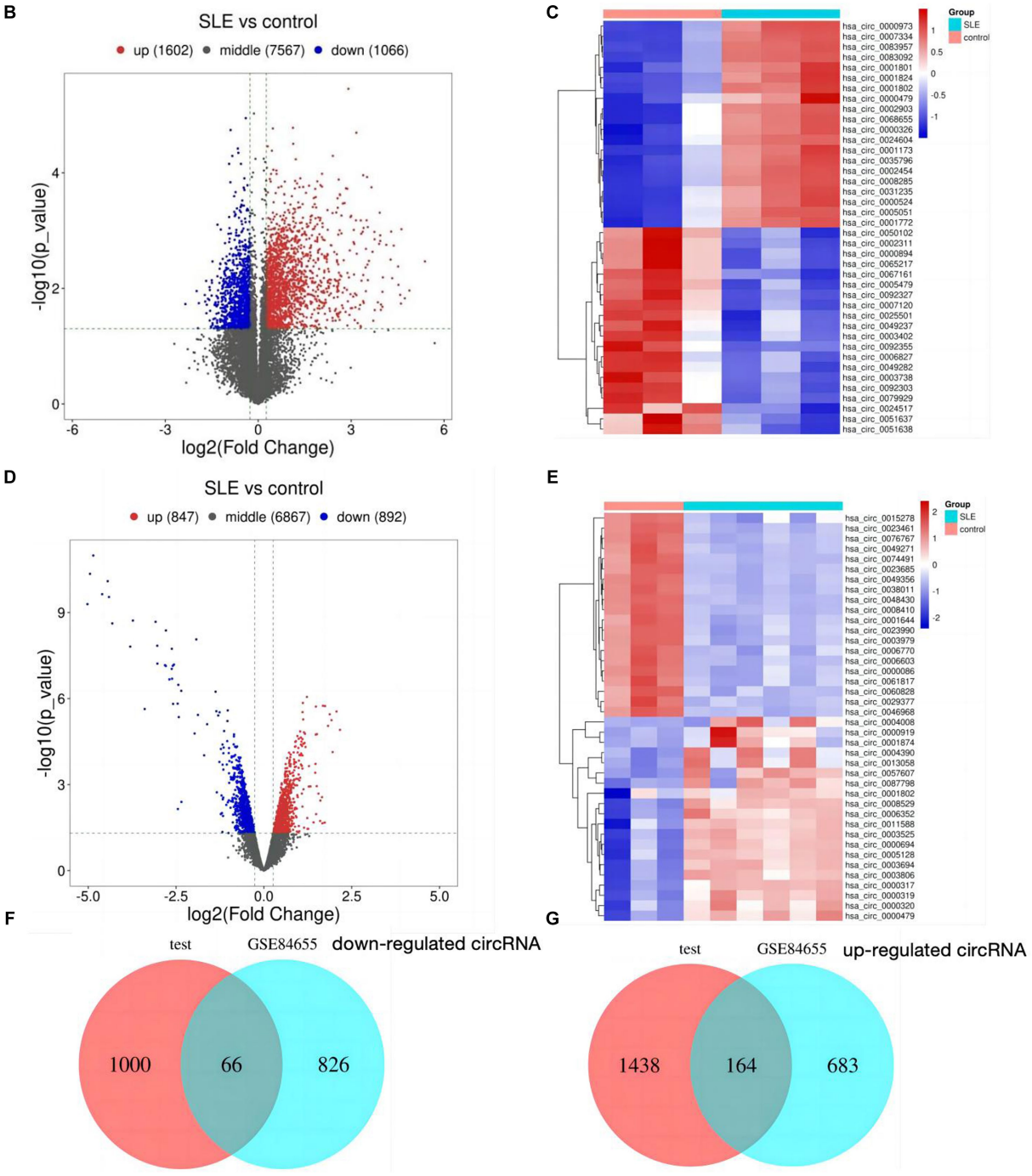

FIGURE 1
Identification of DEcircRNAs in SLE **(A)** Basic information of circRNA datasets. **(B)** Volcano plots for different expression of circRNAs of bone-marrow in SLE. **(C)** Heatmap plot showing the top 20 DEcircRNAs in our dataset. **(D)** Volcano plots for DEcircRNAs of GSE84655. **(E)** Heatmap plot showing the top 20 DEcircRNAs of GSE84655. **(F,G)** The Venn plots of DEcircRNAs of two datasets.

presented that DEGs of ceRNA network of SLE were enriched in malignancies, infection, Lipid and atherosclerosis, apoptosis, endocrine resistance, FoxO signaling, HIF-1 signaling, Estrogen signaling and thyroid hormone signaling (Figure 5D).

A

Basic information of microarray datasets from GEO

Data source	Platform	log2Fold change	P_value	Up-DEGs	Down-DEGs	Sample size (SLE/Control)	Detected RNA type
GSE175840	GPL11154	1.2	<0.05	15	71	5/5	miRNA
GSE175839	GPL11154	1.2	<0.05	167	1916	5/5	mRNA

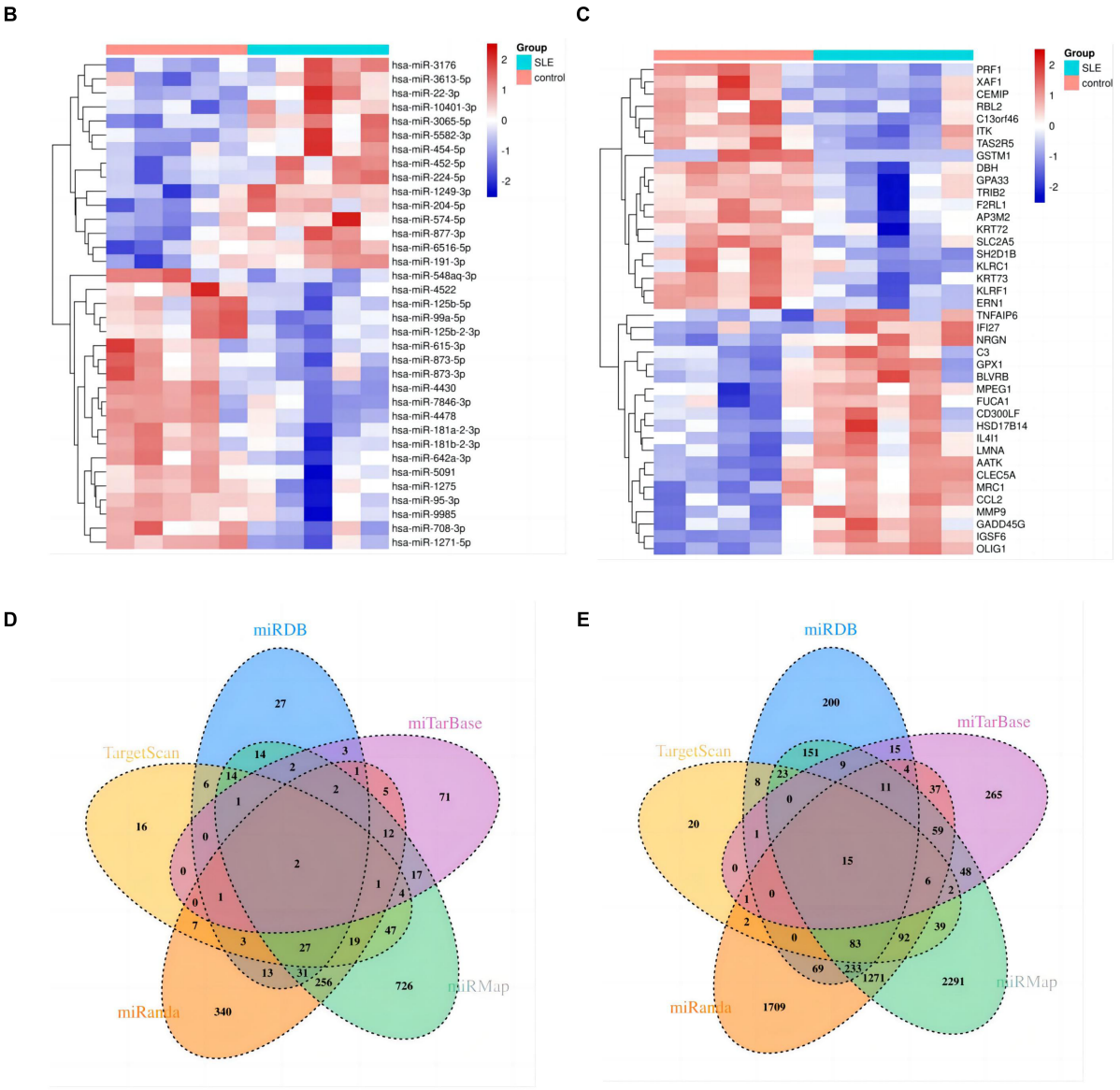

FIGURE 2
Determination of lupus-associated DEmRNAs and DEmiRNA. **(A)** Basic information of miRNAs and mRNA datasets. **(B,C)** Hotmap of the 20 top DEmiRNAs and DEmRNAs. **(D,E)** The Venn plots of predicted miRNAs based on DEmRNAs of GSE175839.

Identification of hub genes

After obtaining the target mRNA of ceRNA network in SLE, 51 DEmRNAs were imported into the PPI network composed of 51nodes and 22edges (Figure 6A). Following the identification of the vital functions of hub genes in the network, we used MOCDE plugin of cytoscape for further analysis, and 9 hub genes were explored (HMOX1, SIRT1, ESR1, AKT3, CDKN18, KDM3A, CHD9, NCOA1, ZNF609).

Immune infiltration characteristics of hub gene SIRT1and ESR1

When screening hub genes (Figure 6B), ESR1 and SIRT1 were the most frequently associated protein-protein interactions in the PPI network. To further investigate the mechanism of SIRT1 and ESR1 involvement in lupus pathogenesis, we analyzed SIRT1 and ESR1 using GSEA and also found that SIRT1 and ESR1 were involved in

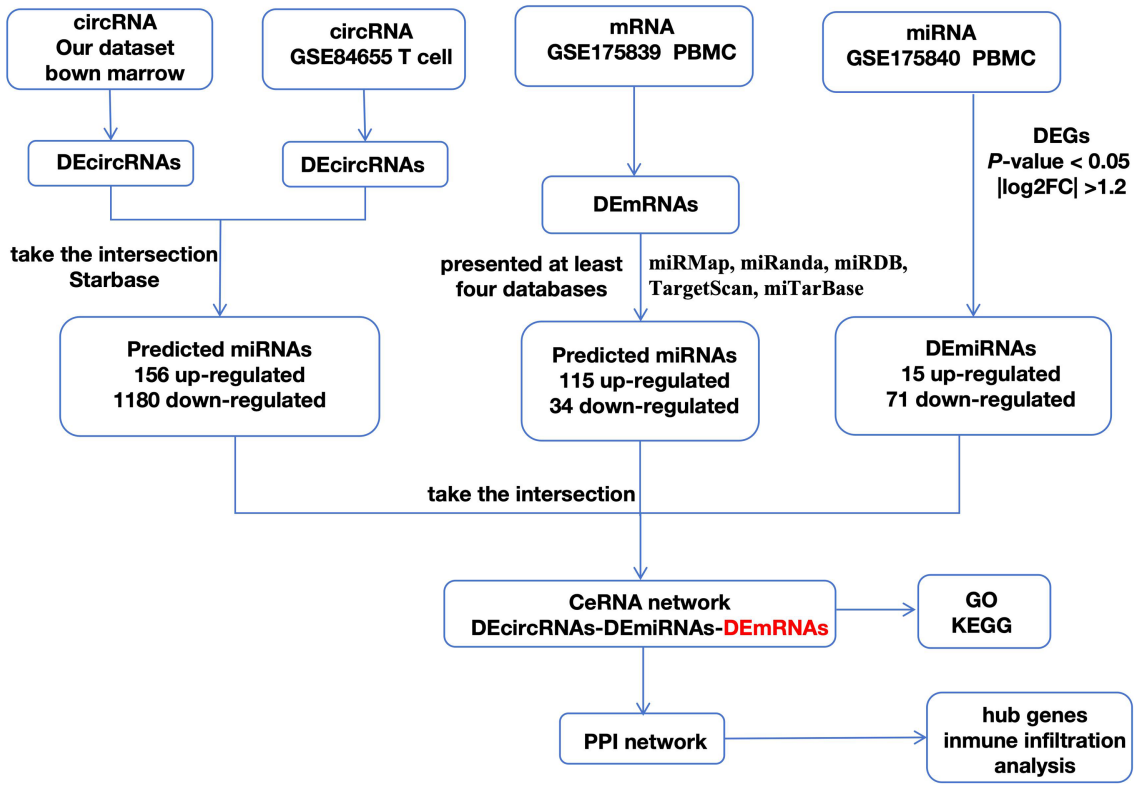

FIGURE 3
A flowchart of DEGs analysis.

TABLE 1 Different expression of circRNAs-miRNAs-mRNAs in SLE.

DE circRNAs	Interacted DEmiRNAs	Targeted mRNAs
hsa_circ_0000345 hsa_circ_0001345	hsa-miR-22-3p hsa-miR-224-5p hsa-miR-452-5p	CHD9, CCNJL, PHF8, POGK, AKT3, BCL9L, C5orf24, ZNF609, MSL2, CYTH3, EPB41L2, ESR1, RIC8B, NCOA1, PDS5A, PDSS1, MPZL3, FNBP4, PRPF38A, RAB5B, ZBTB39, TRUB1, KDM3A, KHNYN, SYNE1, TRIB2, WDR82, WWC2, RSBN1, SIRT1, C18orf25, INTS8, EPM2AIP1, ZNF644, ADD3, PGM2L1, SF3B1, CHD2, CASD1, CDKN1B
hsa_circ_0000787 hsa_circ_0006669 hsa_circ_0001146 hsa_circ_0001022 hsa_circ_0007927 hsa_circ_0000347 hsa_circ_0000227 hsa_circ_0000228 hsa_circ_0001599	hsa-miR-642a-5p hsa-miR-374a-3p hsa-miR-26b-5p hsa-miR-26a-5p hsa-miR-200b-3p hsa-miR-1271-5p	MAP2K3, TNS3, DDIT3, SORT1, MARCKS, CKAP4, IER5, SHCBP1, BID, SCARF1, HMOX1

many biological processes of immune cells (Figures 7A,B), which had been demonstrated in SLE. Immune cell infiltration also confirmed the presence of statistically significant differences in CD4+ memory T cells, naive B cells and M0 Macrophages between SLE patients and controls (Figure 7C). Spearman correlation analysis was subsequently performed to demonstrate the relationship between DEOSRGs and various immune cell subsets. As shown in Figure 7D, SIRT1 was positively correlated with CD4+ memory T cells ($r = 0.733$, $p = 0.021$) and naive B cells ($r = 0.672$, $p = 0.039$), while it was negatively correlated with M0 Macrophages ($r = -0.661$, $p = 0.044$). Meanwhile, the immune infiltration analysis (Figure 7E) indicated that ESR1 had a positive correlation with CD4+ memory T cells ($r = 0.87$, $p = 0.0027$) and NK cells ($r = 0.77$, $p = 0.014$), but a negative correlation with regulatory T cells ($r = -0.79$, $p = 0.0098$), eosinophils ($r = -0.83$, $p = 0.0056$), and M0 macrophages ($r = -0.89$, $p = 0.0013$). Using the CIBERSORT method, we evaluated the immune infiltration features between SLE patients and controls. The bar plot depicted the relative percent of diverse immune cell subsets in each sample (Figure 7F). These results revealed that SIRT1 and ESR1 may be crucial to the progression in SLE.

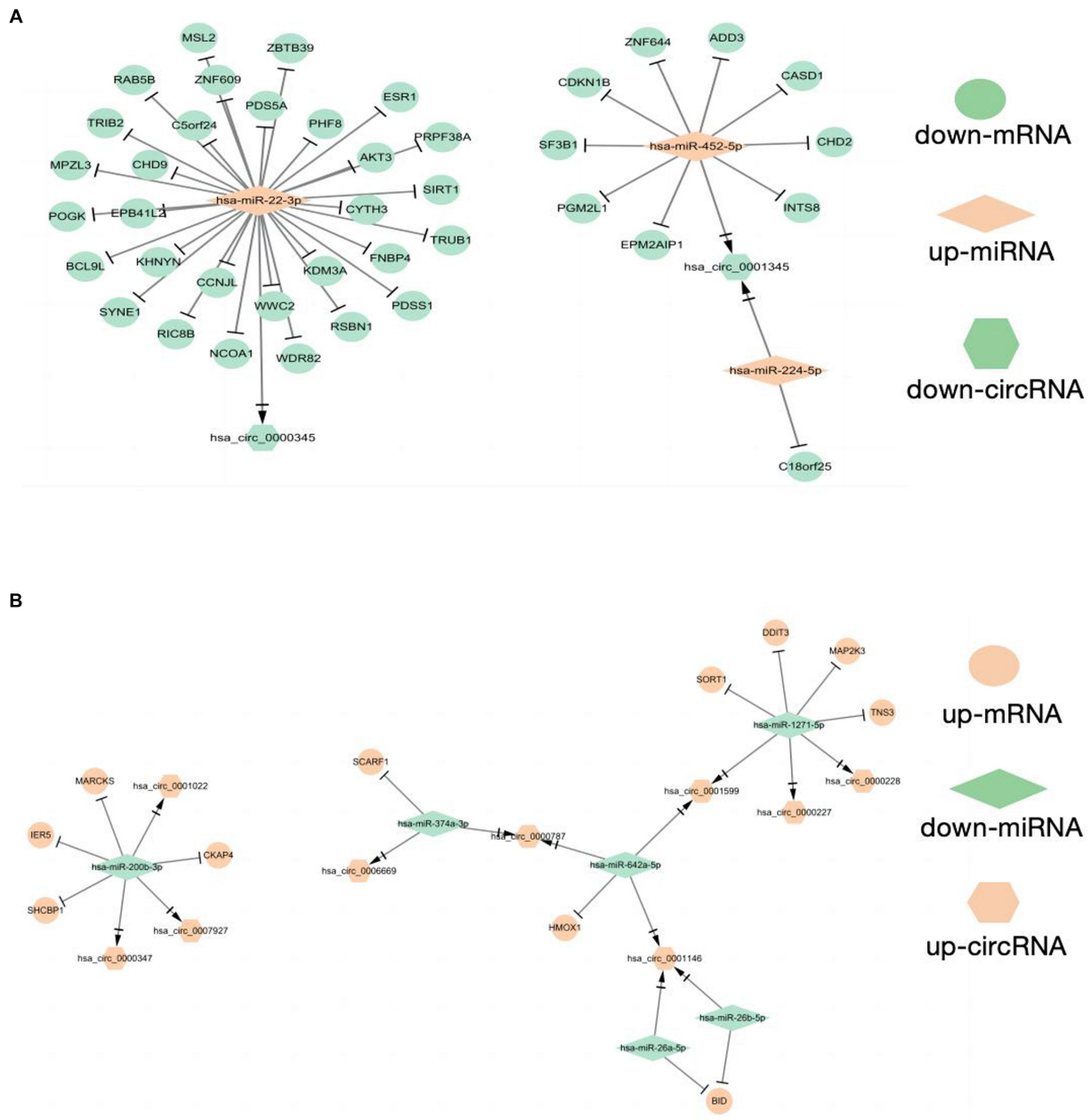

FIGURE 4
Construction of CeRNAs networks **(A)** CircRNA(down)-miRNA(up)-mRNA(down) regulatory network **(B)** CircRNA(up)-miRNA(down)-mRNA(up) regulatory network.

Discussion

The expression profiles of non-coding RNAs (ncRNAs), which include miRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), have attracted increasing attention in various diseases due to the rapid growth of bioinformatics (26, 27). Compared with healthy individuals, most circRNAs were found to be abundant in peripheral blood, serum, T cells, peripheral blood mononuclear cells and kidney tissue, indicating their possible roles. In summary, circRNA dysregulation may have implications for key molecular processes involved in the pathogenesis of systemic lupus erythematosus (28). Therefore, a more comprehensive understanding of the importance of circRNAs in SLE is required. We used microarray data analysis to construct the circRNA-miRNA-mRNA network. The mRNAs in the network were used to identify significantly altered GO terms and the KEGG signaling pathway. The hub genes were then identified using the PPI network. In order to open up a new perspective on the therapy of SLE, certain effective substances have also been discovered.

Based on the results of functional enrichment analysis, we hypothesized that in SLE, the aberrant circRNAs utilize protein and RNA binding to regulate chromosome organization, chromatin organization, and negatively regulate adipocyte differentiation, as well as positively regulate colorectal migration of blood vessels. According to KEGG signaling pathway analysis, it is linked to cancer-related signaling pathways. Since the immunological and genetic pathways of SLE pathogenesis and the use of immunosuppressive drugs (ISDs) in

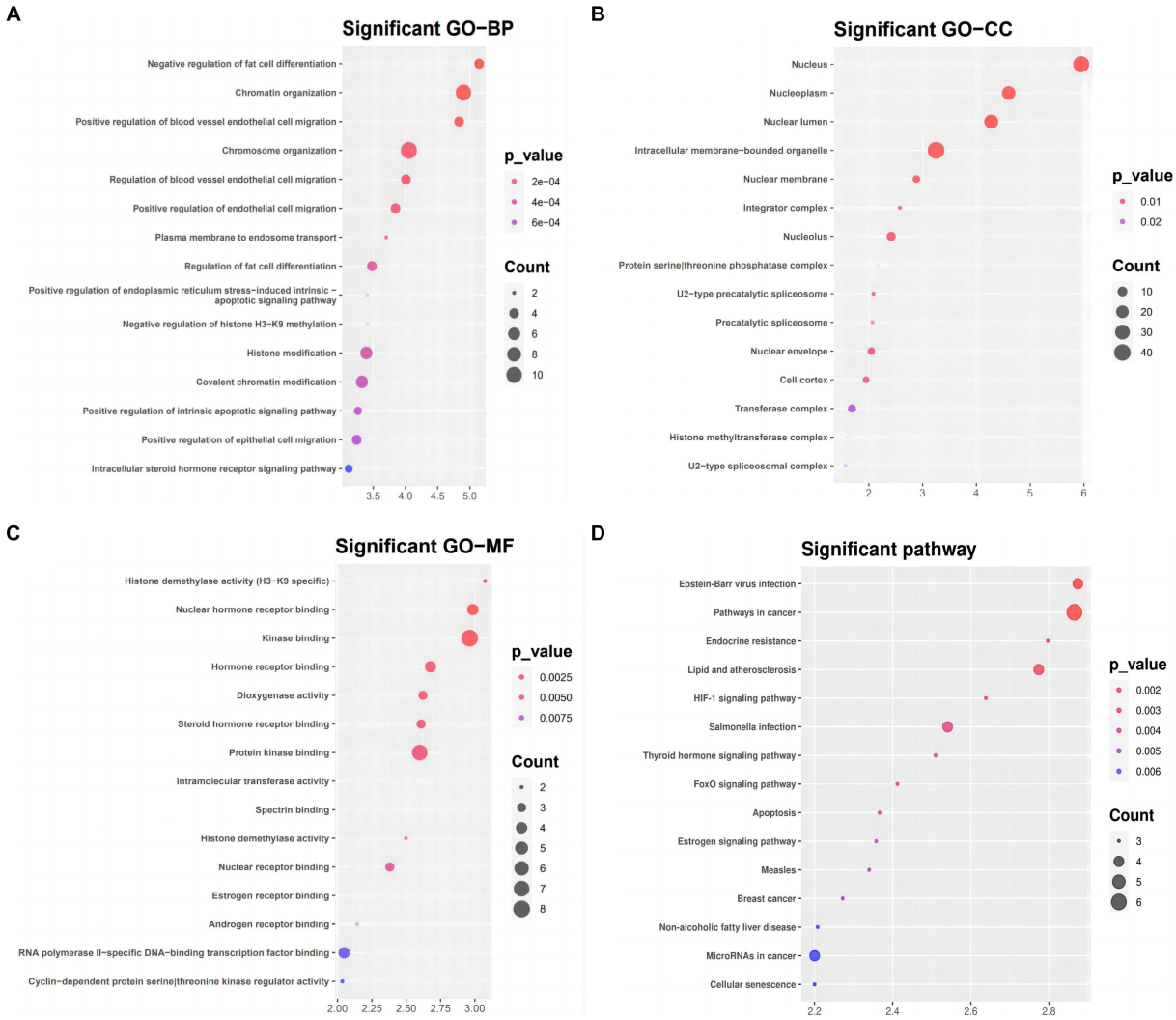

FIGURE 5
GO term and KEGG pathways enrichment analysis **(A)** Biological process (BP), **(B)** cellular component (CC), **(C)** molecular function (MF) of the GO annotation **(D)** KEGG pathways of DEmRNA.

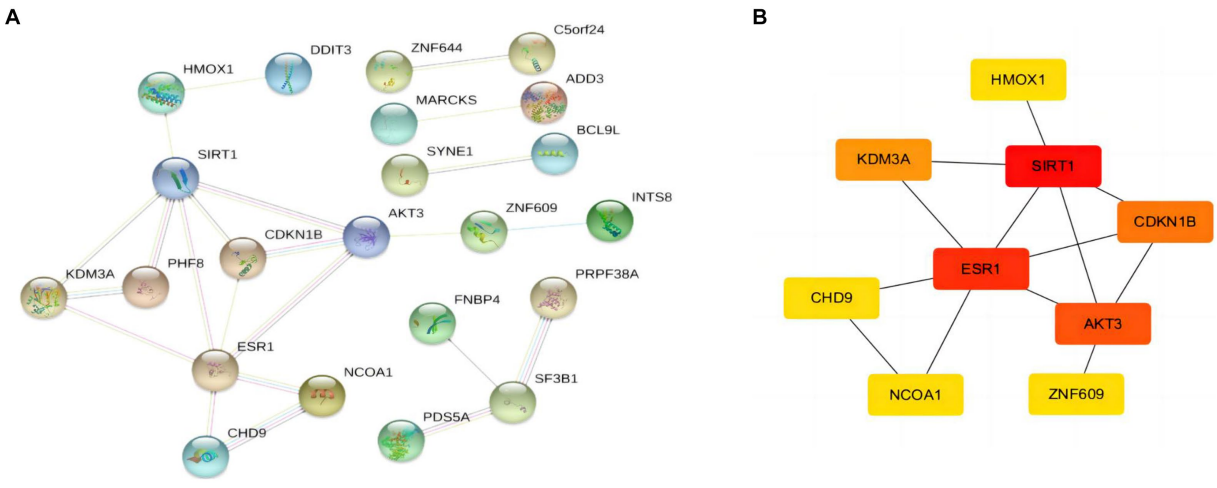

FIGURE 6
PPI network and hubgenes of targeted DEmRNAs in SLE **(A)** PPI network of DEmRNAs **(B)** The top 9 hubgenes analysed by Cytoscape.

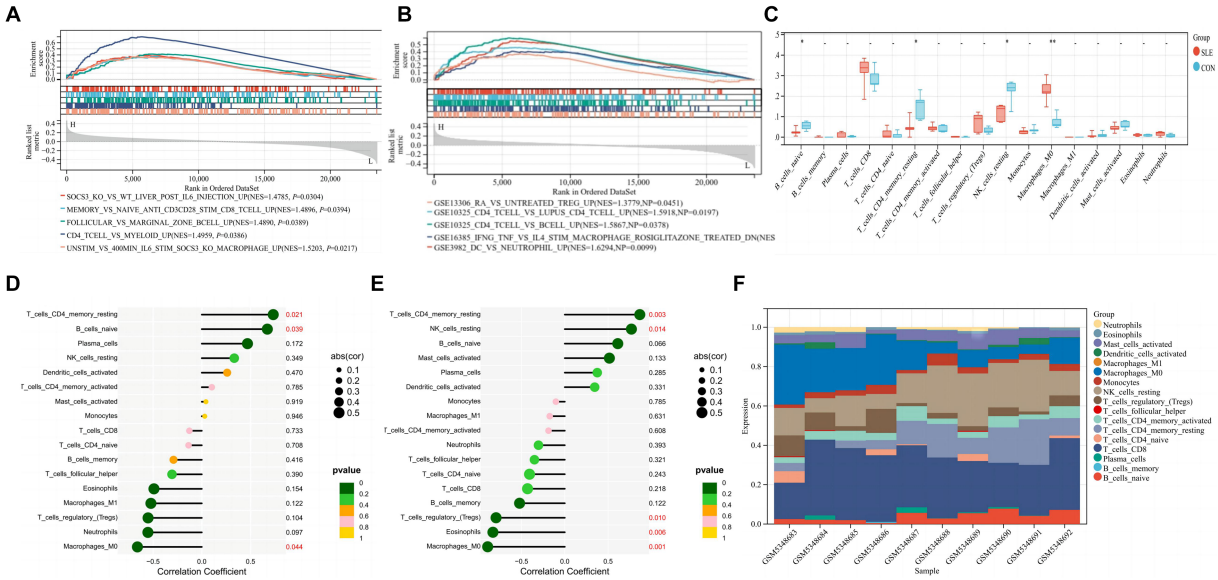

FIGURE 7
Immune infiltration characteristics between SLE patients and controls. **(A,B)** GSEA analysis for SIRT1 and ESR1 in SLE group. **(C)** Infiltrating difference of immune cells between SLE patients and controls in the box plot. **(D,E)** Spearman correlation between immune cell subsets for SIRT1 and ESR1. The color of dots denotes the P value. The size of dots denotes the strength of correlation. **(F)** The relative percent of immune cell subsets in a bar plot. Statistical significance at the level of * <0.05, ** <0.01, and *** <0.001. SLE: Systemic lupus erythematosus; CON: Controls.

treatment may increase the likelihood of such changes, the relevant literature has shown (29) that the risk of malignancy in SLE is considerable. In KEGG pathway analysis, it can be found that ESR1 is mainly related to endocrine resistance, the cancer signaling pathway, the estrogen signaling pathway, and Epstein-Barr virus infection, while SIRT1 is mainly related to the FoxO signaling pathway and Salmonella infection. Salmonella infection disrupts SIRT1/AMPK checkpoint control of mTOR to impair autophagy (30), and alterations of autophagy contribute to the progression of various autoimmune diseases, including systemic lupus erythematosus (SLE) (31). In the FoxO signaling pathway, such observations with Foxo3a in helper T cells imply disease relevance in SLE (32). However, the SIRT1-regulated signaling pathway of these hub genes (ESR1) in autoimmune diseases has not been reported. Whether it is related to the pathogenesis of SLE needs to be confirmed by further experiments.

SLE is more prevalent in women of reproductive age between the ages of 15 and 40, with a male-to-female incidence ratio of 1: 9. As a result, it has been suggested that estrogen may play a role in the development of SLE. In lymphoid nuclei, estrogen primarily interacts with estrogen receptor types 1 and 2 (ESR1 and ESR2, respectively) to carry out biological actions (33). Further evidence that ESR1, not ESR2, produces the lupus phenotype can be found in male mice lacking functioning ESR1. These mice are resistant to developing the lupus phenotype (34). Recent studies demonstrating that ESR1 promotes SLE in F1-generation female mice of the lupus mouse model (35) lend weight to this idea. In a previous investigation, Lee et al. (36) found that ESR1 gene polymorphisms were related to the age at which SLE first manifested in Korean patients; however, this study only included female participants. ESR1 rs2234693 and rs9340799 were revealed to be strongly linked with SLE susceptibility by Wang et al. (37). When Zhou et al. (38) compared the frequencies of C allele rs2234693 and G allele rs9340799 in the ESR1 gene between SLE patients and non-SLE controls, they found that the frequency of the C allele was considerably higher. Faslodex, an ESR1 antagonist, significantly decreased SLEDAI scores in SLE patients in a limited clinical trial (39). These findings support the idea that estrogen plays a part in the development of SLE.

SIRT1 is an NAD^+-dependent monomeric protein that plays a significant role in important cellular activities, including cell differentiation, apoptosis, metabolism, aging, and immunological response. In Consiglio's research, the SIRT1 promoter variant rs3758391 was found to be connected with SLE incidence, and the rs3758391 T allele may be linked to higher SLEDAI and lupus nephritis scores (40). Anti-dsDNA antibodies are well documented to fluctuate with SLE activity and to be closely associated with the symptoms of severe lupus (41, 42). Olivares et al. found that serum anti-dsDNA antibody levels in LN patients were significantly correlated with urinary SIRT1 mRNA levels, which is a valuable marker of renal injury (43). Systemic lupus erythematosus (SLE) patients had higher levels of the plasma protein SIRT1 (SIRT1) compared to healthy controls, and there was a significant correlation between the plasma SIRT1 concentration and disease activity, according to research by Yang et al. (44).

The final circRNA-miRNA-mRNA network contained HSA circ 0000345 and HSA miR-22-3p, which we identified. Only pertinent literature has demonstrated that there is currently no relationship between this circRNA and autoimmune disorders (45, 46). By suppressing HSA circ 0000345, HSA circ 0000345 plays a critical role in the treatment of atherosclerosis. The involvement of HSA circ 0000345 in endothelial cell damage is strongly related to the onset and progression of atherosclerosis. Down-regulation of HSA circ 0000345 has been demonstrated in the literature (47) to have an anti-tumor impact. However, there are no studies indicating that abnormal expression of HSA circ 0000345 contributes to the

development of SLE. Future studies should verify the expression status of HSA circ 0000345 in SLE patients and its function. Additionally, Previous studies have demonstrated that miR-22-3p expression levels are increased in the peripheral blood of patients with lupus (48, 49), which is consistent with our findings. In contrast, compared to controls, SLE with lupus nephritis (LN) patients exhibited a downregulation of miR-22-3p (50). The disparate results may be attributed to the varying cell subtypes examined. Further experimentation is required to confirm its status and function.

Nevertheless, it is necessary to acknowledge the limitations of this work. First, the sample size of bone marrow for high-throughput sequencing was limited due to the difficulty in accessing bone marrow specimens from SLE patients and limited research funding. Additionally, the candidate genes screened were not validated with a large enough sample size, which resulted in a lack of rigor and precision. To address these issues, we plan to expand the sample size and validate the function and mechanism of action of the hub genes in SLE in further experiments.

Conclusion

In summary, a novel circRNA-miRNA-mRNA ceRNA network (circ 0000345- miR-22-3-P-ESR1/SIRT1) was identified in SLE by bioinformatics and comprehensive analysis, which could be served as a possible biomarker or therapeutic target for SLE.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: GEO, accession GSE266651, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE266651.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Fujian Provincial Hospital (Approval ID: K-2021-11-019). 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

JH: Writing – original draft. YD: Software, Visualization, Writing – original draft. JL: Data curation, Investigation, Writing – review & editing. HL: Writing – review & editing. FG: Methodology, Supervision, Writing – review & editing. ZC: Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – review & editing. YW: Conceptualization, Data curation, Investigation, Software, Visualization, 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.

Publisher's note

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The causal relationship between immune cells and Sjögren's syndrome: a univariate, multivariate, bidirectional Mendelian randomized study

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Introduction: Immune cells are involved in the onset and progression of Sjögren's syndrome (SS). This study explored the causal relationship between immune signature cells and SS, which has not been fully elucidated.

Methods: We conducted univariate, multivariate, and bidirectional Mendelian randomization to investigate the causal relationship between 731 immunological feature characteristic cells and SS pairs and explore the interaction of immune cells in SS.

Results: After false discovery rate correction, six immune cells were significantly associated with SS risk. Among them, four contributed to SS (CD24 on memory B cell, CD27 on IgD + CD24 + B cell, CD28 on CD39+ secreting CD4 Treg cell, and CD80 on CD62L + mDC); two appeared to reduce SS risk (CD3 on CD39 + CD8 + T cell and CD38 on IgD + CD38 + B cell). Pleiotropy and heterogeneity were not observed. Three immune cells exerted independent effects for SS (CD27 on IgD + CD24 + B cell, CD80 on CD62L + mDC, and CD38 on IgD + CD38 + B cell); two were risk factors (CD27 on IgD + CD24 + B cell and CD80 on CD62L + mDC); and one was a protective factor (CD38 on IgD + CD38 + B cell). Twenty-three immune cells showed a reverse causal relationship with SS.

Conclusion: These findings demonstrate the influence of immune cells on SS risk and the effects of SS on immune cells, providing new clues for further research on the mechanisms underlying SS.

KEYWORDS

Mendelian randomization, immune cells, Sjögren's syndrome, causal relationship, B cell

Introduction

Sjögren's syndrome (SS) is a heterogeneous, etiological, systemic autoimmune disease characterized by chronic inflammation and dysfunction of the exocrine glands. SS can affect different organs and tissues and is usually accompanied by sicca symptoms, such as fatigue, chronic pain, and multiple organ-related symptoms (1, 2). SS is the second most common autoimmune rheumatic disease, affecting between 0.4 and 3.1 million individuals (3). SS causes a health burden for patients and substantial social disease costs, necessitating early diagnosis and active intervention to improve the prognosis of SS.

The pathogenesis of SS is very complex and has not been fully elucidated. Increasingly more evidence has shown that immune system dysfunction plays a crucial role in the

etiology of SS, and the interaction between inflammation and genetic and environmental variables influences the occurrence, development, and resulting tissue damage of SS (4). Studies have shown that disturbances of the innate immune barrier involving the interferon (IFN) pathway in the early stages of SS disease are involved in the etiology of SS (5, 6). Vogelsang et al. reported a significant reduction in myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) in the peripheral blood as well as the presence of pDC in salivary glands of SS patients compared to healthy controls (7). Zhao et al. reported that plasma cell-type DCs were enriched in the minor salivary gland of SS, inducing CXCR5(+) CD19(+) B cells to accumulate by secreting type I IFN (8). In addition, the adaptive immune system plays an integral role in the development of SS, and polyclonal overactivation of B cells and proliferation of Th1 and Th17 cells contribute to the progression of the disease (9). Epithelial function is also involved in the complex etiology of SS (10). However, the results of studies on the causal relationship between SS and immune cells have been inconsistent to date, which may be due to insufficient sample sizes, confounding factors, and biases.

Recent advances in large-scale genome-wide association studies (GWASs) and Mendelian randomization (MR) methods have made it possible to assess causal relationships between immune cells and disease outcomes. Compared to other statistical methods, MR can reduce the bias caused by confounding and reverse causation (11, 12). In this study, our research applied univariate, multivariate, and bidirectional MR analyses to investigate the impact of multiple variables and the causal relationship between immune cells and SS risk to clarify the association between immune cell characteristics and SS.

Materials and methods

Study design

Our study investigated the causal associations between 731 immune cells and SS based on MR analysis. In the process of our research, MR analysis was used to observe three core assumptions to ensure unbiased causal effects: (1) Genetic variants are closely related to the exposure, (2) genetic variants are not associated with potential confounders, and (3) genetic variants affect outcomes only by the exposure pathway (13). Data summaries about immune cells and SS were acquired from publicly accessible GWAS. Therefore, there was no need to obtain ethical approval. The workflow of our research is shown in Figure 1.

GWAS data source for 731 immune cells

The GWAS data for the 731 immune cells, which were acquired from a public catalog (GCST90001391-GCST90002121), were based on a study of the genetic characteristics of immune cells. The study aimed to examine the effects of 22 million genetic variations on 731 immune cell cells among 3,757 Sardinians and further verify the relationships among autoimmune illnesses and immunological characteristics. According to this study, we could understand the types of GWAS data, which include 389 median fluorescence intensity measurements representing surface antigen levels, 192 relative cell counts, 118 absolute cell counts, and 32 morphological characteristics (14).

GWAS data source for Sjögren's syndrome

We chose the GWAS data for SS from the FinnGen dataset[1] for MR analysis (dataset finn-b-M13_SJOGREN), including 16,380,454 single-nucleotide polymorphisms (SNPs), and the GWAS was performed on 214,435 Europeans (nCase = 1,290, nControl = 213,145).

Instrumental variable selection

Recent research indicated that when selecting important SNPs for various immune trait cells, we should choose a loose cutoff value of $p < 1 \times 10^{-5}$ (15). According to the 1,000 Genomes Projects reference panel, the linkage disequilibrium r^2 threshold was set to be <0.001 within a 10,000-kb distance to remove the genetic linkage imbalance effect. In the reverse MR analysis, we chose a stricter standard value of $p < 5 \times 10^{-8}$ and $r^2 < 0.001$ within a 10,000-kb distance. Finally, SNPs with low F statistics (<10) were removed to avoid weak instrumental bias, and the rest were used as instrumental variables for MR analysis.

Statistical analysis

Our study mainly adopted the inverse-variance weighting (IVW) method to observe the causal relationship between the 731 immune cell types and SS, while four additional supplemental methods were used, including MR-Egger, weighted median, weighted mode, and simple mode. We comprehensively analyzed the results of five methods to ensure the reliability of the results. At the same time, multiple methods were used to observe for possible pleiotropy and heterogeneity among the results. Cochran's Q-test was utilized to assess for heterogeneity, the MR-Egger intercept was utilized to address and account for pleiotropy, the leave-one-out analysis was utilized to assess robustness, and the MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) test was used to assess for horizontal pleiotropy and detect value bias.

In our study, R statistical software (version 4.3.2) was used to complete all statistical analyses using "Two Sample MR" (version 0.5.8) packages and the MR-PRESSO package (version 1.0).

Results

The causal effect of immune cells on Sjögren's syndrome

Through univariable MR analysis, 22 immune cells were found to be causally related to SS risk. After correcting for the false discovery rate (FDR), we identified six immune cells that have a causal relationship with SS, and they were, respectively, distributed in three

1 https://gwas.mrcieu.ac.uk/datasets/finn-b-M13_SJOGREN/

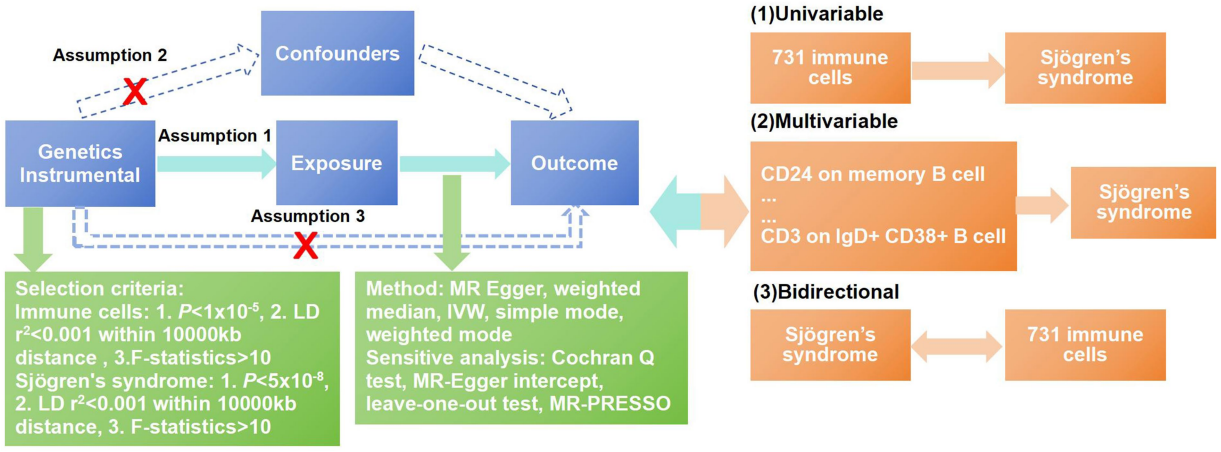

FIGURE 1
Workflow of the MR analysis. In this study, univariate Mendelian randomization (MR) was conducted to investigate the causal associations between immune cells and SS, and five methods were adopted to ensure the reliability of the result, including MR-Egger, weighted median, inverse-variance weighting (IVW), weighted mode and simple mode. The MR-PRESSO test, Cochran's Q-test, MR-Egger intercept analysis, and leave-one-out test were used to observe the pleiotropy and heterogeneity of the results. To overcome the interference between immune cells, we used multivariable MR analysis to further correct the independent effects of these associated immune cells for SS. At last, a bidirectional MR analysis was conducted to explore the reverse causal relationships between immune cells and SS.

B cells, two regulatory T cells (Tregs), and one classical DC (cDC). The result of applying the IVW method indicated that CD24 on memory B cell (odds ratio [OR] = 1.094, 95% confidence interval (CI) 1.026–1.165, $p = 0.06$, positive FDR [P_{FDR}] = 0.031) increased the risk of SS, and the results of the other four methods were similar: MR-Egger (OR = 1.138, 95%CI 1.048–1.236, $p = 0.005$), weighted median (OR = 1.105, 95%CI 1.000–1.220, $p = 0.049$), simple mode (OR = 1.051, 95%CI 0.881–1.254, $p = 0.584$), and weighted mode (OR = 1.122, 95%CI 1.032–1.219, $p = 0.011$). CD27 on IgD+ CD24+ B cell (OR = 1.115, 95%CI 1.044–1.191, $p = 0.001$, $P_{FDR} = 0.027$) also increased the risk of SS, and the results of the other four methods were similar: MR-Egger (OR = 1.153, 95%CI 1.053–1.262, $p = 0.004$), weighted median (OR = 1.151, 95%CI 1.041–1.271, $p = 0.006$), simple mode (OR = 1.005,95%CI 0.839–1.204, $p = 0.958$), and weighted mode (OR = 1.167,95%CI 1.064–1.280, $p = 0.003$). CD28 on CD39+ secreting CD4 regulatory T cell (OR = 1.096, 95%CI 1.006–1.195, $p = 0.036$, $P_{FDR} = 0.050$) was a risk factor for SS, and the results of the other four methods were similar: MR-Egger (OR = 1.070, 95%CI 0.946–1.210, $p = 0.295$), weighted median (OR = 1.049, 95%CI 0.929–1.183, $p = 0.440$), simple mode (OR = 1.156, 95%CI 0.953–1.403, $p = 0.155$), and weighted mode (OR = 1.046, 95%CI 0.933–1.172, $p = 0.451$). CD80 on CD62L+ mDC (OR = 1.102, 95%CI 1.033–1.176, $p = 0.003$, $P_{FDR} = 0.023$) was also a risk factor, and the results of the other four methods were similar: MR-Egger (OR = 1.090, 95%CI 0.998–1.189, $p = 0.067$), weighted median (OR = 1.078, 95%CI 0.987–1.178, $p = 0.097$), simple mode (OR = 1.066, 95%CI 0.916–1.241, $p = 0.414$), and weighted mode (OR = 1.086, 95%CI 1.002–1.177, $p = 0.055$). In addition, CD3 on CD39+ CD8+ T cell (OR = 0.884, 95%CI 0.782–1.000, $p = 0.049$, $P_{FDR} = 0.049$) reduced the risk of SS, and the results of the other four methods were similar: MR-Egger (OR = 0.874, 95%CI 0.645–1.185, $p = 0.402$), weighted median (OR = 0.894, 95%CI 0.750–1.065, $p = 0.208$), simple mode (OR = 0.953, 95%CI 0.752–1.208, $p = 0.699$), and weighted mode (OR = 0.886, 95%CI 0.736–1.066, $p = 0.220$). CD38 on IgD+ CD38+ B cell (OR = 0.861, 95%CI 0.781–0.949, $p = 0.003$, $P_{FDR} = 0.028$) reduced the risk of SS, and the results of the other four methods were similar: MR-Egger (OR = 0.833, 95%CI 0.707–0.981, $p = 0.045$), weighted median (OR = 0.867, 95%CI 0.754–0.998, $p = 0.046$), simple mode (OR = 0.789, 95%CI 0.633–0.983, $p = 0.051$), and weighted mode (OR = 0.859, 95%CI 0.748–0.987, $p = 0.048$) (Table 1). Furthermore, we used Cochran's Q-test to assess heterogeneity and the MR-Egger intercept to account for pleiotropy; and the p-values of all results were greater than 0.05 (Table 2). The leave-one-out analysis showed that these data were stable. Finally, we did not observe any horizontal pleiotropy nor biased values through the MR-PRESSO test (Table 2) (Supplementary material S1).

Multivariable MR analysis

To further assess the independent causal effect of immune cells on SS, we conducted a multivariate MR analysis (Figure 2). We found that only three immune cells exerted independent effects for SS: CD27 on IgD+ CD24+ B cell (OR = 1.111, 95%CI 1.038–1.189, $p = 0.002$); CD80 on CD62L+ mDC (OR = 1.105, 95%CI 1.034–1.181, $p = 0.003$) increased the risk of SS; and CD38 on IgD+ CD38+ B cell (OR = 0.893, 95%CI 0.818–0.975, $p = 0.011$) reduced the risk of SS. Remarkably, our results of multivariate MR analysis were broadly consistent with previous analyses, indicating that our results were highly reliable.

Exploration of the causal effect of SS on immune cells

We conducted the MR analysis to explore the causal effect of SS on immune cells. We found that 25 immune cells had important relationships with SS. After correcting for FDR, 23 immune cells (Figure 3) were identified to have reverse causal associations with SS, including B cell (16 cells), Treg (2 cells), cDC (2 cells), the maturation

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TABLE 1 Causal effects of immune cells on Sjögren's syndrome.

Exposure	Outcome	Method	OR (95% CI)	*p*-value	Adjust *p*
CD3 on CD39+ CD8br	Sjögren's syndrome	MR-Egger	0.874 (0.645–1.185)	0.40214	
		Weighted median	0.894 (0.750–1.065)	0.20806	
		IVW	0.884 (0.782–1.000)	0.04949	0.04949
		Simple mode	0.953 (0.752–1.208)	0.69922	
		Weighted mode	0.886(0.736–1.066)	0.22004	
CD38 on IgD+ CD38br		MR-Egger	0.833 (0.707–0.981)	0.04509	
		Weighted median	0.867 (0.754–0.998)	0.04612	
		IVW	0.861 (0.781–0.949)	0.00251	0.02759
		Simple mode	0.789 (0.633–0.983)	0.05086	
		Weighted mode	0.859(0.748–0.987)	0.04764	
CD80 on CD62L+ myeloid DC		MR-Egger	1.090 (0.998–1.189)	0.06653	
		Weighted median	1.078(0.987–1.178)	0.09682	
		IVW	1.102 (1.033–1.176)	0.00315	0.02311
		Simple mode	1.066(0.916–1.241)	0.41419	
		Weighted mode	1.086 (1.002–1.177)	0.05544	
CD27 on IgD+ CD24+		MR-Egger	1.153(1.053–1.262)	0.00445	
		Weighted median	1.151 (1.041–1.271)	0.00586	
		IVW	1.115(1.044–1.191)	0.00124	0.02718
		Simple mode	1.005 (0.839–1.204)	0.95787	
		Weighted mode	1.167(1.064–1.280)	0.00268	
CD28 on CD39+ secreting Treg		MR-Egger	1.070(0.946–1.210)	0.29487	
		Weighted median	1.049 (0.929–1.183)	0.43962	
		IVW	1.096 (1.006–1.195)	0.03611	0.04965
		Simple mode	1.156(0.953–1.403)	0.15501	
		Weighted mode	1.046 (0.933–1.172)	0.45098	
CD24 on memory B cell		MR-Egger	1.138 (1.048–1.236)	0.00462	
		Weighted median	1.105 (1.000–1.220)	0.04889	
		IVW	1.094 (1.026–1.165)	0.00562	0.03090
		Simple mode	1.051 (0.881–1.254)	0.58393	
		Weighted mode	1.122 (1.032–1.219)	0.01125	

MR, Mendelian randomization; IVW, inverse-variance weighting; CI, confidence interval; DC, dendritic cell; br, bright; Treg, regulatory T.

TABLE 2 Sensitivity analysis results of causal effects of immune cells on Sjögren's syndrome.

Immune cell	Egger intercept	Cochran's Q-test		MR-PRESSO
		MR-Egger	IVW	
CD24 on memory B cell	0.152	0.965	0.940	0.943
CD27 on IgD+ CD24+ B cell	0.300	0.757	0.748	0.773
CD28 on CD39+ secreting CD4 regulatory T cell	0.580	0.225	0.255	0.228
CD3 on CD39+ CD8+ T cell	0.937	0.408	0.485	0.529
CD38 on IgD+ CD38+ B cell	0.627	0.403	0.456	0.536
CD80 on CD62L+ myeloid dendritic cell	0.696	0.241	0.279	0.296

MR, Mendelian randomization; IVW, inverse-variance weighting.

stage of T cells (2 cells), and mDC (1 cell). However, we did not observe a bidirectional causal relationship between immune cells and SS. SS was a risk factor for 13 types of immune cells, including BAFF-R on IgD+ CD38- B cell (OR = 1.224, 95%CI 1.036–1.446, $p = 0.017$, $P_{FDR} = 0.033$), BAFF-R on B cell (OR = 1.214, 95%CI 1.027–1.434, $p = 0.023$, $P_{FDR} = 0.035$), CD123 on pDC (OR = 1.252, 95%CI

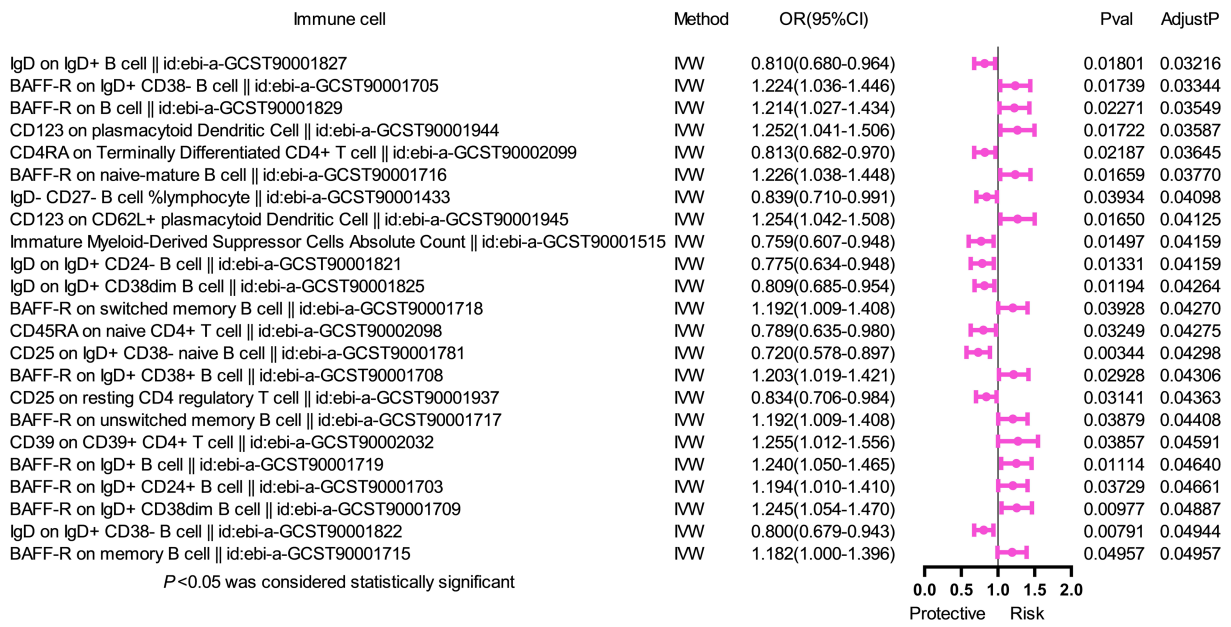

FIGURE 2
Forest plot of multivariable MR analysis between six identified immune cells and Sjögren's syndrome. Multivariate MR analysis showed that three immunophenotypes remained statistically significant, while the other phenotypes lost significance. Two immunophenotypes (CD27 on IgD+ CD24+ B cell and CD80 on CD62L+ myeloid dendritic cell) were risk factors, and one immunophenotype (CD38 on IgD+ CD38+ B cell) was a protective factor.

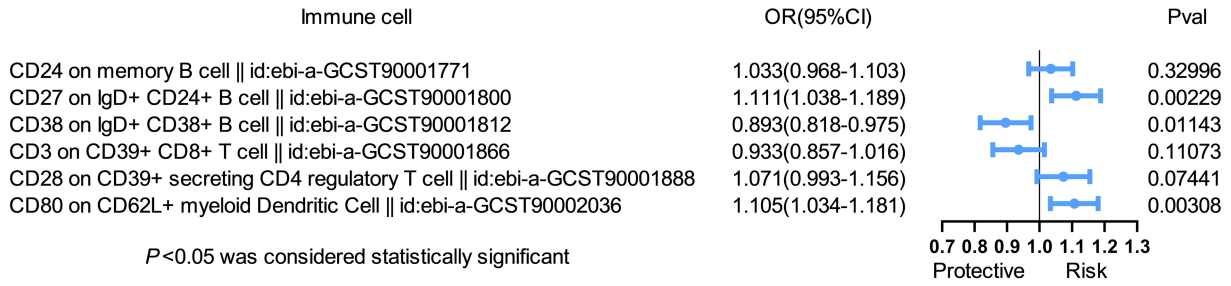

FIGURE 3
Forest plot with MR analysis showed that SS affected 25 immune cells. MR analysis showed that SS affected 25 immune cells. After correcting for FDR, 23 immune cells, belonging to B cell (16 cells), Treg (2 cells), cDC (2 cells), the maturation stage of T cells (2 cells), and myeloid cells (1 cell) were identified to have a reverse causal relationship with SS.

1.041–1.506, $p = 0.017$, $P_{FDR} = 0.036$), BAFF-R on naive-mature B cell (OR = 1.226, 95%CI 1.038–1.448, $p = 0.017$, $P_{FDR} = 0.038$), CD123 on CD62L+ pDC (OR = 1.254, 95%CI 1.042–1.508, $p = 0.017$, $P_{FDR} = 0.041$), BAFF-R on switched memory B cell (OR = 1.192, 95%CI 1.009–1.408, $p = 0.039$, $P_{FDR} = 0.043$), BAFF-R on IgD+ CD38+ B cell (OR = 1.203, 95%CI 1.019–1.421, $p = 0.029$, $P_{FDR} = 0.043$), BAFF-R on unswitched memory B cell (OR = 1.192,95%CI 1.009–1.408, $p = 0.039$, $P_{FDR} = 0.044$), CD39 on CD39+ CD4+ T cell (OR = 1.255,95%CI 1.012–1.556, $p = 0.039$, $P_{FDR} = 0.046$), BAFF-R on IgD+ B cell (OR = 1.240,95%CI 1.050–1.465, $p = 0.011$, $P_{FDR} = 0.046$), BAFF-R on IgD+ CD24+ B cell (OR = 1.194,95%CI 1.010–1.410, $p = 0.037$, $P_{FDR} = 0.047$), BAFF-R on IgD+ CD38dim B cell (OR = 1.245,95%CI 1.054–1.470, $p = 0.010$, $P_{FDR} = 0.049$), and BAFF-R on memory B cell (OR = 1.182,95%CI 1.000–1.396, $p = 0.050$, $P_{FDR} = 0.050$). In addition, SS was a protective factor for 10 types of immune cells, including IgD on IgD+ B cell (OR = 0.810, 95%CI 0.680–0.964, $p = 0.018$, $P_{FDR} = 0.032$), CD4RA on terminally differentiated CD4+ T cell (OR = 0.813, 95%CI 0.682–0.970, $p = 0.022$, $P_{FDR} = 0.036$), IgD- CD27- B cell %lymphocyte (OR = 0.839,95%CI 0.710–0.991, $p = 0.039$, $P_{FDR} = 0.041$), immature myeloid-derived suppressor cells absolute count (OR = 0.759,95%CI 0.607–0.948, $p = 0.015$, $P_{FDR} = 0.042$), IgD on IgD+ CD24- B cell (OR = 0.775,95%CI 0.634–0.948, $p = 0.013$, $P_{FDR} = 0.042$), IgD on IgD+ CD38dim B cell (OR = 0.809,95%CI 0.685–0.954, $p = 0.012$, $P_{FDR} = 0.043$), CD45RA on naive CD4+ T cell (OR = 0.789,95%CI 0.635–0.980, $p = 0.032$, $P_{FDR} = 0.043$), CD25 on IgD+ CD38- naive B cell (OR = 0.720,95%CI 0.578–0.897, $p = 0.003$, $P_{FDR} = 0.043$), CD25 on resting CD4 regulatory T cell (OR = 0.834,95%CI 0.706–0.984, $p = 0.031$, $P_{FDR} = 0.044$), and IgD on IgD+ CD38-B cell (OR = 0.800,95%CI 0.679–0.943, $p = 0.008$, $P_{FDR} = 0.049$). In the same way, the results of the other four methods were similar to those of the IVW method (Supplementary material S2). Furthermore, we found no evidence of heterogeneity and pleiotropy

via Cochran's Q-test and MR-Egger intercept analysis (Table 3), so we could assume that there was no horizontal pleiotropy. Finally, the leave-one-out test suggested that the result of reverse MR analysis was also stable (Supplementary material S2).

Discussion

In our study, we conducted MR to investigate the causal relationship between 731 immune cells and SS. In this study, we found a strong causal relationship between six immune cells for SS ($P_{FDR} < 0.05$) and 23 immune cells for SS ($P_{FDR} < 0.05$). Our results provide further insight into the causal relationship between immune cells and SS. As far as we know, this is the first univariate, bivariate, and bidirectional MR analysis performed to investigate the link between immune cells and SS.

We observed that two types of T cells were significantly associated with the risk of SS, with elevated CD28 on CD39+ secreting CD4 regulatory T cells increasing the risk of SS, and elevated CD3 on CD39+ CD8+ T cells decreasing the risk of SS. CD39 plays an important role in the immune system. CD39+ CD8+ T cells inhibit the production of IFN-γ by CD39- CD8+ T cells through paracrine secretion of adenosine, which operates through the A2A receptor (16). Early disturbance of the innate immune barrier involving the IFN pathway in SS disease is associated with the etiology of SS (6). IFN-γ induces salivary gland epithelial cell ferroptosis in SS (17). An increase in the number of CD39+ CD8+ T cells found in Crohn's patients is correlated with enhanced signal transduction of reactive oxygen species (ROS) (16). The ROS/pSTAT4/important protein aquaporin 5 axis affects salivary dysfunction in SS (18). Although some studies have focused on Tregs and SS, the role of Tregs in the occurrence and development of SS remains elusive. Sarigul et al. reported that the increase of Foxp3+ Treg cells in the peripheral blood of SS patients was positively correlated with a higher grade of infiltration at the salivary glands (19). Alunno et al. reported an expansion of CD4+ CD25- GITR+ regulatory T cell subsets in the peripheral blood of patients with primary SS (pSS)', which was correlated with the degree of disease activity (20). The correlation between CD28 on CD39+ secreting CD4 regulatory T cell and SS has not been clarified at present. In other autoimmune diseases, an increase in peripheral CD39-expressing T regulatory cells has been associated with relapsing–remitting multiple sclerosis (21). In addition, in patients with type 2 diabetes, elevated CD39+ Treg cells are associated with hyperglycemia, overweight, and obesity (22). The collaboration of CD39 and CD73 results in the conversion of ATP to ADP and adenosine 5′-monophosphate (cAMP), ultimately generating

TABLE 3 Sensitivity analysis results of causal effects of Sjögren's syndrome on immune cells.

Immune cell	Egger intercept	Cochran's Q-test	
		MR-Egger	IVW
IgD on IgD+ B cell	0.655	0.202	0.330
BAFF-R on IgD+ CD38- B cell	0.601	0.990	0.769
BAFF-R on B cell	0.451	0.611	0.445
CD123 on plasmacytoid dendritic cell	0.939	0.668	0.908
CD4RA on terminally differentiated CD4+ T cell	0.678	0.607	0.752
BAFF-R on naive-mature B cell	0.464	0.535	0.441
IgD- CD27- B cell % lymphocyte	0.922	0.882	0.982
CD123 on CD62L+ plasmacytoid dendritic cell	0.952	0.634	0.890
Immature myeloid-derived suppressor cells' absolute count	0.581	0.826	0.723
IgD on IgD+ CD24- B cell	0.642	0.144	0.225
IgD on IgD+ CD38dim B cell	0.644	0.313	0.491
BAFF-R on switched memory B cell	0.631	0.791	0.779
CD45RA on naive CD4+ T cell	0.798	0.090	0.204
CD25 on IgD+ CD38- naive B cell	0.858	0.165	0.363
BAFF-R on IgD+ CD38+ B cell	0.666	0.930	0.843
CD25 on resting CD4 regulatory T cell	0.722	0.552	0.751
BAFF-R on unswitched memory B cell	0.644	0.905	0.816
CD39 on CD39+ CD4+ T cell	0.889	0.104	0.256
BAFF-R on IgD+ B cell	0.445	0.572	0.419
BAFF-R on IgD+ CD24+ B cell	0.597	0.878	0.755
BAFF-R on IgD+ CD38dim B cell	0.429	0.676	0.419
IgD on IgD+ CD38- B cell	0.627	0.466	0.615
BAFF-R on memory B cell	0.594	0.954	0.759

MR, Mendelian randomization; IVW, inverse-variance weighting.

adenosine (23, 24). Adenosine plays an immunosuppressive role when interacting with A2A and A2B receptors but stimulates an immune response when interacting with A1 and A3 receptors (25–27). These findings may be the reason why CD28 on CD39+ secreting CD4 regulatory T cell is a risk factor for SS, whereas CD3 on CD39+ CD8+ T cell plays a protective role in SS. However, further experiments are needed to clarify the relevant mechanisms. cAMP can activate the mitogen-activated protein kinase (MAPK) pathway, thereby inhibiting nicotinamide adenine dinucleotide phosphate oxidase and alleviating TGF-B1-induced salivary gland fibrosis. CD28 on CD39+ secreting CD4 regulatory T cell and CD3 on CD39+ CD8+ T cell may affect the pathogenesis and development of SS through adenosine (and its derivatives) or IFN, which needs to be clarified by validation and functional evaluation experiments.

In addition, our results showed that three phenotypes of B cells were associated with SS risk. Elevations of CD24 on memory B cells and CD27 on IgD+ CD24+ B cells increased the risk of SS, while CD38 on IgD+ CD38+ B cells decreased the risk of SS. Polyclonal over-proliferation of B cells is one of the immunological features of SS (9). CD24 expression on pro-B cells plays a role in the selection and development of B cells in bone marrow. In memory B cells, there was a strong positive correlation between CD24 expression and phosphorylation flow (phosphorylation of AMPK-pAMPK), especially in IgD+ IgM+ memory B cells (28). MAPKs are downstream of many immune and cytokine receptors, such as toll-like receptors, interleukin (IL)-1R, tumor necrosis factor receptor, colony-stimulating factor 1 receptor, IL-17R, epidermal cell growth factor, fibroblast growth factor, and vascular endothelial growth factor (29). These immune and cellular factors are involved in the occurrence or development of SS and its complications, to varying degrees (30–34).

Peripheral blood IgD+ CD38+ B cells, also known as naïve B cells, were significantly higher in SS patients than in healthy donors and higher in women than men (35, 36). An increased proportion of CD38 high IgD+ B cells in pSS is involved in IgG overproduction, including autoantibodies, and correlates with disease progression (37). In patients with pSS treated with iguratimod, CD38+ IgD+ B cells and BAFF-R were significantly reduced, while disease activity scores were decreased (38). This is not consistent with our findings, suggesting that IgD+ CD38+ B cells reduce the risk of SS, but the results of MR analysis could not fully reveal the relationship between immune cells and SS and need to be confirmed in combination with experiments. It is also possible that interference between immune cells causes their true role in SS to be obscured.

Furthermore, our results showed that the elevation of CD80 on CD62L+ mDCs was positively correlated with the risk of SS and remained significantly correlated after adjusting for multivariate MR. mDCs undergo many activities that contribute to the initiation of immunity. There are two main subtypes of human DCs: mDCs and pDCs. pDCs primarily drive innate inflammatory responses to pathogens by secreting large amounts of IFN-alpha (IFNα), whereas mDCs are specifically used for antigen presentation to guide adaptive responses (39, 40). CD80 on CD62L+ is the more mature mDCs phenotype, and mature DCs are stimulators of T cell immune response, whereas immature DCs support T cell tolerance (41). Compared to healthy controls, pDC and mDC2 in the peripheral blood of pSS patients were significantly reduced (40). Compared to non-S'S dry eye and healthy volunteers, SS' dry eye showed significantly higher DC density, larger DC size, and more DC dendrites with a larger DC field. Moreover, DC density and morphological parameters were significantly correlated with the degree of salivary gland pathology, serum antibody titer, and ocular surface damage (42). However, there is no literature on the change in CD80 on CD62L+ mDC expression level in SS, which is worthy of further exploration.

Our results showed that SS was a risk factor or protective factor for 23 immunophenotypes, including 16 B cells, 4 T cells, 2 cDC cells, and 1 mDC. Our results confirm that SS is a B cell-associated disease at the genetic level. BAFF is a member of the TNF superfamily by binding to the transmembrane activator, calcium modulator, and cyclophilin ligand interactor or BAFF-R on B cells. BAFF supports B cell development, differentiation, and survival, especially for plasma and plasma cells, and plays a key role in the pathogenesis of B cell-associated autoimmune diseases. BAFF therapy targeting B cells in SS' helps reduce disease activity in SS and restores normalization of B cell frequency, phenotype, and function (43, 44). The results of this study provide potential indicators and clues for the early diagnosis and activity assessment of SS, which can be further explored and validated in future clinical cases.

Considered together, our findings demonstrate that immune cells play a potential causal role in SS, providing an important auxiliary role for clarifying diagnosis and therapeutic strategies, as well as providing directions for the development of new drugs. Our MR analysis offered several advantages. First, we used univariate, multivariate, and bidirectional MR to mitigate confounding factors and reverse causation. Second, our study adopted five methods to ensure the reliability of the result, including MR-Egger, weighted median, IVW, weighted mode, and simple mode. At the same time, multiple methods were used to observe pleiotropy and heterogeneity of the results, such as the MR-PRESSO test, Cochran's Q-test, MR-Egger intercept analysis, and leave-one-out test, to ensure that our MR results were robust and reliable, with no apparent bias from other sources of pleiotropy. However, our study has limitations. First, in this study, multifactor, bidirectional MR analysis was performed, and the results showed that six types of immune cells may be associated with the risk of SS. However, MR analysis could not reveal the causal relationship between immune cells and SS, but it provides new avenues for studying the mechanisms of SS. Further experiments are needed to evaluate and validate the function of the selected immune cells by analyzing patient clinical data and biological samples. Second, the GWAS data used in this study were derived from European populations, so may not be directly applicable to other populations. Third, GWAS data were currently unable to distinguish between pSS and secondary SS, which made it impossible to perform subgroup stratification analyses of the SS population. Fourth, the limited sample size could introduce bias, so a larger sample is needed to obtain reliable results.

Conclusion

This study used univariate, multivariate, and bidirectional MR analysis to investigate the causal relationship between several immune cells and SS and to clarify that immune cells affect the progression of SS in a complex pattern. These findings improve our understanding of the interaction between immune cells and SS risk, providing new avenues for studying the prevention, diagnosis, and treatment of

SS. Nevertheless, further experiments are needed to elucidate the underlying mechanisms.

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

WZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MH: Writing – original draft, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization. YZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. JP: Conceptualization, Software, Writing – original draft. FQ: Methodology, Project administration, Writing – original draft. XL: Conceptualization, Investigation, Writing – review & editing. LZ: Data curation, Formal analysis, Writing – original draft. LL: Conceptualization, Software, 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2024.1408562/full#supplementary-material

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Case report: Formation and recurrence of inflammatory pseudotumor after metal-on-metal hip arthroplasty

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The metal-on-metal (MoM) artificial hip joint is a prosthesis used in early hip arthroplasty, particularly for hip resurfacing and total hip arthroplasty. However, abrasion and corrosion of MoM bearings result in the production of metal ions, such as cobalt and chromium, thereby inducing several complications such as inflammatory pseudotumor, aseptic inflammation, and allergy to metal ions (delayed type IV hypersensitivity). In this case report, we present a patient who was hospitalized for recurrence of a mass in the right inguinal area. In 2010, the patient underwent right MoM total hip arthroplasty for right femoral head necrosis and exhibited a good postoperative recovery. In 2019, the patient experienced pain in the right hip with activity limitation without any evident triggers, and a palpable mass was observed in the right inguinal area. A large periprosthetic mass was resected under general anesthesia, and the patient recovered well after the operation. Based on post-surgery imaging and pathological examinations, the mass was diagnosed as a periprosthetic inflammatory pseudotumor. In 2021, the inflammatory pseudotumor recurred at the same site. He then underwent right total hip revision surgery under epidural anesthesia and recovered well after surgery. No recurrence was noted at moderate follow-up. The incidence of inflammatory pseudotumors is high in MoM hip arthroplasty. Early revision is necessary in patients who meet the indications for revision, while regular postoperative follow-up is crucial.

KEYWORDS

metal-on-metal (MoM) bearings, inflammatory pseudotumor, prosthesis revision, total hip arthroplasty, adverse reaction to metal debris

Introduction

Although metal-on-metal (MOM) hip implants have now been discontinued, the proportion of MoM hip arthroplasty was high in early-stage joint replacements. It was the preferred choice for young patients and patients with high activity levels because it was associated with a lower incidence of dislocation, increased joint load bearing, and the avoidance of osteolysis caused by polymeric materials such as polyethylene (1). However, MoM hip arthroplasty has recently been found to be associated with varying degrees of periarticular inflammatory pseudotumors. These pseudotumors manifest as cystic or solid soft tissue masses composed of inflammatory cells from necrotic tissue that are connected to the hip joint (2). On clinical examination, the patients may present with a palpable mass on the anterior or lateral hip surface, or even in the iliac fossa. Pain predominantly occurs in the

inguinal region and occasionally radiates to the greater trochanter and lower extremities. In patients with pseudotumors, severe pain may cause them to adopt a limping gait. Over time, the patients may develop hip instability (subluxation, dislocation). Some patients may experience other symptoms such as stiffness in the lower extremities, decreased range of motion, and weakness of the abductor muscles (3). These sterile masses in the tissue surrounding the prosthesis are clinically known as inflammatory pseudotumors and are believed to result from an adverse reaction to metal ions released during the wear and tear of metal-bearing surfaces. Soft tissue inflammatory reactions in response to metal debris are collectively called adverse reactions to metal debris (ARMD) and include inflammatory pseudotumors, aseptic lymphocytic vasculitis-associated lesions (ALVALs), and metallosis (4).

In 27–32% of patients with MoM hip arthroplasty, asymptomatic pseudotumors can be detected on ultrasound and MRI scans (5, 6). Many regulatory agencies have recommended that the initial screening test be an ultrasound or metal artifact reduction sequence (MARS) MRI. Ultrasound is a cost-effective and readily available modality that is less affected by the presence of adjacent metal prostheses, with the key limitation being its user dependency (7). Because of its high specificity and sensitivity in detecting these responses and its versatility in assisting with preoperative planning and longitudinal comparisons, MARS MRI has been recommended as a first-line modality for evaluating the soft tissue surrounding the prosthesis in patients with MoM implants (8). A pseudotumor can be definitively diagnosed based on the pathological tissue obtained during revision surgery.

During a 7-year follow-up of 1,419 patients with MoM hip resurfacing, the incidence of pseudotumors was as high as 3.4%, and the incidence of asymptomatic pseudotumors has been estimated to be 4% (9). In a cross-sectional study of 148 hip joints from 111 patients, pseudotumors were present in 13 of 30 (43%) MoM THAs, 13 of 47 (27%) MoM RHAs, and 29 of 71 (41%) metal-on-polyethylene (MoP) THAs, which shows a statistically similar prevalence (10). However, pseudotumors in ceramic-on-ceramic (CoC) bearings and ceramic-on-polyethylene (CoP) bearings are rarely reported. Patients with inflammatory pseudotumors usually have poor clinical outcomes, with most of them requiring revision surgery. This severely affects the longevity of the prosthesis and the patient's joint function (3).

Studies on revision surgery for recurrent inflammatory pseudotumors are rare. This report contributes to the research by presenting a case of successful revision surgery in a patient experiencing recurrence following inflammatory pseudotumor resection.

Case report

A 64-year-old male patient was hospitalized complaining of recurrent right groin swelling and right hip pain with movement for 6 months. He had a history of hypertension and coronary artery disease. The patient underwent a right MoM total hip arthroplasty (ASR XL Acetabular System, Johnson & Johnson Medical Ltd., Shanghai, China) in our hospital in 2010 for right femoral head necrosis, a left CoP total hip arthroplasty (CORAIL Total Hip System, Johnson & Johnson Medical Ltd., Shanghai, China) in our hospital in 2017 for a traumatic left femoral neck fracture, and resection of a large periprosthetic mass in 2019. This mass was diagnosed as an inflammatory pseudotumor. The pseudotumor recurred at the same site and he underwent revision surgery (CORAIL Revision Hip System, Johnson & Johnson Medical Ltd., Shanghai, China) in 2021. As of 2024, the pseudotumor had not recurred at the 3-year follow-up.

Examination

At physical examination, the spine had a good physiological curvature with no deformity. The patient reported no compression or percussion pain in the spinous processes. The patient experienced pain in the right hip with a limited range of motion. The right lower limb was swollen, and a large mass was palpated from the right lower abdomen to the right inguinal area without obvious compression pain. The remaining limbs exhibited no joint redness, joint ankylosis, muscle tenderness, muscle atrophy, or varicose veins in the lower limbs. Peripheral circulation, limb sensation, and muscle tone were good. The Harris Hip Score (HHS) was 87 points (The observation indices primarily included four aspects: pain, function, deformity, and joint mobility. Excellent: 90–100; good: 80–89; acceptable: 70–79; and poor: ≤69) (11).

During the laboratory examination, the glucose level decreased to 1.54 mmol/L, whereas the levels of lactate dehydrogenase, adenosine deaminase, chlorine, protein, and the erythrocyte sedimentation rate increased to 2226.0 and 67 U/L, 100.8 mmol/L, 39,357.00 mg/L, and 28 mm/h, respectively. The blood cobalt (Co) and chromium (Cr) ion concentrations determined by inductively coupled plasma/mass spectrometer (ICP/MS) techniques were 12.5 and 9.2 μg/L, respectively, on 12 April 2021.

On ultrasonography [Diagnostic Ultrasound System and Transducer, Philips Healthcare (Suzhou) Co. Ltd., Suzhou, China], a solid mass measuring 17.2 cm × 8.8 cm was observed extending from the right lower abdomen to the right groin and around the right hip (Figure 1A). It was predominantly cystic, with clear borders and irregular margins. X-ray (DigitalDiagnost DR, Philips Healthcare Co. Ltd., Suzhou, China) revealed changes following right artificial hip arthroplasty (Figure 1B). Computed tomography (CT) (SOMATOM Definition AS, Siemens Shanghai Medical Equipment Ltd., Shanghai, China) showed a mass in the right iliac fossa and right hip perimuscular space, changes following bilateral hip arthroplasty, and degenerative changes in the bilateral hip joints (Figures 1C,D). On magnetic resonance imaging (MRI) (MAGNETOM Avanto 1.5 T, Siemens Shanghai Medical Equipment Ltd., Shanghai, China), changes were observed following bilateral hip arthroplasty, and large abnormal signals were noted around the right hip joint (Figures 1E,F). An abscess was formed.

Final diagnosis

A periprosthetic, inflammatory pseudotumor was noted after right hip arthroplasty.

Treatment

Following successful epidural anesthesia, the patient underwent surgery in which a posterior lateral approach was used to expose and incise the joint capsule. The incision was made by cutting through the skin, subcutaneous tissue, and fascia one layer at a time. During the

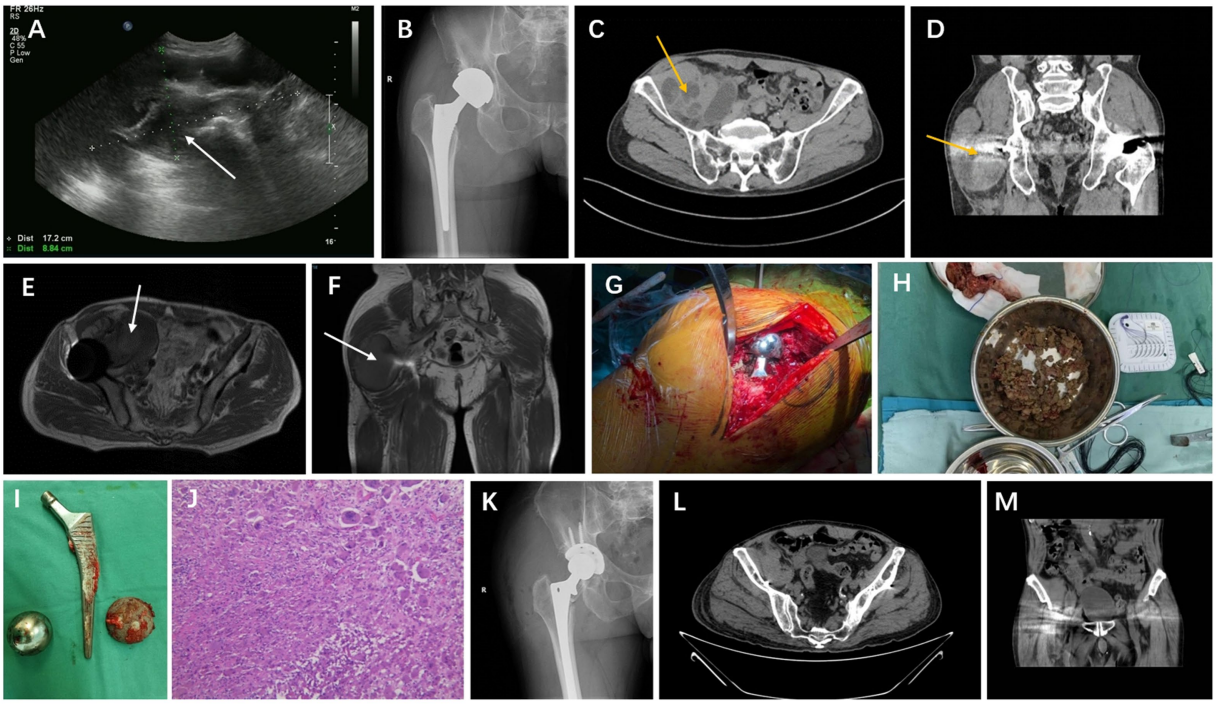

FIGURE 1
A 64-year-old male patient underwent revision surgery for recurrent inflammatory pseudotumor after MOM hip arthroplasty. **(A–F)** Preoperative ultrasonography, X-ray of the hip, CT horizontal plane, CT coronal plane, MRI horizontal plane, and MRI coronal plane; **(G)** intraoperative exposure of the prosthesis; **(H)** intraoperatively resected inflammatory pseudotumor; **(I)** removed MOM prosthesis; **(J)** pathological examination of resected material, H&E, magnification, 200X; **(K–M)** review of the X-ray, CT horizontal plane, and CT coronal plane after revision surgery.

operation, the prosthesis was in good condition (Figure 1G), with no signs of loosening. However, a grayish-black silt-like material and necrotic connective tissue were significantly observed around the prosthesis (Figure 1H). The dislocated hip was surgically treated to remove the femoral head and stem (Figure 1I). The acetabulum and proximal femur were exposed, followed by glenoid labrum resection. The pseudotumor and necrotic tissues were also removed and subjected to postoperative pathological examination and culture (Figure 1J). After the acetabulum was polished with a grinding file, a 55-mm metal cup (Pinnacle Revision Acetabular Cup System, Johnson & Johnson Medical Ltd., Shanghai, China), two fixation screws, and a polymer polyethylene liner were implanted. Following successful trial molding, a 12-gage revision stem (CORAIL Revision Hip Stem, Johnson & Johnson Medical Ltd., Shanghai, China) was installed, and good hip movement was noted after repositioning. Saline irrigation was performed, followed by the repair of the joint capsule. After a drain was placed, the incision was closed. Following the operation, the patient was transferred back to the ward, where he was treated for inflammation, dehydration, thrombosis prevention, pain relief, and nutritional support, including administration of cephalosporins, non-steroidal anti-inflammatory drugs, low-molecular-weight heparin, and glycerol fructose. Subsequently, postoperative X-rays of the hip joint and pelvic CT scans were repeated (Figures 1K–M).

Outcome and follow-up

The fibrous tissue capsule wall exhibited synovial tissue hyperplasia with numerous multinucleated giant cells, foam cells, and acute and chronic inflammatory cell infiltration, along with significant coagulative necrosis.

A smear from the secretion culture was subjected to Gram staining. This stained smear revealed a small number of neutrophils with no bacteria. The culture remained sterile for 4 days.

On the second day after surgery, the drain was removed, and the patient began rehabilitation exercises, including ankle pump exercises, quadriceps exercises, hip abduction, and flexion. Repeat each set of movements 10-15 times, 2-3 times per day. On the fourth day, the patient could stand with the assistance of a walking aid and perform daily activities independently. To prevent postoperative dislocation, internal rotation and retraction of the affected limb beyond the midline and hip flexion beyond 90° were prohibited for 3 months after surgery. He was discharged from the hospital 1 week after admission, and moderate follow-up displayed no complications such as the recurrence of inflammatory pseudotumors, periprosthetic infection, or osteolysis. Figure 2 presents the timeline of the patient's diagnosis, treatment, and follow-up.

Discussion

Metal-on-metal interfaces have been extensively used in the early stages of clinical application in young patients with high demands on joint mobility and quality of life. These interfaces have the advantage of lowering the joint dislocation rate, increasing the joint's range of motion, and reducing osteolysis (12). However, stable and unstable prostheses cannot avoid wear and metal corrosion after long-term use. The resulting metal debris and metal ions can trigger several immune

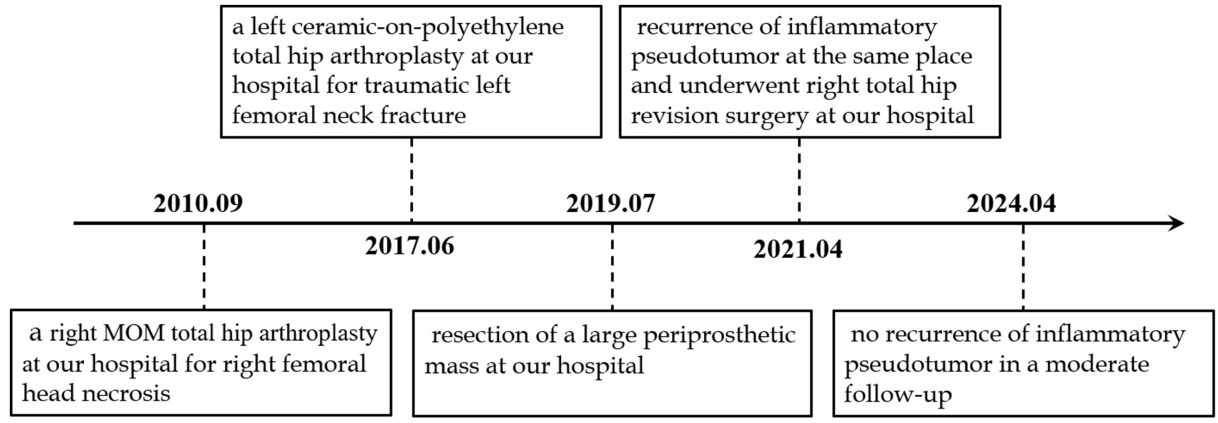

FIGURE 2
Timeline of the patient's diagnosis with the relevant data about the treatment and follow-up.

and hypersensitivity reactions, leading to clinical problems such as inflammatory pseudotumors, heavy metal toxicity, and delayed allergic reactions, and eventual joint revision is inevitable (13). Although MoM prostheses are now rarely used because of their high complication and revision rates, 80% of prostheses are still retained in patients. An increasing number of MoM patients are expected to require revision in the future (14).

By introducing an inorganic artificial joint to replace the damaged, deformed synovial joint, total hip arthroplasty modifies the original tissue type and structure of the hip joint. However, it removes existing pathological factors while introducing new ones. In MoM joint prostheses, corrosion or abrasion of the metal material can produce metal debris and ions, as well as metal oxides such as Co, Cr, titanium, molybdenum, and nickel. This leads to increased serum and urine metal ion concentrations in the patient (15), further causing massive lymphocytic infiltration of the tissues surrounding the prosthesis, which is observed on pathological examination as tissue necrosis, deposition of wear particles with macrophage infiltration, diffuse lymphocytic infiltration, and ALVALs (a type IV hypersensitivity reaction) (16, 17). The joint capsule and periprosthetic area are first exposed to these types of wear particles and noxious irritants, such as metal ions, and aseptic inflammation also begins in these areas. When the joint fluid is dispersed to the surrounding tissues or absorbed through the lymphatic system, the wear particles and metal ions are carried, and the locally produced inflammatory factors enter the surrounding tissues or the circulatory system. Under the action of these various inflammatory factors, the joint capsule wall undergoes tissue necrosis and fibrosis becoming hardened and less elastic. Thus, the joint capsule is more likely to rupture under the effect of fluid pressure. After rupture, the fluid enters the surrounding soft tissue space and props up a new space or directly infiltrates and invades the surrounding tissues (18). A cystic mass or cystic solid mass, known as an inflammatory pseudotumor, is finally observed on MRI or ultrasound.

Several recent clinical follow-up studies have confirmed that the prosthesis failure rate after MoM total hip arthroplasty has significantly increased, with rates as high as 34% at 5 years. Laaksonen et al. (19) offered a 2010 Depuy recall of the ASR XL MoM THA product line, which demonstrated a 13% postoperative revision rate. A meta-analysis study (20) reported that the incidence of inflammatory pseudotumors is up to 6.5% in MoM hip arthroplasty. Our case patient was admitted to the hospital 9 years after MoM hip arthroplasty because of the worsening symptoms of hip pain and limited mobility. Based on imaging and pathological findings, the patient was diagnosed as having a periarticular inflammatory pseudotumor. Revision surgery was not completed until 11 years after the surgery. However, the inflammatory pseudotumor may have formed before the onset of symptoms. The inflammatory pseudotumor, osteolysis, and aseptic loosening can be clinically asymptomatic and are not criteria for prosthesis failure. However, the absence of clinical symptoms does not mean irrelevance, and these negative events will progressively lead to THA failure (21). Apart from revision, no proven effective interventions can help cease the progression of periprosthetic lesions (2). The main indications for revision are a pseudo-grade III lesion pseudotumor (solid pseudotumor) observed on imaging (22) (Grade I: cyst wall thickness of <3 mm; Grade II: cyst wall thickness of >3 mm; and Grade III: lesions were predominantly solid lesions, where the largest dimension of the solid components was greater than the diameter of the cystic components); hip symptoms exhibiting elevated whole-blood metal ion concentrations; and persistent or progressive hip symptoms. These symptoms primarily include groin pain that occasionally radiates to the greater trochanter and lower thighs, a feeling of instability, hip dysfunction, and popping sounds (23). Due to the lack of clear guidelines and the complexity of the patient's condition, risk factors for patient management and prognosis in the clinic, such as in asymptomatic or mildly symptomatic patients with anomalies, have not been assessed accurately (19). In such cases, determining a revision surgical plan, as well as pre- and post-surgical patient management, can be challenging for surgeons.

The MoM prosthesis was comparable to other types of prostheses in terms of clinical and functional results (24). However, the release of metal debris or ions into the bloodstream because of the wear of metal bearings increases the risk of higher serum concentrations of Cr and Co ions in patients implanted

with MoM prostheses (25). In the United Kingdom, the Medicines and Healthcare Products Regulatory Agency has indicated blood Cr and Co ion concentrations of >7 μg/L as a high-risk threshold for ARMD (26). In 25 patients who had MoM bearings removed, metal ion levels decreased by 90% at 12 weeks after the MoM implant was removed (27). Because of individual differences and differences in initial ion concentrations, the trend of decreasing serum metal ion concentrations varies for each patient after revision surgery. The serum concentrations of Cr and Co ions in our case patient were consistently above the threshold before the revision surgery. Moreover, metal ion concentrations exhibited no significant decrease in the short term after the bearing was replaced until 6 months later when the concentrations were reduced below the threshold. However, some studies have noted that serum metal ion concentrations and pseudotumor formation are not significantly related, which has raised concerns about the reliability of these concentrations as a suitable screening test (25, 28). Therefore, metal ion analysis should not be used alone for evaluating patients with MoM hip implants. Clinical symptoms, blood test results, and radiographic studies must all be carefully considered while predicting prosthesis failure (29).

Pseudotumor revision is significantly associated with postoperative complications, with up to 50% of patients experiencing severe complications and one-third of patients requiring further revisions (30). Women with small femoral heads, acetabular cup implant inclination >55°, and primary hip dysplasia have a worse prognosis (9). The most common postoperative complications are dislocation, pseudotumor recurrence, and aseptic loosening (31). The pseudotumor recurrence rate after revision resection may be as high as 30% (6). Various surgical, individual, and implant factors contribute to pseudotumor recurrence (19). Intraoperative incomplete debridement, or residual metal content, is the main cause of pseudotumor recurrence (32). However, in clinical practice, because the pseudotumor is connected to the joint capsule, the extent and degree of debridement need to be carefully selected to protect crucial neurovascular tissues (32). This results in an incomplete resection of the pseudotumor. In addition, extensive debridement can cause joint instability and increase the dislocation risk (33). Therefore, the surgeon must completely remove the diseased tissue and metal fragments. Otherwise, the risk of pseudotumor recurrence increases (34). In the present case, the pseudotumor recurred 2 years after the first inflammatory pseudotumor was resected. The patient was followed up every year by telephone and outpatient service after the second revision surgery to check on the status of his imaging and to learn about his postoperative joint function, and no pseudotumor recurrence has been reported so far. The frequency of follow-up needs to be individualized based on the implant risk stratification and the clinical status of the patient. According to studies (20, 35), annual follow-up is sufficient for patients with moderate- to high-risk implants. Follow-up should include history, clinical examination, functional scores, blood metal ion measurements, and ultrasound (20). If clinical concerns exist, a MARS MRI can be conducted. For patients with low-risk implants, a less intensive follow-up is required, such as annual questionnaires and 5-year clinical reviews (34, 36).

In the present case, the patient's pseudotumor recurred 2 years after the first inflammatory pseudotumor was resected, and no pseudotumor recurrence was observed on imaging examinations since the second revision surgery. A medium follow-up revealed that the metal ion concentrations in the patient's body were steadily remained below the high-risk threshold 6 months after the second revision surgery. Therefore, continuous postoperative imaging and laboratory examination are quite necessary to determine pseudotumor recurrence (37).

Patients who develop inflammatory pseudotumors following MoM arthroplasty and meet the indications for revision surgery should undergo early revision to prevent further osteolysis and the occurrence of pathological fractures and to reduce the complexity of revision surger (35). The surgeon needs to accurately diagnose and judge whether the patient meets the indications for revision surgery through clinical symptoms, imaging data, and laboratory data. The use of monolithic revision and ceramic-to-polyethylene interfaces in revision surgery also results in better clinical outcomes (38). Ceramic interfaces are currently popular in healthcare settings. However, ceramic-to-polyethylene and metal-to-polyethylene interfaces used in revision MoM hip arthroplasty reduced the incidence of adverse outcomes by 70 and 63%, respectively, compared with ceramic-to-ceramic interfaces (39). In addition, regular follow-up after early MoM arthroplasty and revision is crucial for detecting and understanding the size and extent of pseudotumor formation through ultrasound and MRI. To achieve a clear diagnosis, appropriate laboratory tests and pathological biopsy are necessary because distinguishing inflammatory pseudotumors from infections, tumors, and other diseases is sometimes difficult (40).

In conclusion, the occurrence of inflammatory pseudotumors in MoM hip arthroplasty is significant and is related to prosthesis-produced metal debris. Early revision in patients who meet the indications for revision is essential to avoid adverse factors, such as aseptic loosening, that affect the prognosis of revision, and regular postoperative follow-up is vital. Collecting patient and implanted prosthesis data through collaboration between healthcare providers and regulators for constructing huge data centers and developing clinical predictive models is critical for clinical decision-making.

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 Ethics Committee of the Second Affiliated Hospital of Nanjing Medical University. 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

MM: Formal analysis, Writing – original draft. CX: Formal analysis, Writing – original draft. PX: Formal analysis, Writing – review & editing. XW: Formal analysis, Writing – review & editing. LF: Formal analysis, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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Polyneuropathy in systemic sclerosis: exploring the causes and biomarkers

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Introduction: Systemic sclerosis (SSc) is a rare autoimmune disease with multiple organ involvement; however, the contribution of the nervous system (NS) remains relatively understudied. There are no specific data on the role of the autoimmune response and inflammation in the development of peripheral nerve system (PNS) damage in SSc and markers to assess this damage have yet to be identified.

Objectives: The primary objective of this study was to define the autoimmune mechanisms that lead to neuropathy by identifying antibodies (Abs) that target certain component of the NS or are associated with SSc. The secondary objective was to identify markers of NS damage that correlate with the detection and progression of polyneuropathy (PNP).

Methods: This study included patients diagnosed with SSc who met ACR/EULAR 2013 classification criteria at two leading Latvian hospitals between January 2016 and December 2021. Patients underwent a nerve conduction study (NCS). The SSc-associated Abs, Abs against myelin-associated glycoprotein (MAG) and anti-ganglioside Abs (GM1, GM2, GD1a, GD1b and GQ1b) were analysed. Potential serum PNS biomarkers—neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), fibroblast growth factor 21 (FGF21) and growth/differentiation factor 15 (GDF15)—were measured.

Results: We recruited 103 Caucasian patients diagnosed with SSc. SSc-associated Abs did not differ significantly between patients with and without PNP ($p > 0.05$). Anti-MAG and anti-ganglioside Abs in patients with PNP did not present a significant increase above the reference range. NfL, GFAP and GDF15 were significantly elevated in the presence of PNP ($p < 0.05$), with a moderate to high effect size ($r = 0.36–0.65$). Our regression analysis revealed a strong

association between the HAQ-DI score, older age, male gender and the risk of developing PNP.

Conclusion: The development of PNP in patients with SSc is most likely due to ageing, natural progression and the sequelae of the disease. Several serum biomarkers—NfL, GFAP and GDF15—could be used as relevant diagnostic biomarkers for PNP in patients with SSc. Future studies are warranted to validate the diagnostic efficacy of these biomarkers and to unravel the complex interplay of factors leading to PNP in patients with SSc.

KEYWORDS

systemic sclerosis, scleroderma, polyneuropathy, nervous system, autoimmune, serum biomarkers

1 Introduction

Systemic sclerosis (SSc) is a rare autoimmune disease with known autoantibodies that help establish a diagnosis and affect the prognosis (1–3). Although multiple organ involvement is widely acknowledged and studied, the contribution of the nervous system (NS) remains relatively understudied (4–6). In most recent classification criteria, NS damage was not included in point assessment, again highlighting its undefined role in SSc (7). Although a few studies have been conducted to establish the prevalence and type of NS involvement in SSc, mostly focusing on peripheral nervous system (PNS), they differed widely in numbers, partly because the authors used different methods of assessing NS damage. Over time, NS involvement in SSc has become more frequent, especially in recent studies, with a range from 17 to 40% (5, 8–11).

While only a few studies have evaluated the prevalence of NS involvement in SSc, there is even less research regarding the true pathogenesis of neuropathy in this rare disease. Most symptoms in patients with SSc can be explained by microvascular damage, the autoimmune response and inflammation, and fibrosis with variable severity (12, 13). The first and to this day the most accepted cause for neuropathy development in SSc is ischaemic damage of the NS (8, 14). Thus, it would be logical to conclude that patients with severe Raynaud's disease, pitting scars and ischaemic skin lesions should develop neuropathy, but the proportion of patients without nerve damage contradicts this view, suggesting that other mechanisms are involved in the pathogenesis of neuropathy in SSc (15, 16).

There are no specific data on the role of the autoimmune response and inflammation in the development of neuropathy in SSc. In many systemic connective tissue diseases, the idea of studying specific antibodies (Abs) against various nerve structures comes from research performed in immune-mediated polyneuropathies (PNP) like Guillain–Barré syndrome (17, 18). This approach is still understudied in SSc and could lead to new insights into neuropathy pathogenesis and a future change in treatment tactics.

Another understudied issue is biomarkers for the progression and severity of SSc. Several biomarkers are used to measure and monitor the severity of lung and skin damage in SSc; however, markers to assess PNS damage and its progression have yet to be identified (19, 20). Neurofilament light chain (NfL) has proved to be useful biomarker for PNP, given that it is related to metabolic and genetic disorders, but it has not been studied in SSc (21, 22). There are other known biomarkers that are mostly or partly secreted from Schwann cells that can associated with PNS damage due to various diseases, including growth/differentiation factor 15 (GDF15) studied in diabetic neuropathies and glial fibrillary acidic protein (GFAP) associated with inflammatory PNP (23, 24).

The primary objective of this study was to define the autoimmune mechanisms that lead to neuropathy by identifying Abs that target certain component of the NS or are associated with SSc. The secondary objective was to identify markers of NS damage that correlate with the detection and progression of PNP.

2 Materials and methods

2.1 Subjects

This study included patients diagnosed with SSc who met the American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) 2013 classification criteria and who received a consultation by rheumatologists at two leading Latvian hospitals between January 2016 and December 2021 (7). Using the hospital databases, patients with diagnostic codes M34.0–M34.9 based on the International Classification of Diseases, 10th Revision (ICD-10) were selected. Patients with connective tissue diseases other than SSc and patients with localised scleroderma were excluded. The age at disease onset was defined as the time of onset of the first non-Raynaud's SSc symptom. The skin condition was evaluated according to the modified Rodnan skin score (mRSS) by a rheumatologist (25).

This study was approved by the Rīga Stradiņš University medical ethics committee (Institutional Review Board reference no 22-2/481/2021). All participants provided written informed consent.

2.2 Methods

The enrolled subjects underwent a uniform evaluation of the PNS. First, the patients underwent a nerve conduction study (NCS) by a certified neurophysiology expert. Motor and sensory conduction were evaluated according to the PNP examination protocol (26). Each patient underwent an NCS of the bilateral upper extremities (the motor and sensory components of the

ulnar and median nerves) and the bilateral lower extremities (the motor component of the peroneal and tibial nerves and the sensory component of the sural nerve) to determine nerve conduction latency, amplitude, and velocity. The patients with abnormal NCS results—considering the normal values used in Latvian clinical practice—in more than one attribute for two separate nerves were diagnosed as having PNP. The patients were divided in two groups according to the NCS results. The first group included patients with PNP, while the second included patients without PNP.

The patients were also evaluated with the Health Assessment Questionnaire Disability Index (HAQ-DI). The use of personal assistance or assistive devices were acknowledged. The scores from each of the eight sections were added together and then divided by eight to obtain the functional disability index. In addition, blood was collected from each patient. After separating the serum, aliquots were stored at −80°C prior to analyses.

The SSc-associated Abs were analysed using a commercial line immunoblot assay (EUROLINE Systemic Sclerosis Profile, Euroimmun). The EUROLINE Systemic Sclerosis (Nucleoli) Profile (IgG) contains 13 recombinant antigens: DNA-topoisomerase I (Scl-70), centromere proteins A and B (CENP-A and CENP-B, respectively), RNA polymerase III (subunits RP11 and RP155), fibrillarin, NOR-90, Th/To, PM-Scl-100, PM-Scl-75, Ku, platelet-derived growth factor receptor (PDGFR) and Ro-52. The detection and interpretation were carried out electronically using the Euroimmun EUROLineScan programme. A signal intensity of 0–5 (negative) and 6–10 (borderline) was considered negative, while a signal intensity of ≥11 was considered positive.

Several nervous system–specific Abs—namely Abs against myelin-associated glycoprotein (MAG) and anti-ganglioside Abs (GM1, GM2, GD1a, GD1b and GQ1b)—were evaluated with GanglioCombi® MAG enzyme-linked immunosorbent assay (ELISA) kits (Bühlmann Laboratories). A signal intensity of 0–29 (negative) and 30–49 (borderline) was considered negative, while a signal intensity of ≥50 was considered positive. These Abs were assessed in patients with PNP first. If the data suggested a significant change in these patients, then the other groups were evaluated.

Two potential serum PNS biomarkers—NfL and GFAP—were measured with a Single molecule array (Simoa) assay (Quanterix, Billerica, MA, United States). Fibroblast growth factor 21 (FGF21) and GDF15 were measured using commercially available ELISAs according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, United States). All measurements were performed in one round of experiments using one batch of reagents by board-certified laboratory technicians who were blinded to the clinical data. The intra-assay coefficients of variation, determined using internal control samples, were below 10%.

2.3 Data analysis

The data distribution was assessed with a normal Q–Q plot and the Shapiro–Wilk test. The Mann–Whitney U test was used to compare SSc-associated Abs, NfL, GFAP, GDP-15 and FGF21 between patients with and without PNP. Additionally, this test was used to compare NfL between the control group and patients with PNP. Differences in SSc-associated Abs between patients with and without PNP were assessed with the chi-square test of homogeneity or Fisher's test.

A binomial logistic regression was conducted to determine factors (age, sex, SSc duration, mRSS and HAQ-DI) related to patients with and without PNP. Forward and backward stepwise regression methods were used to build the model. All possible models and interactions were calculated. The Akaike information criterion (AIC) was used to select the best model. Additionally, receiver operating characteristic (ROC) curve analysis, to determine the area under the curve (AUC), was conducted to evaluate the performance of the regression model as binary classifier. An AUC >0.7 was considered to indicate good performance in distinguishing between patients with and without PNP. The Youden index was used to identify the optimal cut-off point.

3 Results

We initially recruited 103 Caucasian patients diagnosed with SSc (18 men and 85 women). Table 1 summarises the sex-specific clinical and Ab characteristics in these patients.

Among the 103 patients recruited for this study, three declined to undergo an NCS. Following the NCS, the remaining cohort of 100

TABLE 1 Sex-specific clinical and antibody characteristics in patients with systemic sclerosis.

		Men	Women	Total
Descriptive statistic	Number of patients	18	85	103
	Mean (standard deviation) age in years	60.06 (14.92)	61.66 (11.95)	61.38 (12.46)
	Mean (standard deviation) disease duration in years	8.95 (6.33)	15.14 (9.87)	14.06 (9.62)
Symptoms	Raynaud's phenomenon, *n* (%)	16 (88.88%)	71 (83.52%)	87 (84.46%)
	Mean (standard deviation) Modified Rodnan skin score	10.36 (12.95)	10.67 (8.78)	10.63 (9.41)
SSc-associated antibodies	Classical antibodies* *n* (%)	8 (44.44%)	52 (65.82%)	60 (61.86%)
	Scl-70 n (%)	4 (22.22%)	18 (22.78%)	22 (22.68%)
	CENP-A and CENP-B *n* (%)	4 (22.22%)	31 (39.24%)	35 (36.08%)
	RP11 and RP155 n (%)	0	3 (3.80%)	3 (3.09%)
	Novel antibodies** *n* (%)	9 (50%)	35 (44.30%)	44 (45.36%)

*Scl-70 (topoisomerase I), CENP-A and CENP-B (centromere proteins A and B, respectively), RP11 and RP155 (RNA polymerase III). **Fibrillarin, NOR-90, Th/To, PM-Scl-100, PM-Scl-75, Ku, platelet-derived growth factor receptor (PDGFR) and Ro-52.

TABLE 2 Demographic, clinical and neurophysiological characteristics and comparisons of patients with systemic sclerosis and with or without polyneuropathy (PNP).

Variable	SSc without PNP 57 (57%)	SSc with PNP 43 (43%)	*p*-value
Sex, n (%)			0.0
Male	5 (29.41%)	12 (70.59%)	
Female	52 (62.65%)	31 (37.35%)	
Mean (standard deviation) age in years	57.30 (12.24)	67.07 (10.47)	<0.001
Mean (standard deviation) disease duration in years	12.48 (8.68)	16.26 (10.51)	0.049
Mean (standard deviation) modified Rodnan skin score	8.05 (9.14)	7.36 (9.67)	0.715
Raynaud's phenomenon, n (%)	51 (89.47%)	36 (83.7%)	0.860
Mean (standard deviation) nerve conduction study results			
Nervus peroneus			
Amplitude (mV)	3.32 (1.79)	2.10 (1.28)	<0.001
Velocity (m/s)	45.2 (11.1)	41.7 (3.43)	<0.001
Nervus tibialis			
Amplitude (mV)	8.38 (2.84)	4.90 (2.84)	< 0.001
Velocity (m/s)	46.5(2.58)	40.8 (3.20)	<0.001
Nervus suralis			
Amplitude (mV)	11.7 (6.54)	7.54 (4.73)	0.002
Velocity (m/s)	47.2 (12.2)	41.1 (1.75)	<0.001

patients with SSc was stratified into subgroups based on the presence or absence of PNP. We identified PNP in 43 patients, representing 43% of the cohort. Within this subset, 15 patients had sensory-motor demyelinating PNP, while 28 had sensory-motor axonal demyelinating PNP. Table 2 illustrates the distinctions in demographic, clinical, and neurophysiological characteristics between patients with SSc and with or without PNP.

We assessed SSc-associated Abs in 97 patients; they did not differ significantly between patients with and without PNP ($p > 0.05$). We assessed anti-MAG and anti-ganglioside Abs in 24 patients. All 24 patients had PNP based on the NCS results, but they did not present a significant increase in the Abs above the reference range.

We assessed potential PNS serum biomarkers—NfL, GFAP, GDF15 and FGF21—in 68 patients, 30 with PNP, 38 without PNP. Table 3 summarises the comparison of serum biomarkers concentration between patients with and without PNP. NfL, GFAP and GDF15 were significantly elevated in the presence of PNP ($p < 0.05$), with a moderate to high effect size ($r = 0.36–0.65$). We observed the most pronounced difference for NfL, with significantly lower levels in control subjects (median = 5.2, interquartile range [IQR] 4.3–7.4) compared with those with PNP (median = 15.3, IQR 11.8–25.0; U = 35.0, $p < 0.001$, $r = 0.93$).

The final binomial logistic model was significant ($\chi^2(3) = 30.8$, $p < 0.001$; Table 4). The AUC was 0.81, indicating strong performance in distinguishing between patients with and without PNP (Figure 1).

Our regression analysis revealed a strong association between the HAQ-DI score and the risk of developing PNP. A 1-point increase in the HAQ-DI score was significantly associated with a 95% higher likelihood of PNP (95% confidence interval [CI] 13–236%; $p < 0.001$). Based on the Youden index, individuals with an HAQ-DI score exceeding 0.63 had a greater than 50% probability of developing PNP. Age was also a significant predictor of PNP development. Each additional year of age was associated with a 9% increase in PNP risk (95% CI 4–14%; $p < 0.001$). Using the Youden index, individuals aged ≥63 years had a > 50% chance of developing PNP. Furthermore, we observed a significant sex difference in PNP risk. Women were 86% less likely to develop PNP compared with men (95% CI 46–97%; $p < 0.001$). Finally, we removed SSc duration and the mRSS from the final regression model due to their lack of statistical significance to the model.

TABLE 3 Comparison of biomarker levels in patients with systemic sclerosis (SSc) and with or without polyneuropathy (PNP).

Parameter	SSc without PNP 38 (55.88%)	SSc with PNP 30 (44.11%)	*p*-value	*r*
	Median (interquartile range)	Median (interquartile range)		
NfL, pg/mL	9.8 (6.0–13.1)	15.3 (11.8–25.0)	<0.001	0.62
GFAP, pg/mL	77.1 (43.9–99.0)	100.5 (67.8–159.8)	0.011	0.36
GDF15, pg/mL	964.5 (705–1,389)	1681.5 (1303–2049)	<0.001	0.65
FGF21, pg/mL	130.7 (65.3–372.5)	148.3 (99.5–287.5)	0.501	NA

FGF21 Fibroblast growth factor 21; GDF15, growth/differentiation factor 15; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; NA, not applicable; r, effect size (Cohen's r).

4 Discussion

To our knowledge, this is one of the few studies on SSc that focuses on the involvement of the PNS, analysing both the prevalence of this complication and its pathogenesis and biomarkers of severity. We found a higher prevalence of PNP in SSc compared to data from other studies, possibly due to detailed and targeted assessment of the

TABLE 4 Results of final regression model showing the patient's age, sex, and health assessment questionnaire disability index score as predictors of developing polyneuropathy.

					95% confidence interval of the odds ratio	
Predictor	Estimate	Z	*p*-value	Odds ratio	Lower	Upper
Intercept	−4.73	−3.11	0.002	0.01	0.001	0.17
Health assessment questionnaire disability index	0.67	2.39	0.017	1.95	1.13	3.36
Age	0.08	3.43	<0.001	1.09	1.04	1.14
Sex						
Female–Male	−2.00	−2.84	0.005	0.14	0.03	0.54

References: Dependent variable—patients without PNP; sex—male.

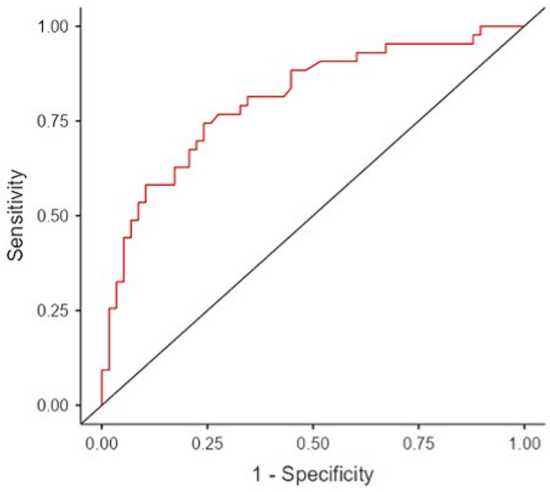

FIGURE 1
Performance of the final binomial logistic model based on receiver operating curve analysis that predicts polyneuropathy in patients with systemic sclerosis.

PNS. Moreover, the materials and methods used in these studies provide a wider range of results. A systematic review of 113 studies found a neuropathy prevalence of 27.37%, including 26% ($n = 556/2143$) with small fibre neuropathy and 10.8% ($n = 231/2143$) with large fibre neuropathy, however, titles and abstracts were not selected according to strict criteria for the neuropathies assessed (8). Confirmatory diagnostic tests for PNP in SSc varied according to study design (27–33). Some studies performed electrophysiological examinations, while others used imaging techniques, biopsies or other methods (4, 27–34). We believe that the high prevalence of PNP in our study can be explained by the fact that we worked with a relatively large study group and that all subjects were assessed using both clinical symptoms and electrophysiological methods, where motor and sensory components were studied on multiple nerves in each limb.

Historically, the classical SSc-specific or SSc-associated Abs—anti-topoisomerase Abs (ATAs), anti-centromere Abs (ACAs) and anti-RNA polymerase Abs (ARAs)—have received the most attention (35). Currently, novel Abs are assessed in addition to the classical Abs, and their presence in different clinical phenotypes remains a research goal (36). Only a few studies have evaluated the association of these classical Abs with neuropathies in SSc, and the results have varied greatly. In a 1994 study, 35% of patients with SSc presented neurological symptoms, and 73% of them had either ARAs or ATAs, but not ACAs (37). On the contrary, in a 2021 systemic review, the authors mentioned that ACAs are a risk factor for non-compression neuropathies in patients SSc (8). Similarly, in Brazilian study of 63 patients with SSc, seven were diagnosed with PNP, of whom 6 had ACAs and 1 had ARAs (38). In a Spanish study, ARAs, ATAs and ACAs were present in patients with SSc and PNP, but the authors did not provide the statistical analysis (39).

Expanded SSc-associated Ab panels have started to play an increasingly important role in research and clinical practice. Although there is wide spectrum of clinical phenotypes in SSc, information regarding NS involvement is frequently missing (40). We could not find published data about expanded SSc-associated Abs in patients with SSc and NS damage. Interestingly, none of our patients was positive for anti-PDGFR Abs, and only one patient was positive for anti-Fib Abs. The most common SSc-associated Abs were anti-Ro52 Abs, ACAs and ATA. Only three patients (3%) were positive for ARAs, a lower frequency than for Abs that are not included in the SSc classification criteria: anti-Ku, anti-PM100, anti-Th/To and anti-NOR90 Abs. We did not find significant association between any of the SSc-associated Abs and the presence of PNP.

In autoimmune neuropathies, gangliosides are one of the most frequent targets of Abs (41). Gangliosides are nerve fibre glycoproteins that play an important role in both impulse transmission and nerve fibre regeneration. Anti-ganglioside Abs are often detected in the serum of patients with Guillain–Barré syndrome (37–78% of the cases) (42). They have been studied in patients with systemic lupus erythematosus and neuropsychiatric manifestations: the authors detected Abs more frequently in patients with neuropsychiatric manifestations compared with the asymptomatic group (43). There are very few studies on anti-ganglioside Abs in patients with SSc. In 1994, 34 patients with scleroderma, of whom 28 had PNP, were evaluated for the presence of anti-GM1 Abs. The levels were lower in these patients compared with healthy individuals, and there was no association with the development PNP (44). In our study, performed almost 30 years later, we also could not find a significant association between anti-MAG or anti-ganglioside Abs and the development of PNP in patients with SSc. Due to the lack of data on the association between PNP in SSc and specific nervous system–specific Abs we initially determined Abs only in a subset of patients with definite PNP, randomly selected. We would most likely not expect a significant change if Abs were detected in all patients with PNP, and even if they were detected at low titres, these data would only show false positives and unnecessarily confound the overall significance of the study.

In this study, no Abs were associated with a more frequent development of PNP in patients with SSc. At present, immune-mediated peripheral nerve damage in SSc remains questionable. In the treatment of PNP in patients with SSc, the role of immunosuppressive drugs remains equivocal and, according to our data, there is no reason to expect them to be efficacious. Additional research is necessary to predict PNS damage in patients with SSc so that they can be managed appropriately.

In recent years, successful new candidate serum biomarkers have been identified for SSc-associated interstitial lung disease (ILD), including surfactant protein D (SP-D), Krebs von den Lungen 6 glycoprotein (KL-6), CCL18 and intercellular adhesion molecule 1 (ICAM-1) (45, 46). For ILD, there has been a focus on searching for biomarkers in SSc that are also related to skin involvement and vascular injury (20, 47). Unfortunately, researchers have not yet evaluated serum biomarkers for PNS damage in patients with SSc. Thus, we chose to evaluate the most promising biomarkers based on the connection to the PNS. Of these four serum biomarkers—NfL, GFAP, GDF15 and FGF21—three of them showed promise as candidate PNP serum biomarkers in patients with SSc.

NfL stand out as novel biomarker for early diabetic sensorimotor PNP; there are possible similarities in vascular injury in both diabetic PNP and PNP in SSc (21). Our findings confirmed the already established significant role of NfL as a serum biomarker for neuropathies of different aetiologies (48).

A less-studied biomarker in PNP is GFAP, which has mostly been associated with central NS damage due to its predominant secretion from astrocytes. However, studies have demonstrated the presence of GFAP in the PNS (49, 50). Researchers have reported elevated serum GFAP levels in chronic neuropathies like chronic sensory-motor axonal neuropathy and chronic inflammatory demyelinating PNP (24). Unlike NfL, GFAP has not been widely evaluated in diabetic neuropathies, reducing the likelihood of linking this biomarker to neuropathy caused by vascular injury. We did not find any studies of GFAP in SSc, but serum GFAP was significantly elevated in patients with SSc and PNP.

GDF15 and FGF21 have less association with the NS. GDF15 is a cytokine belonging to the transforming growth factor beta superfamily. Elevated GDF15 levels are observed in inflammation, myocardial ischaemia and tumours (51). Serum GDF15 levels were elevated in patients with pulmonary hypertension (PH) and SSc compared with patients with SSc but not PH, as well as in patients with SSc, ILD and more pronounced skin lesions (52–54). There is evidence of increased GDF15 secretion by Schwann cells in nerve injury, and increased GDF15 levels have been found in patients with diabetic neuropathy, mainly with more pronounced manifestations of metabolic syndrome (23, 55, 56). We found elevated serum GDF15 levels in the patients with SSc and PNP compared with the patients with SSc but not PNP. Of note, there have been no other studies that evaluated this serum biomarker in patients with SSc and neuropathies.

Only FGF21 showed no significant change between the SSc with PNP and the SSc without PNP groups. This pleiotropic hormone—considered to be a major regulator of energy homeostasis—is mainly synthesised in the liver, pancreas and adipose tissue (57, 58). Recently, researchers have shown that FGF21 has regenerative capability in the PNS by suppressing oxidative stress, and the FGF21 levels were elevated in patients with diabetic neuropathy after aerobic training (59, 60). While there have been no studies on FGF21 levels in patients with SSc, we found that FGF21 levels did not change significantly in patients with SSc and PNP, indicating that FGF21 has less of a connection to the NS compared with other biomarkers. FGF21 expression is significantly increased in the muscles of mice with mitochondrial myopathies, where its levels are directly related to the presence of cytochrome oxidase negative fibres, a marker associated with the severity of the disease. This observation underscores the relevance of FGF21 in muscle pathology, especially under conditions characterised by damaged mitochondrial function (61, 62).

We found that the axonal demyelinating form of PNP was the most common in our patients with SSc. The absence of significant correlations between Abs and PNP has led us to consider alternative pathogenic mechanisms. Comparisons between the patients with and without PNP showed several intriguing differences: the patients with PNP were generally older, with an average age of 67 years compared with 57 years, and it was more prevalent in men (66% compared with 36%). These observations indicate that ageing, metabolic factors and ischaemic mechanisms may contribute significantly to the emergence of axon neuropathies, reflecting the patterns observed in cases of idiopathic PNP. In the literature, researchers have noted a higher prevalence of idiopathic PNP in people aged >60 years. Similar results have been reported in studies focusing on chronic axon idiopathic PNP in people aged >60 years, with a 3:2 male-to-female ratio (63, 64). As the name suggests, the condition is idiopathic, and metabolic factors are most strongly considered to be involved in the aetiology, but microvasculopathy identified in biopsies shows a different pattern than in diabetic neuropathies (64, 65). These coincidences lead us to suspect sequential development of PNP in patients with SSc over time, associated with ageing and a logical progression of the disease with more pronounced vasculopathy and metabolic factor–associated effects. Our regression analysis confirmed this view: it showed that age is a significant predictor of PNP development.

A deeper look into the serum biomarkers we evaluated in patients with SSc revealed three biomarkers associated with PNP. NfL and GFAP had already been shown to be associated with axonal injury, strengthening our above hypothesis of the development of PNP in SSc (24, 66). On the other hand, GDF15 and FGF21 have mostly been associated with mitochondrial stress and subsequent metabolic changes (67, 68). Interestingly, they behaved differently in our study. While the FGF21 levels were slightly higher in patients with SSc and PNP, the difference was not significant. The GDF15 levels were significantly elevated in patients with SSc and PNP, similarly to patients with diabetic neuropathies, were metabolic damage plays an important role (23). We believe additional studies that detect muscle damage and loss are needed to further investigate the role of mitochondrial damage and metabolic markers in patients with SSc.

Our results suggest that the use of serum biomarkers in clinical environments may facilitate early identification of PNS damage in patients with SSc. By dynamically monitoring biomarkers such as the NfL, GFAP and GDF15, it could be possible to detect deterioration of nerve function without further electrophysiological testing. However, research focusing on hereditary neuropathy has challenged the effectiveness of neurofilament fluctuations as indicators of disease progression, suggesting that these markers may not be suitable for tracking slow-moving diseases due to their lack of specificity and their tendency to reflect general rather than specific nerve damage (69).

A strength of this study is the choice of the group of interest: PNP is one of the complications of SSc that seems to have been neglected. To our knowledge, this is the first study that has extensively defined serum tests of different significance in patients with SSc and PNP. Moreover, we analysed both the immune pathogenesis of PNP and the reflection of nervous system damage in serum biomarkers in a univariate manner. However, several limitations must be acknowledged. First, the study did not include a healthy control group, which might have provided more evidence for our findings linking the development of PNP in SSc patients also to natural ageing. Secondly, this study focused on the development of neuropathy as the main complication of SSc, without providing a full description of the patients' other organ involvement such as ILD, PH and others. We included the presence of Raynaud's phenomenon, which partially characterises vasculopathy, and the mRSS, which partially characterises disease severity by skin involvement, but it would also be very useful to include more clinical symptoms. However, the relationship of the different clinical manifestations of the disease to the involvement of the PNS must be demonstrated in future projects.

5 Conclusion

There was no association between SSc-associated or other inflammatory neuropathy-associated Abs and the development of PNP in patients with SSc. The development of PNP in patients with SSc is most likely due to ageing, natural progression and the sequelae of the disease. Several serum biomarkers—NfL, GFAP and GDF15—could be used as relevant diagnostic biomarkers for PNP in patients with SSc. Future studies are warranted to validate the diagnostic efficacy of these biomarkers and to unravel the complex interplay of factors leading to PNP in patients with SSc. This endeavour should ultimately pave the way for novel therapeutic strategies and a more nuanced understanding of this multifaceted 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 the Rīga Stradiņš University medical ethics committee (Institutional Review Board reference no 22-2/481/2021). All participants provided written informed consent. 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

KI: Writing – original draft, Writing – review & editing. MZ: Writing – review & editing. KB: Writing – review & editing. HZ: Writing – review & editing. NK: Writing – original draft, Writing – review & editing. VĶ: 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.

Publisher's note

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Progress of rituximab in the treatment of systemic lupus erythematosus and lupus nephritis

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with heterogeneous clinical manifestations, often leading to significant morbidity and mortality, particularly due to lupus nephritis (LN). The standard therapeutic approach involving mycophenolate mofetil, cyclophosphamide, and glucocorticoids has shown limitations due to cumulative toxicity and side effects. The introduction of biologic agents, especially rituximab (RTX), a chimeric monoclonal antibody targeting CD20+ B cells, has revolutionized the treatment landscape. This review synthesized the current understanding of B cells' role in SLE and LN and evaluates RTX's therapeutic impact. B cells contribute to disease pathogenesis through autoantibody production and immune complex formation, leading to tissue damage. RTX's mechanisms of action, including Complement-Dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and induction of apoptosis, have demonstrated efficacy in both SLE and LN treatment. Clinical studies have reported remission rates and improved renal outcomes with RTX use, although challenges such as human anti-chimeric antibody development and optimal dosing persist. The review emphasized the need for continued research to elucidate RTX's long-term benefits and risks, and to explore personalized treatment strategies that incorporate B cell biology for better disease management in SLE and LN.

KEYWORDS

rituximab, systemic lupus erythematosus, lupus nephritis, B cells, treatment

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterized by a recurrent-remission course and a broad spectrum of clinical manifestations (1, 2). Globally, the incidence and prevalence of SLE vary significantly by geographical location, with the highest rates observed in North America, while Africa and Australia report the lowest. Factors such as age, gender, and ethnicity play crucial roles in determining clinical outcomes and disease management. Notably, SLE is more prevalent among females; however, disease progression tends to be more severe and rapid in males, resulting in a poorer prognosis. These differences may be attributed to varying environmental factors and genomic differences (3).

To date, the pathogenesis of SLE remains unclear. It can be associated with the interplay of genetic susceptibility, environmental factors, immune system irregularities, and hormonal

influences (4, 5). In pathological conditions, patients with SLE typically produce a large number of antibodies, leading to the formation of antigen–antibody complexes. These complexes deposit in the kidneys, causing renal damage, and ultimately resulting in lupus nephritis (LN) (6, 7). Consequently, LN is a severe complication and one of the most common clinical manifestations of SLE, as well as one of the leading causes of mortality among SLE patients. Approximately 60% of individuals with SLE may develop LN, and 5 ~ 20% of those with LN progress to renal failure within 10 years (8–12). Therefore, the primary goals of LN treatment are to control disease activity, prevent relapses and progression, and avert the development of end-stage renal disease.

First-line treatments for LN typically include mycophenolate mofetil (MMF) or cyclophosphamide (CYC) combined with glucocorticoids (GC). Maintenance therapy typically involves MMF or azathioprine (AZA) combined with low-dose GC. The remission rate after 1 year of LN treatment ranges from 30.4 to 66.2%, with a good renal response being associated with improved disease prognosis (13, 14). Despite the introduction of emerging immunosuppressants such as tacrolimus (TAC) and MMF, the complex pathophysiological characteristics of SLE and LN and the clinical difficulty of controlling the disease with a single drug pose significant challenges to treatment. These challenges are further compounded by the notable side effects and cumulative toxicity of immunosuppressive drugs, including ovarian failure, bone marrow suppression, gastrointestinal symptoms, teratogenicity, and an increased risk of malignancies (15). Glucocorticoids (GC) are the cornerstone of SLE and LN treatment. However, prolonged use can lead to various serious long-term adverse effects and an increased risk of infections. It is also associated with an elevated risk of early cardiovascular disease, with SLE patients experiencing a 2- to 4-fold increase in the risk of coronary artery events (16). Studies have indicated that cumulative GC doses are significantly associated with an increased risk of cataracts and osteoporosis with fractures. Additionally, prolonged GC use elevates the risk of ischemic necrosis, diabetes, and hypertension (17). Furthermore, the current treatment regimens for SLE and LN remain limited in their efficacy, with up to 28% of patients eventually progressing to end-stage renal disease (ESRD) or death (18). Therefore, additional therapeutic strategies are needed to improve the prognosis of LN patients. Given the crucial role of B cells in the development and progression of dysregulated immune responses, the use of B cell-depleting agents in the treatment of SLE and LN remains a topic of ongoing debate. Rituximab (RTX), an anti-CD20 monoclonal antibody (mAb), has been extensively used in SLE and other rheumatologic disorders. Thus, we synthesized the current understanding of the role of B cells in SLE and LN, and further summarized the progress of RTX in the treatment of SLE and LN.

Role of B cells in the pathogenesis and progression of SLE and LN

In patients with SLE and LN, B cells play a multifaceted pathogenic role, characterized by abnormalities in their differentiation and function. B cells contribute to immune damage through multiple mechanisms, including the production of autoantibodies, which can induce immune injury via antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Additionally, B cells play a crucial role by producing various cytokines, presenting antigens, regulating immune responses, and providing co-stimulatory signals, highlighting their significant involvement in the pathogenesis of SLE and LN (19, 20). As a B cell hyperactivity-driven, non-organ-specific autoimmune disease, SLE is characterized by several B cell abnormalities. These abnormalities primarily function to promote the production of autoantibodies (21). Autoimmune dysregulation leading to the production of autoantibodies against various cellular components is a hallmark of the disease, particularly against nuclear antigens. Over 95% of patients possess autoantibodies targeting antinuclear antigens (antinuclear antibody, ANA) (22). Autoantibodies, as the core pathological feature of the disease, include characteristic antibodies against double-stranded DNA (dsDNA), histones, and the entire nucleosome, as well as antibodies against RNA-binding proteins. These proteins notably include Sm, U1RNP, Ro (SS-A), La (SS-B), and hnRNP A2 (RA33), along with phospholipids (e.g., cardiolipin) or phospholipid-binding proteins like β-2-glycoprotein-I. Under normal circumstances, these charged antibodies tend to form immune complexes to some extent, which deposit in the glomerular basement membrane (GBM) of the kidneys, thereby progressing to LN (23). As early as 1967, dsDNA antibodies were discovered in renal tissue biopsies from LN patients, and high specificity for SLE (24, 25). Moreover, the reciprocal changes in elevated dsDNA antibodies and serum complement levels (C3 and C4) serves as markers for onset, classification, diagnosis, and disease activity assessment in SLE and LN. These levels also correlate with treatment response, making autoantibodies valuable as therapeutic diagnostic biomarkers for outcome measurement in routine clinical practice and clinical trials (26, 27). Advancements in technology have enabled more sophisticated and precise analyses of autoantibodies, providing new insights into SLE pathogenesis and positioning it at the forefront of autoimmune mechanism research.

Self-reactive B cells, a type of lymphocyte, produce autoantibodies leading to autoimmune diseases. The mechanisms behind the generation of self-reactive B cells remain unclear, but potential mechanisms include: (1) During B cell development in the bone marrow, abnormalities in the central checkpoint may lead to a vast diversity in the pre-B cell receptor repertoire. This results in the failure of apoptosis of self-reactive B cells, allowing their survival; (2) T cell-dependent B cells, upon antigen and T cell stimulation, enter the germinal center. During the process of somatic hypermutation, abnormalities in negative selection can result in the production of self-reactive B cells (28). Under normal conditions, the survival and activation of self-reactive B cells in the body are regulated by multiple checkpoints. The immune system effectively modulates self-reactive B cells through negative selection mechanisms, including clonal deletion, clonal anergy, and receptor editing, which suppress their proliferation and promote B cell immune tolerance. Consequently, the development of self-reactive B cells is effectively inhibited.

However, when central and peripheral checkpoints become dysfunctional due to factors such as abnormal levels of B lymphocyte stimulatory factors, defects in inhibitory receptors on self-reactive B cells, lowered activation thresholds for B cells, impaired clearance of apoptotic products, and genetic abnormalities, self-reactive B cells can become activated and proliferate. This breakdown in B cell self-tolerance leads to the production of autoantibodies against self-antigens, resulting in the onset of autoimmune diseases such as SLE and LN (29, 30). Yurasov et al.

discovered that in patients with SLE, the number of self-reactive mature naive B cells was double that of healthy individuals. This increase was accompanied by a higher quantity of polyreactive antibodies (30). Additionally, high levels of B lymphocyte activating factor (BAFF) from the tumor necrosis factor family and type I interferons (IFNs) may enhance the survival of self-reactive B cells (31). Therefore, B cell abnormalities, particularly self-reactive B cells, play a crucial role in the pathogenesis of SLE and LN. Given the role of B cells in the development of dysregulated immune responses, B cell depletion therapy has been pioneered in patients with refractory SLE and LN.

B cell-targeted therapy

To date, targeted therapies against autoreactive B cells have become significant therapeutic strategies for systemic lupus erythematosus and various other autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and autoimmune thrombocytopenia. The mechanisms of B cell-targeted therapies can be broadly categorized into the following strategies: elimination of autoreactive B cells, blockade of extracellular soluble factors or receptors, intrinsic blockade of B cell activation pathways, and receptor editing (32). Numerous emerging agents are currently under development based on these mechanisms.

In terms of eliminating autoreactive B cells, the predominant approaches involve monoclonal antibodies and CAR-T cell therapy. Monoclonal antibodies target various CD molecules expressed at different stages of B cell development and maturation. These antibodies mediate B cell elimination through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity. Examples include anti-CD19 antibodies such as inebilizumab, obexelimab, and tafasitamab; anti-CD20 antibodies like rituximab, ocrelizumab, and obinutuzumab; anti-CD22 antibody epratuzumab; and anti-CD38 antibody daratumumab. CAR-T cell therapy involves the genetic modification of T cells to express chimeric antigen receptors (CARs) that recognize B cell surface molecules, thereby leveraging T cell-mediated cytotoxicity for effective B cell elimination (33). Examples include Anti-CD19 CAR-T cells and BCMA-CD19 compound CAR-T cells.

Regarding the blockade of extracellular soluble factors or receptors, recent research has focused on agents that inhibit BAFF (B-cell activating factor). BAFF, a member of the TNF superfamily, plays a critical role in preventing B cell apoptosis and promoting B cell differentiation (34). Relevant therapeutic agents include belimumab, tabalumab, blisibimod, and ianalumab. Telitacicept is a dual-target agent that inhibits both BAFF and APRIL, with analogous mechanism agents including atacicept and povetacicept.

The elevated expression of type I interferons is associated with both innate and adaptive immune dysfunctions in systemic lupus erythematosus (35). For instance, anifrolumab, a type I IFN receptor inhibitor. Other agents targeting this pathway include rontalizumab and sifalimumab.

Beyond BAFF and IFN-α, other cytokine-targeted therapeutics are under development, such as IL-12 and IL-23 inhibitors like ustekinumab and IL-6/sIL-6R inhibitors including sirukumab, tocilizumab, and vobarilizumab (a soluble IL-6 receptor). CD40 ligand inhibitors, such as dapirolizumab pegol, are also being explored.

For the intrinsic blockade of B cell activation pathways, relevant drugs include SYK inhibitors (cevidoplenib, lanraplenib), BTK inhibitors (rilzabrutinib, fenebrutinib, Evobrutinib), and proteasome inhibitors (bortezomib). In the domain of receptor editing, research is ongoing to use edited CAR-Treg cells to suppress autoreactive B cell functions. Additionally, theoretically, receptor editing of autoreactive B cells could potentially avoid autoreactive binding, though no such therapeutics have yet been developed, which might be a future exploration direction.

Mechanism of action of RTX in B cell-targeted therapy

The anti-CD20 monoclonal antibody RTX is the first to receive FDA approval for the treatment of CD20-positive B cell malignancies, such as non-Hodgkin lymphoma and chronic lymphocytic leukemia. Subsequently, its therapeutic reach has expanded to autoimmune diseases, including rheumatoid arthritis (RA), SLE, and LN (36–38). The therapeutic effectiveness of rituximab is based on its impact on B cells. Consequently, the recognition that B cells play a more important part in autoimmune diseases than last believed has resulted in its growing usage for off-label reasons. The multifaceted mechanisms by which RTX treats SLE and LN include: (1) Complement-Dependent Cytotoxicity (CDC): RTX effectively binds to C1q, activating complement *in vitro*, leading to a cascade reaction and the formation of a membrane attack complex, inducing lysis of CD20+ B cells; (2) Inhibition of Cell Proliferation and Induction of Apoptosis: RTX cross-linking by cells expressing Fc receptors induces apoptosis through the activation of the Caspase-3 signaling pathway, leading to B lymphocyte apoptosis. Additionally, RTX can directly induce cell death via Fab-mediated pathways; (3) Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC): RTX induces the aggregation of monocytes, macrophages, and natural killer cells through the binding of their Fc receptors to the Fc fragment of RTX, leading to the lysis of B lymphocytes (39). Although ADCC is identified as the primary action mechanism of RTX, complement-mediated cytotoxicity also plays a significant role; (4) Follicular regulatory T (Tfr) cells are a specific subset of regulatory T cells concentrated mostly in the germinal center (GC), which act as regulators of GC responses. They may interfere with the identification mechanism of Tfh cells and B cells, trigger Tfh death, and inhibit B cell activity. Using RTX to rebuild GC responses might also contribute to the treatment of SLE (40). Additionally, RTX can affect the function and number of T cells, further contributing to its therapeutic profile. B lymphocytes may function as antigen presentation cells (APC) for T lymphocytes, resulting in a pro-inflammatory response via generating cytokines.

Progress in RTX drug research for SLE and LN treatment

The clinical studies that have been published regarding the treatment of SLE and LN related to RTX are shown in Table 1 (41–68).

Clinical usage and dosage of RTX

In August 1997, the chimeric mouse/human monoclonal antibody (mAb) RTX, which targets the B cell CD20 receptor, was approved for

TABLE 1 Published clinical studies on the treatment of SLE and LN related to RTX.

	Country	Study design	Cases(N)	RTX DOSE	Other drugs use	follow-up time(Month)	Outcome
Sfikakis et al. (41)	Greece	PCS	10	375 mg/m^2 *4	P	12	CR:50% TR:80%
Vigna-Perez et al. (42)	Mexico	PCS	22	2 × 0.5–1 g	NM	3	CR: 23% TR: 55%
Gunnarsson et al. (43)	Sweden	PCS	7	375 mg/m^2 *4	CYC: 2 × 0.5 mg/m2, MTP: 4 × 100–250 mg, P	6	CR: 43% TR: 86%
Lindholm et al. (44)	Sweden	RCS	17	375 mg/m^2 *4	NM	12	CR: 12% TR: 65%
Boletis et al. (45)	Greece	PCS	10	375 mg/m^2 *4	MMF: 2 g/d, P	38	CR: 70% TR: 80%
Melander et al. (46)	United Kingdom	RCS	20	375 mg/m^2 *4	NM(CYC 3pts)	22	CR: 35% TR: 60%
Pepper et al. (47)	United Kingdom	PCS	18	2*1 g	MMF: 1 g/d, MTP: 2 × 500 mg, P	12	CR: 33% TR: 67%
Garcia-carrasco et al. (48)	Mexico	RCS	13	2*1 g	MTP: 2 × 500 mg	6	CR: 38% TR: 76%
Ramos-casals et al. (49)	Spain	RCS	49	375 mg/m^2 *4 or 2*1 g	NM	26	CR: 80%
Catapano et al. (50)	United Kingdom	RCS	11	4 × 375 mg/m2 (4pts) 2 × 1 g (7pts)	CYC: 500 mg MTP: 500–1,000 mg	4	CR: 36% TR: 91%
Jónsdóttir et al. (51)	Sweden	PCS	25	375 mg/m^2 *4	CYC: 2 × 0.5 g, MMF (2pts), P	12	CR: 16% TR: 56% (6 m) CR: 20% TR: 80% (12 m)
Davies et al. (52)	United Kingdom	PCS	18	2*1 g	CYC: 2 × 0.5 g, MTP: 2 × 500 mg	6	CR: 61% TR: 72%
Condon et al. (53)	United Kingdom	PCS	50	2*1 g	MTP: 2 × 500 mg, MMF: 0.5–1.5 g/d	12	CR: 52% TR: 86%
Tsanyan et al. (54)	Russia	PCS	45	1*0.5 g (2pts) 2*0.5 g (16pts) 3*0.5 g (1pts) 4*0.5 g (13pts) 1*1 g (3pts) 2*1 g (11pts)	MTP: 6*250-1000 mg	6	CR: 81% TR: 86%
Contis et al. (55)	France	RCS	17	4*375 mg/m2 (10pts) 2*1 g (7pts)	MTP: 100–750 mg	12	CR: 24% TR: 53%
Kotagiri et al. (56)	Australia	PCS	14	1*375 mg/m2	AZA (6pts), MMF (7pts), CYC(1pts)	6	CR: 14% TR: 79%
Chavarot et al. (57)	France	RCS	15	4*375 mg/m2 (6pts) 2*1 g (9pts)	P	6	CR: 27% TR: 80% (6 m) CR: 47% TR: 60% (12 m)

(Continued)

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TABLE 1 (Continued)

	Country	Study design	Cases(N)	RTX DOSE	Other drugs use	follow-up time(Month)	Outcome
Hogan et al. (58)	France	RCS	12	2*1 g	MTP: 500 mg, MMF: 1200 mg/m^2/d	6	CR: 75% TR: 100% (6 m) CR: 75% TR: 100% (12 m)
Rovin (59)	Latin America	RCT	144	1 g*4d	NM	12	CR:34.7% TR: 59.7%
Zhang et al. (60)	China	RCT	84	375 mg/m2 *4	CTX:800 mg/m, P	12	CR:64.3% TR: 83.3%
Moroni et al. (61)	Italy	RCS	37	1 g*2	CYC:1–2 mg/kg/d,MYC:1–2 g/d or AZA:1–2 mg/kg/d,P	12	CR:70.6% TR: 100%
Goswami et al. (62)	India	RCS	222	1.9 + 0.25 g	LDCyC:500 mg/2 week, HDCyC:750–1,200 mg/m, MMF:1.5–3 g/d	6	CR:72.7% TR: 81.8%
Roccatello et al. (63)	Italy	RCS	60	375 mg/m^2 *8	MMF:2 ~ 3 g /d, CYC: 500 mg, a total of 3,000 mg	12	CR:93.3% TR: 100%
Moroni et al. (64)	Italy	RCS	24	2 g	MMF:1.5–3.0 g/d， P	12	CR:26.4% TR:56.9%
Tanaka Y et al. (65)	Japan	RCS	115	375 mg/m^2 *4 OR 1 g/m^2 *1 ~ 2	HCQ:192.3 ± 144.1(13pts); MMF:963.0 ± 921.7 (27pts); TAC:2.1 ± 1.5 (23pts); AZA:57.3 ± 66.5(9pts); MZR:134.9 ± 109.3(8pts); CYC:124.0 ± 121.0(5pts); MTX:11.0 ± 1.4(week) (2pts), P	NM	CR: 20.8% TR: 52.5%
Tanaka et al. (66)	Japan	RCS	34	1 g*4	P	13	CR:37.65% TR: 32.2%
Iaccarino et al. (67)	Italy	RCS	134	1 g*2(118pts) 375 mg/m^2 *(27pts)	CYC:750 mg*2, P	12	CR:57.8% TR: 84.4%
Condon et al. (53)	United Kingdom	RCS	50	1 g*2	MMF:500 mg*2, MPA:1.2–2.4 mg/L, P	13	CR:52% TR: 86%
Davies et al. (52)	United Kingdom	RCS	18	1 g*2	CYC:500 mg, P	6	CR:61.1% TR: 72.2%
Li et al. (68)	China	RCS	19	1 g	CTX:750 mg, P	12	CR:21.1% TR: 78.9%

AZA, Azathioprine; CR, Complete remission; CS, Control study; CYC, Cyclophosphamide; d, Day; HDCyC, High-dose cyclophosphamide; LDCyC, Low-dose cyclophosphamide; m, Month; MMF, Mycophenolate mofetil; N, The number of patients with available data for analysis; NM, Not mentioned; P, Glucocorticoids; PR, Partial remission; RCS, Retrospective case series; RCT, Randomized controlled trial; RTX, Rituximab; TR, Total remission.

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use in follicular lymphoma. Edwards et al. were pioneers in demonstrating its effectiveness and safety in the treatment of rheumatoid arthritis (RA) (36). In a randomized, double-blind, controlled study involving patients with active rheumatoid arthritis (RA) receiving methotrexate (MTX) treatment, a single course of two infusions of RTX significantly improved disease symptoms at both week 24 and week 48 compared to MTX alone or in combination with cyclophosphamide (CYC) or continuous MTX therapy (37). Additionally, Leandro et al. were the first to publish an open-label study involving six female patients with refractory SLE who were resistant to standard immunosuppressive therapy. This study provided preliminary evidence for the safety and efficacy of RTX in the treatment of refractory SLE (38). To date, the clinical usage and dosing of RTX vary by condition. For lymphoma and pediatric autoimmune diseases, the standard dosage is 375 mg/m3 for 4 weeks. For conditions such as SLE and RA, the dosage often increases to 100 mg administered twice over 2 weeks. The dosing of RTX for treating SLE and LN typically falls between these two regimens (69). However, a multicenter systematic review involving 1,370 patients with systemic autoimmune diseases treated with biologics found that rituximab (RTX) treatment for refractory SLE might be more effective when using the lymphoma treatment regimen (375 mg/m3 for 4 weeks) compared to the two-week doses of two 100 mg (70). However, based solely on the aforementioned review, it is challenging to draw definitive conclusions regarding the relative efficacy of the two regimens. Catapano et al. used both RTX dosing regimens to treat refractory SLE and, although not in a formal comparative setting, did not find significant differences in B cell depletion levels, clinical outcomes, or adverse effects (50). Therefore, the two-week doses of two 100 mg might be more convenient and could become the preferred treatment regimen for patients with SLE and LN.

In clinical practice, RTX is rarely used alone; it is often combined with glucocorticoids or with both glucocorticoids and immunosuppressants. When used in combination therapy, the dose of glucocorticoids is gradually reduced as clinical symptoms improve, significantly enhancing efficacy and reducing the risks of infection, bone marrow suppression, liver function impairment, and secondary malignancies (71, 72).

Efficacy and safety of RTX

To date, extensive clinical research has been conducted on rituximab, and its efficacy is still uncertain. Both randomized controlled trials of rituximab for SLE patients, the EXPLORER and LUNAR studies, failed to meet their primary endpoints. In the EXPLORER study, 257 patients with moderate to severe non-renal SLE were randomly assigned to receive either RTX or placebo treatment. RTX was administered at a dose of 1,000 mg at weeks 0, 2, 24, and 26, against a background treatment of azathioprine (AZA), methotrexate (MTX), or mycophenolic acid (MPA). At week 52, there was no significant difference between the treatment group and the placebo group in terms of the primary endpoint (73). In the LUNAR study, 144 patients with class III or IV lupus nephritis (LN) receiving mycophenolate mofetil (MPA) treatment were randomly assigned to receive either a placebo or rituximab (RTX) treatment. Similarly, in this study, RTX failed to achieve the primary endpoint, and there was no significant difference between the placebo and treatment groups in the proportion of patients achieving complete or partial renal remission (59).

However, numerous clinical trials and case reports have observed significant efficacy and reliable safety of RTX in patients with SLE and LN (74). In the study by Yi et al., patients in the RTX group had lower 24-h urinary protein and SLEDAI scores and a significantly higher complete remission rate than those in the CTX group (75). Looney et al. concluded that RTX relieved symptoms in most patients with refractory severe SLE (76). Ramos et al. found that RTX significantly improved symptoms in 91% of patients with refractory and recurrent LN (77). In addition, several systematic evaluations and network meta-analyses have analyzed the efficacy and safety of RTX in the treatment of LN (78–80), suggesting that RTX has significant clinical efficacy and good safety, making it a promising therapy for the treatment of SLE and LN, particularly for refractory severe SLE and refractory LN. Jens Vikse et al. retrospectively analyzed the clinical data of 70 patients with systemic inflammatory and autoimmune diseases and treated with long-term rituximab (≥16 years). In their study, infections and persistent dysgammaglobulinemia were the most common adverse events, occurring in 34.3 and 25.7%, respectively. End organ damage occurred in two patients, and no opportunistic infections were observed. Three patients died of lethal infection during the observational period. They concluded that long-term rituximab treatment is relatively well tolerated, and that no cumulative side effects were observed (81). In 2012, the American College of Rheumatology recommended RTX as a second-line treatment for refractory class III and IV LN. Additionally, the Chinese guidelines for the diagnosis and treatment of lupus nephritis indicate that for refractory or frequently relapsing LN, RTX can be used in combination therapy (71, 72). Furthermore, the 2019 management recommendations for LN, jointly developed by the European League Against Rheumatism (EULAR) and the European Renal Association-European Dialysis and Transplant Association, proposed that for non-responsive and refractory LN, RTX can also be used either as monotherapy or as an adjunct to mycophenolate mofetil (MMF), mycophenolic acid (MPA), or cyclophosphamide (CYC) (13).

The clinical efficacy of rituximab in treating systemic lupus erythematosus (SLE) demonstrates considerable variability, probably attributable to the following factors: elevated levels of BAFF (B-cell activating factor), B-cell reconstitution and the disease-specific high degree of heterogeneity in lupus. B-cell reconstitution after the infusion of rituximab is associated with increased BAFF levels. Elevated BAFF promote autoreactive B-cell proliferation and survival (82). Additionally, SLE is a highly heterogeneous disease with various pathogenic mechanisms, including autoreactive B-cells, alterations in TLR receptor function, differences in the IFN-α pathway, T-cell dysfunction, etc. (83). This heterogeneity may result in diverse patient responses to treatment, suggesting that a single therapeutic approach may not address all underlying mechanisms.

To overcome these challenges, potential strategies include combination therapy and sequential treatment. The potential of combining anti-B cell and anti-BAFF therapies should be further explored. Besides, in terms of sequential treatment, clinical studies of belimumab administration followed by RTX or RTX administration followed by belimumab are currently under investigation (84, 85).

Conclusion

As an anti-CD20 monoclonal antibody B cell depleting agent, rituximab (RTX) has shown promising efficacy in several retrospective and open-label studies. Despite this, continued monitoring of RTX's

significant biological effects is necessary to evaluate long-term clinical benefits and risks. Considering the pathogenic significance of the B cell family in SLE and LN, targeting B cells and plasma cells presents a highly attractive therapeutic approach for SLE and LN. Understanding and advancing B cell biology in SLE and LN is crucial. In addition to the specific targeting of B cell surface antigens by RTX, treatment failures in SLE and LN have also been noted. This has driven interest in alternative targets for B cell activation, such as B lymphocyte stimulator (BlyS) and B cell activating factor (BAFF), which are expected to become focal points for future research. Additionally, selective targeting of B cell therapies will play a pivotal role in the personalized treatment management of SLE and LN patients. Moving forward, further work is needed to elucidate the full potential of B cell depletion strategies through drugs like RTX in the clinical setting.

Author contributions

SM: Data curation, Formal analysis, Investigation, Software, Writing – original draft. YL: Data curation, Methodology, Writing – original draft. JH: Methodology, Software, Validation, Writing – review & editing. LL: Methodology, Software, Supervision, 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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BMP-4 and fetuin A in systemic sclerosis patients with or without calcinosis

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Introduction: Systemic sclerosis (SSc) is a connective tissue disease at the interface between inflammation and autoimmunity progressively leading to diffuse microvascular and fibrotic involvement of the skin and of multiple internal organs. Approximately, 20-40% of SSc patients suffer from cutaneous calcinosis, a debilitating manifestation due to calcium salt deposition in soft connective tissues, causing pain, ulceration, infection, and deformities, responsible of severe functional limitations. Pathomechanisms are poorly understood as well as markers/molecules capable to predict the risk of patients to develop calcinosis.

Methods: An observational study was performed in 51 female patients, 25 with and 26 without calcinosis to compare clinical and laboratory parameters and to evaluate pro- and anti-calcifying circulating markers and the *in vitro* serum calcification potential (T50). Moreover, calcinosis samples were analyzed to characterize their mineral composition.

Results and discussion: Data demonstrate statistically significant differences in the prevalence of clinical manifestations and ACA and Scl70 autoantibodies in SSc patient with calcinosis compared to those without calcinosis. In SSc patients with calcinosis, serum levels of BMP-4 are higher, fetuin A might be regarded as a potential circulating prognostic marker and a negative correlation was observed between T50 and the global score of clinical manifestations, suggesting a potential predictive role of pro- and anti-calcifying molecules in SSc patients. Furthermore, calcinosis samples were characterized by the co-existence of phosphate and carbonate minerals with different stability and solubility. Further investigations on circulating markers in larger patient cohorts, especially at the early stages and throughout the natural course of the disease, may clarify their pathogenetic role in the SSc-related cutaneous calcinosis.

KEYWORDS

bone morphogenic protein, calcification, fetuin A, scleroderma, serum calcification propensity

Introduction

Systemic sclerosis (SSc) is a connective tissue disease at the interface between inflammation and autoimmunity progressively leading to fibrosis of the skin and of multiple internal organs (1).

Calcinosis is a clinical complications occurring in 20-40% of SSc patients (2–5) being characterized by calcium salt deposition in skin and in subcutaneous tissues causing pain, ulceration, infection, and deformities causing severe functional limitations (6). The underlying mechanisms are still elusive, even if chronic inflammation, trauma, and vascular damage have been hypothesized (7). In the last years studies performed in unrelated diseases (*e.g.*, vascular calcification, chronic kidney disease, ectopic mineralization on genetic basis) demonstrated that pathological calcification is a complex process in which molecular (*e.g.*, fetuin A, osteopontin and BMP-2, -4) and/or different cellular mediators (*e.g.*, smooth muscle cells, circulating vascular progenitor cells and fibroblasts) may play a key role (8, 9).

However, the role of calcification regulatory proteins has been poorly investigated in the pathogenesis of SSc-related calcinosis (10).

Furthermore, the mineral phase of calcinosis has not yet been clearly defined due to contradictory results either describing calcinotic particles preferentially composed of hydroxyapatite (HAP), or of carbonate apatite (11, 12), that has a different stability and solubility compared to HAP.

Therefore, the present observational study aimed to compare clinical and laboratory parameters in SSc patients with (C group) or without (NC group) cutaneous calcinosis. Moreover, circulating markers and *in vitro* serum potential of calcification were evaluated in the two subgroups of patients. Finally, in a few SSc patients, the composition of calcium deposits was also investigated.

Materials and methods

Patients and clinical data

The present observational case control study included 25 SSc patients with cutaneous calcinosis (C group) and 26 without (NC group). All patients gave written informed consent, and the study was approved by the local Institutional Ethical Committee (protocol no. 275/16). The study was performed in adherence with the Declaration of Helsinki. Patients classified according the ACR/EULAR 2013 SSc criteria (13) were consecutively recruited at the Scleroderma Unit of the University-based Scleroderma Unit of the Policlinico of Modena, Italy, from March 2023 to October 2023. Moreover, to avoid comorbidities possibly involving the calcification process, SSc patients with diabetes, osteometabolic diseases, skin cancer and chronic comorbidities (*i.e.*, thyroid-parathyroid pathology, diabetes mellitus, alterations in phosphocalcic metabolism, chronic renal failure) were excluded.

The presence of cutaneous calcification was invariably confirmed by physical examination, prior imaging, or both (6).Baseline information including demographic data, clinical signs and symptoms, organ involvement, capillaroscopic pattern and current therapies were collected.

In all patients of group C calcium deposits were classified according to their shape and consistency on palpation (14). Digital calcinotic samples were obtained from the fingers of 7/25 patients (age range 48y–72y) using a scalpel in sterile conditions, according to standard procedures applied to lower patients' pain and to partially restore mobility (15).

Routine laboratory analyses, including serum levels of calcium, phosphorus, magnesium, alkaline phosphatase (ALP), vitamin D, parathyroid hormone (PTH), and autoantibodies were performed in both patients' subgroups.

A global score was specifically created to evaluate the total burden of clinical manifestations.

Clinical manifestations were evaluated in each patient as a "global score" (GS) resulting for the sum of the occurrence (+1) or absence (0) of telangiectasias, digital ulcers, diffuse subset, interstitial lung disease, PAH, artery hypertension, coronary artery disease, gastro-esophageal reflux disease, esophageal dysfunction, intestinal involvement, osteoporosis, renal involvement.

Measurement of serum calcification propensity

Calcium and phosphate can combine *in vivo* to form amorphous primary calciprotein particles (CPP1), which can subsequently transform in secondary CPP (CPP2) containing crystalline calcium phosphate. The time required for half of the CPP1 to convert into CPP2 is known as T50, which reflects the capacity of serum to resist crystallization of calcium and phosphate (16).

T50 and CPP2 were measured in the serum samples collected from all SSc patients through venipuncture at the same moment of sampling for routine blood chemistry. The T50 and CPP2 were performed at Calciscon (AG, Nidau, Switzerland) according to Pasch and colleagues (16).

Enzyme-linked immunosorbent assay

Serum levels of BMP-2 (ab277085), BMP-4 (ab231930), fetuin-A (ab269372), osteopontin (OPN) (ab269374), osteoprotegerin (OPG) (ab189580) and SPARC/osteonectin (ab220654) were assessed in a blinded manner using commercial kit according to manufacturer's instructions.

Scanning electron microscopy with energy-dispersive spectroscopy

Samples removed from the fingers of patients were firstly observed by SEM (Nova NanoSEM 450, FEI, Hillsboro, OR, USA) with a backscattered electron signal (BSE) to examine the morphology and the distribution of calcified areas. The analysis of

the elements present in the calcinosis samples was performed by EDS, as already described (17).

X-ray diffraction

The mineral phase present in the samples was evaluated by XRD. Spectra were recorded using a Bruker AXS D8 Advance spectrometer (Bruker AXS, MA, United States) with a step size of 0.02, at a scanning rate of 0.1–1.2 s in a 2θ range from 10 to 70. XRD pattern was analyzed using Origin Pro 2020 software (OriginLab).

Raman spectroscopy

Raman spectra were collected with a micro-Raman system (LabRam HR Evolution, HORIBA Scientifc) using 785-nm laser excitation wavelength, 100 × objective (Olympus, numerical aperture, 0.9). The power of the incident laser was 100 mW. The typical spectral resolution was 4 cm^{-1} and 300 scans were applied. Spectra were corrected at baseline to suppress the luminescence background.

The instrument was daily aligned, and intensity calibrated using automated procedures implemented in the instrument start-up process.

The background correction used to reduce fluorescence signals was removed in LabSpec 6 (HORIBA Scientifc) using baseline correction by fitting and subtracting a polynomial function of the 4^{th} order to each spectrum.

The reference spectra of inorganic components were obtained from both literature data (18, 19) and the RRUFF database (https://rruff.info/).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Continuous variables with Gaussian distribution or with skewed distribution are presented as mean ± standard deviation and as median and interquartile range, respectively. Shapiro-Wilk test was applied to evaluate normal distribution of data. The variable comparisons were performed using parametric or no parametric tests for variables with Gaussian or skewed distribution, respectively. The chi-square test was used to compare categorical variables. Pearson correlation analysis was used to assess correlations between variables, and significant correlations were analyzed using linear regression. P values < 0.05 were considered significant.

Results

Patient characteristics

The demographic, clinical, and serological characteristics of patients with (C group) or without (NC group) calcinosis are shown in Table 1. No statistically significant differences in the epidemiological characteristics were observed among groups, with the exception of longer disease duration in the C group (Table 1). Some disease manifestations were recorded at higher prevalence in patients with calcinosis compared to those in the NC group, such as telangiectasias, systemic and artery pulmonary hypertension, gastro-oesophageal reflux, intestinal involvement (Table 1).

In terms of antibody profiles, the percentage of SSc patients positive for anti-centromere (ACA) and anti-topoisomerase (Scl70) autoantibodies differed significantly between NC and C groups. Specifically, a higher percentage of patients in the C group were positive for ACA compared to the NC group (72% *vs* 35%, p=0.0003), contrarily to that observed for serum anti-Scl70 (16% *vs* 54%, p=0.0047). No significant differences between the two groups were observed for anti-nuclear, anti-RNA polymerase III, and SSA-SSB antibodies.

Interesting, no significant differences were found regarding both the capillaroscopic pattern and features.

The GS was significantly higher in C than in the NC group (p= 0.0064) (Table 1).

Circulating promoters and inhibitors of calcinosis

The serum levels of molecules known to favor (*i.e.*, BMP-2, BMP-4 and SPARC) or to inhibit (*i.e.*, OPN, fetuin A and OPG) the calcification process have been investigated in the two groups of patients.

Figure 1 shows that serum levels of both pro- and anti-calcified molecules are not significantly different between NC and C groups, except for BMP-4.

A bivariate correlation analysis was performed between these markers and the GS within the same group of patients. Fetuin A inversely correlated with GS in the C group (Table 2).

Serum calcification propensity

Figures 2A, B shows that T50 value and CPP2 size are similar between the two groups. These two parameters negatively correlated with each other in both groups ($r_{NC\ group}$= -0.601, p= 0.001 and $r_{C\ group}$= -0.479, p= 0.015). A linear relationship exists between the two variables in both patient groups (Figure 2C). Whereas no correlation was found between T50 and GS in the NC patient group, a significant negative correlation was observed in the C patient group, showing a linear relationship (Figure 2D). CPP2 size did not correlate with the GS in both NC and C patient groups ($r_{NC\ group}$= -0.236, p= 0.246 and $r_{C\ group}$= 0.384, p= 0.058).

It is known that T50 and CPP2 can be influenced by several factors such as fetuin-A, phosphate, calcium, magnesium and albumin (16). Correlation between T50 value or CPP2 size with markers of mineral metabolism (*i.e.*, Ca, P, Mg, ALP and PTH) and with inhibitors of mineralization (*i.e.*, serum albumin, fetuin A and OPG) were evaluated within the same group of patients. No correlations with T50 were found in NC and in the C group (data

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TABLE 1 Clinical and laboratory characteristics of SSc patients (pt) with (C group) or without calcinosis (NC group).

	NC group (26 pt)	C group (25 pt)	p value
Age (years), mean ± SD	63 ± 10	67 ± 13	n.s.
Female, n (%)	26 (100%)	25 (100%)	n.s.
Body mass index, mean ± SD	23 ± 2.9	21.6 ± 3.0	n.s.
Smoke history, n (%)	10 (38%)	8 (32%)	n.s.
SSc subtype			
-Limited (lcSSc)	18 (69%)	21 (84%)	n.s.
-Diffuse (dcSSc)	8 (31%)	4 (16%)	n.s.
Duration of Raynaud's phenomenon (years), mean ± SD	18 ± 11	22 ± 13	n.s.
Disease duration at recruitment (years), mean ± SD	13 ± 8.0	20 ± 10	**0.0093**
Telangiectasias, n (%)	13 (50%)	23 (92%)	**0.0010**
Interstitial lung disease, n (%)	14 (54%)	13 (52%)	n.s.
Pulmonary arterial hypertension, n (%)	0 (0%)	5 (20%)	**0.0001**
Artery hypertension, n (%)	7 (27%)	14 (56%)	**0.0349**
Coronary artery disease, n (%)	0 (0%)	1 (0.4%)	n.s.
Hyperlipidemia, n (%)	15 (58%)	20 (80%)	n.s.
Gastro-oesophageal reflux disease, n (%)	11 (42%)	22 (88%)	**0.0006**
Osteoporosis, n (%)	11 (42%)	13 (52%)	n.s.
Oesophageal dysfunction, n (%)	12 (46%)	17 (68%)	n.s.
Intestinal involvement, n (%)	4 (15%)	10 (40%)	**0.0489**
Digital ulcers, n (%)	10 (38%)	17 (68%)	n.s.
Hyperlipidemia, n (%)	15 (58%)	20 (80%)	n.s.
Serum calcium (mg/dL) (8.5-10.5 mg/dL), mean ± SD	9.26 ± 0.27	9.28 ± 0.52	n.s.
Serum phosphate (mg/dL) (2.5-5.1 mg/dL), mean ± SD	3.83 ± 0.52	3.48 ± 0.43	n.s.
Serum Mg (mg/dL) (1,6-2,6 mg/dl), median (IQR)	1.90 (1.80 – 2.00)	1.90 (1.80-2.05)	n.s.
25-hydroxyvitamin D (ng/mL), mean ± SD	37.5 ± 10.4	36.3 ± 13.6	n.s.
Parathyroid hormone (pg/mL) (6.5 - 36.8 pg/mL) median (IQR)	30.7 (23.2–46.7)	30.60 (25.2–43.2)	n.s.
Alkaline phosphatase unit/L (38 - 126 unit/L), median (IQR)	67.0 (54.5–73.0)	62.0 (46.5–73.0)	n.s.
HDL (mg/dL) > 43, mean ± SD	62.0 ± 14.1	61.0 ± 16.0	n.s.
LDL (mg/dL) < 115, mean ± SD	120 ± 21.6	123 ± 33	n.s.
Albumin g/dL (3.5 - 5.0 g/dL), mean ± SD	4.01 ± 0.23	3.94 ± 0.41	n.s.
C-reactive protein mg/dL (0 - 0.7 mg/dL), median (IQR)	0.30 (0.27–0.52)	0.30 (0.2-0.85)	n.s.

(Continued)

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TABLE 1 Continued

	NC group (26 pt)	C group (25 pt)	p value
SSc subtype			
eGFR (mL/min/1.73 m2) < 60 n (%)	5 (19%)	2 (8%)	n.s.
Global score, mean ± SD	3.85 ± 2.20	5.64 ± 2.28	**0.0064**
Treatment:			
Vitamin D use, n (%)	26 (100%)	25 (100%)	n.s.
Phosphate binder use, n (%)	0 (0%)	0 (0%)	n.s.
Lipid-lowering medication use, n (%)	7 (27%)	11 (44%)	n.s.
Bisphosphonate use, n (%)	2 (8%)	4 (16%)	n.s.
Denosumab use, n (%)	2 (8%)	5 (20%)	n.s.

Bold significant values. n.s., no significant.

not shown). CPP2 radius did not correlate with any molecule analyzed in the NC group (data not shown); on the contrary, CPP2 correlated negatively with fetuin A (r= -0.428, p= 0.03) and serum albumin (r= - 0.396, p= 0.04), but positively with OPG (r= 0.399, p= 0.04) in the C group. A linear relationship was observed in all cases (Figures 3A–C).

Analysis of calcinosis samples

Calcinosis samples were mainly characterized by dense and compact material except for the sample #4, that appears as a toothpaste-like fluid. The size of crystals was very heterogeneous depending on the samples (Figure 4).

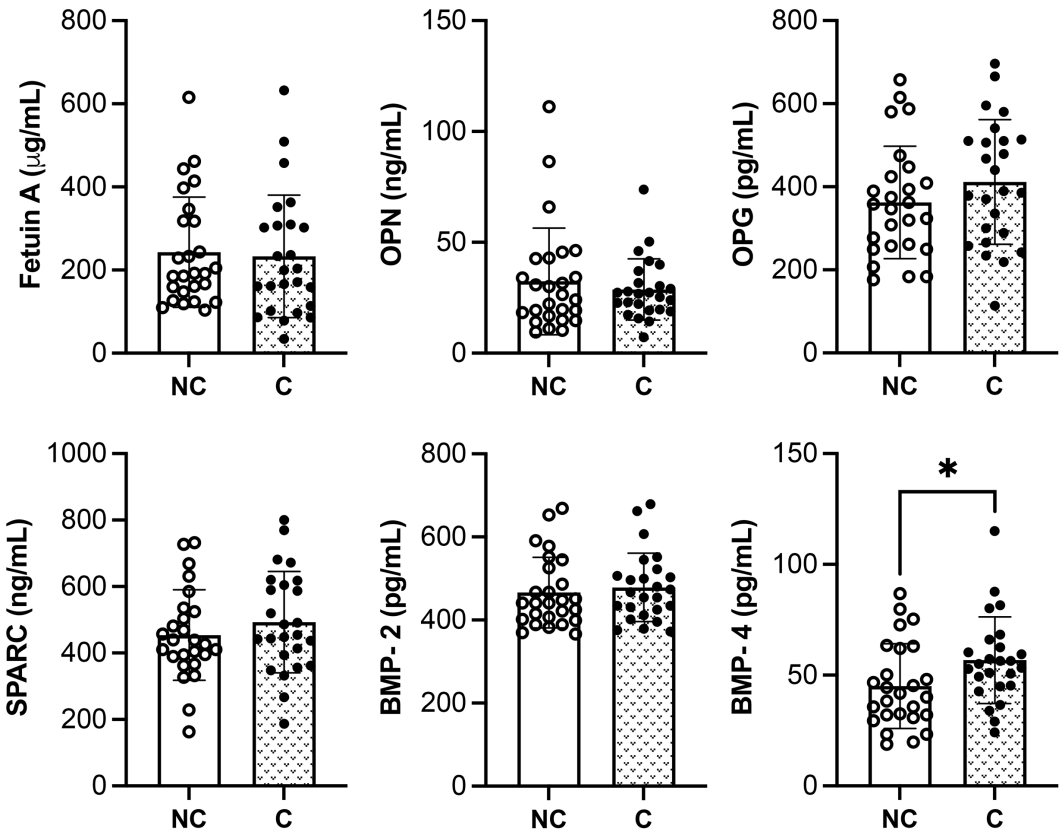

FIGURE 1
Quantitative analysis of pro- and anti-calcified molecules in SSc patients with (C) or without calcinosis (NC). The results are shown as mean ± standard deviation (n= 26 NC patients, n= 25 C patients). *p<0.05. OPN, osteopontin; OPG, osteoprotegerin; SPARC, osteonectin; BMP, bone morphogenetic protein.

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TABLE 2 Correlation of clinical manifestations (global score) with serum anti- and pro-calcifying molecules in SSc patients with (C) and without (NC) calcinosis.

Variables	Correlation with global score			
	NC group		C group	
	r	P value	*r*	P value
Fetuin A	0.021	0.92	**-0.400**	**0.04**
BMP-2	-0.034	0.87	-0.026	0.90
BMP-4	-0.172	0.40	0.017	0.94
OPN	0.182	0.42	0.041	0.85
OPG	0.180	0.46	0.227	0.27
SPARC	-0.176	0.39	0.024	0.91

Pearson correlation analysis was used. BMP, bone morphogenetic protein; OPN, osteopontin; OPG, osteoprotegerin; SPARC, osteonectin.
Significant correlation is in bold.

Small spherical mineral particles were concentrated (#3) or spread (#4) into the samples, whereas in other specimens (*i.e.*, #1, #2, #5, #6 and #7) calcification was in form of small spots with a tendency to fuse into large mineralized areas with a jagged surface.

Element analyses were performed by EDS on the entire surface of the samples. P and Ca were not detected in the absence of mineralization (Figure 4, last panel). In calcified areas, high peaks of P and Ca were always observed, whereas ions such as Na, Cl, S, and Mg were detected only in some samples (Figure 4), partially or totally substituting ions present in calcium-phosphate minerals. These results indicate that P and Ca deposition is not homogeneous in calcinosis samples.

EDS spectra of calcinotic particles allow to obtain information about the elements present in the sample, but do not provide data on the chemical composition and on the crystals' properties.

By X-ray scattering (XRD), samples #2, #4, #5 and #7 exhibit peaks characteristic of HAP located at 2θ = 25.92° (002), 31.79° (121), 39.73° (310), 46.69° (222), 49.52° (123), 53.19° (004) and of calcite located at 2θ = 29.27 (104), 47.07 (018) and 48.34 (116) (Figure 5A). Three out of seven samples (#1, #3, #6) were characterized by a higher proportion of organic components compared to the inorganic ones, thus making it challenging to accurately identify the mineral phase through XRD analysis. Therefore, these samples underwent analysis by Raman spectroscopy. Figure 5B shows peaks at 430 cm^{-1} and 577 cm^{-1}, corresponding to symmetric bending mode of v_2 PO_4^{3-} and the triply degenerate asymmetric bending vibration of v_4 PO_4^{3-}, respectively. A prominent peak is observed at 959 cm^{-1}, ascribed to v_1 fundamental vibration mode arising from PO_4^{3-} group. Moreover, a peak at 750 cm^{-1} and at 1075 cm^{1-} (corresponding to v_4 and symmetric vibration mode v_1 from carbonate, respectively) are present in all samples, in particular in sample #3. The B-type carbonate presence indicates, therefore, the partial substitution of PO_4^{3-} with CO_3^{2}. CO_3^{2-} carbonate anions can partially substitute also hydroxide ions (OH) (A-type), however A-type, characterized by two peaks at 1106 cm^{1-} and 1018 cm^{1-}, was not detected in any samples. These findings indicate the co-existence of phosphate and carbonate minerals in the same calcinosis sample.

In addition, peaks are observed at 851 cm^{-1} (C–C proline, hydroxyproline), 1003 cm^{-1} (phenylalanine ring breathing); 1250-1350 cm^{-1} (amide III); 1452 cm^{-1} (H–C–H union); 1600-1800 cm^{-1} (C=O stretching, amide I), indicating the presence of organic matrix (20, 21).

Discussion

Calcinosis is a manifestation of SSc characterized by the deposition of calcium minerals in the skin and in subcutaneous tissues (4).

Some Authors report an association between calcinosis either with dcSSc (22) or lcSSc (10), whereas other studies (23), including this one, did not found differences in the prevalence of calcinosis between SSc cutaneous subtypes. To explain these discrepancies, it has been suggested that different results can depend on ethnicity (*e.g.*, Caucasians *vs* African-American) and/or on geographic regions (4, 24). Moreover, as already reported (25), calcinosis occurs after several years (*i.e.*, 7-10 years) from SSc diagnosis.

Regarding internal organ involvement, patients with calcinosis develop more frequently arterial hypertension, gastro-esophageal reflux disease, intestinal involvement, and pulmonary arterial

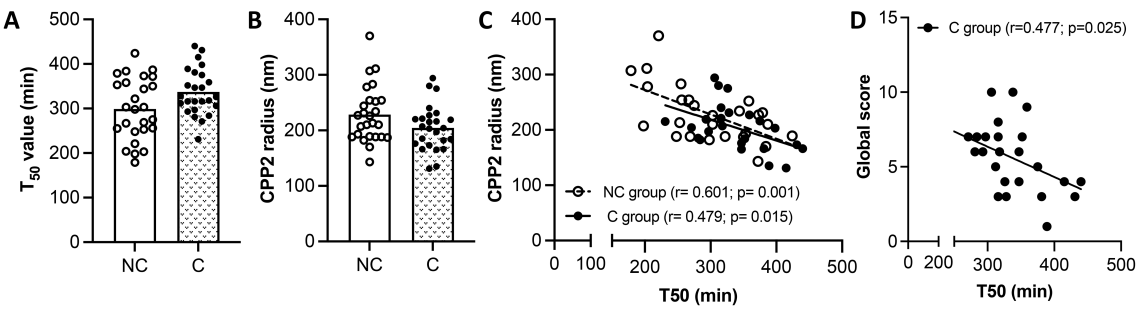

FIGURE 2
Measurement of serum calcification propensity. **(A, B)** Half-maximal time (T50), and secondary calciprotein particles size (CPP2) in SSc patients with (C group) and without (NC group) calcinosis. The results are shown as mean ± standard deviation (n= 26 NC patients, n=25 C patients). **(C)** Linear regression between T50 and CPP2 size in NC and C groups. **(D)** Linear regression between T50 and global score in NC and C patient groups.

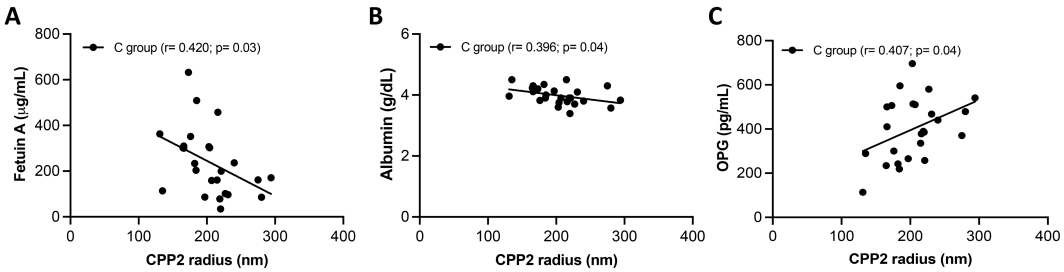

FIGURE 3
Associations between secondary calciprotein particles size (CPP2) and serum inhibitors of calcification. A linear relationship was found between CPP2 size and fetuin A **(A)**, albumin **(B)** and, and osteoprotegerin (OPG) **(C)** in SSc patient with calcinosis (C group).

hypertension (PAH). This observation confirms previous data showing that calcinosis is associated with a more severe disease course (26). Interestingly, PAH is a serious complication of SSc affecting 8-15% of patients (27) and has been related to inflammation and calcification (28). In agreement with data from the literature, our patients show a significant association between PAH and calcinosis. It is worth noting that 20% of our SSc patients in the C group, but none in NC group, were complicated by PAH, although it cannot be excluded a possible contribution of longer disease duration characterizing this patient subgroup. Several studies have underlined some clinical characteristics and serological markers associated with the development of PAH in SSc patients, such as the presence of calcinosis, or telangiectasias, or lcSSc, and/or serum ACA (26, 29–32). In our study, both ACA seropositivity and telangiectasias are significantly more frequent in patients with calcinosis compared to those without.

In several pathological contexts, ectopic calcification has been shown to be associated with a systemic imbalance between pro- and anti-calcifying molecules (33). Since, scattered and sometimes contradictory data are present for SSc patients, we have investigated several circulating calcification-related molecules in both C and NC groups of patients.

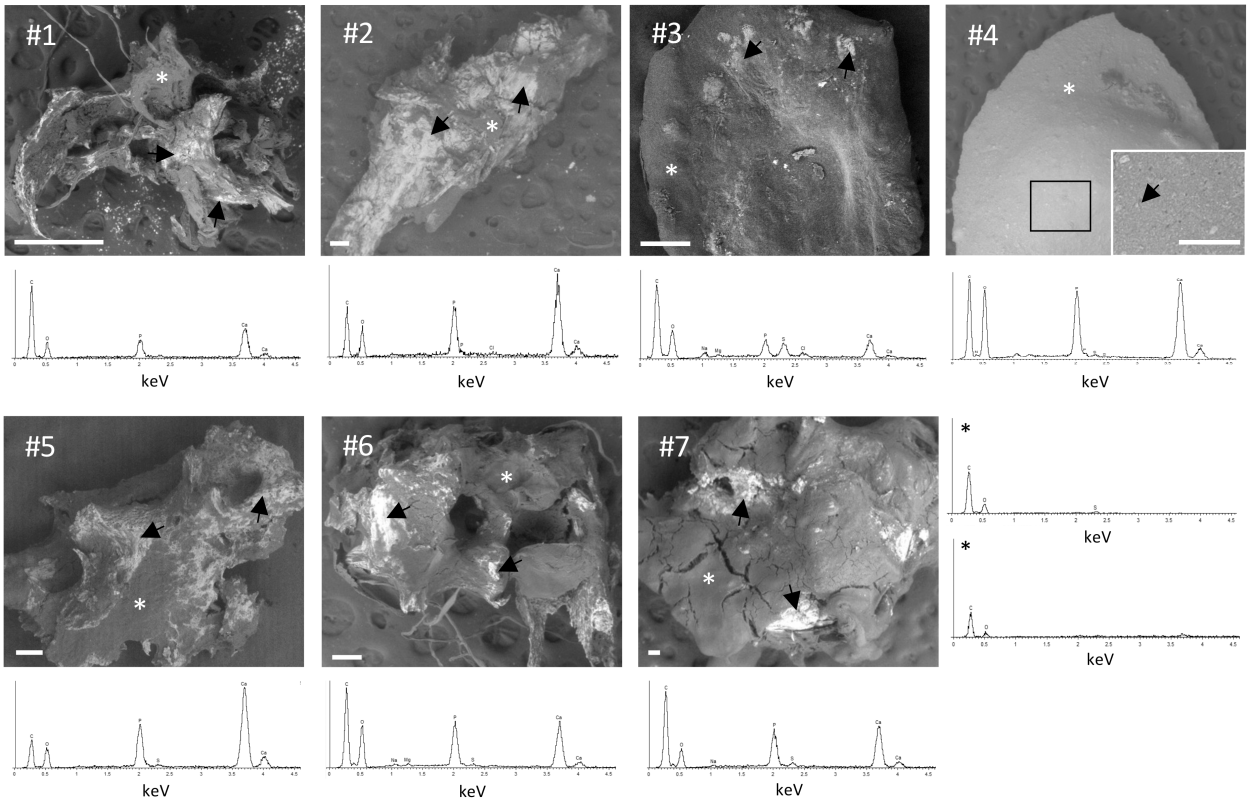

FIGURE 4
Scanning electron microscopy (SEM)/energy dispersive spectroscopy (EDS) analysis of calcinosis samples. Unfixed and unstained calcinosis samples were observed by SEM. Representative EDS spectra obtained in areas marked with an arrow show peaks of P and Ca. Two representative EDS spectra in areas marked with asterisk (*) display the absence of peaks of P and Ca. Scale bar= 100 μm.

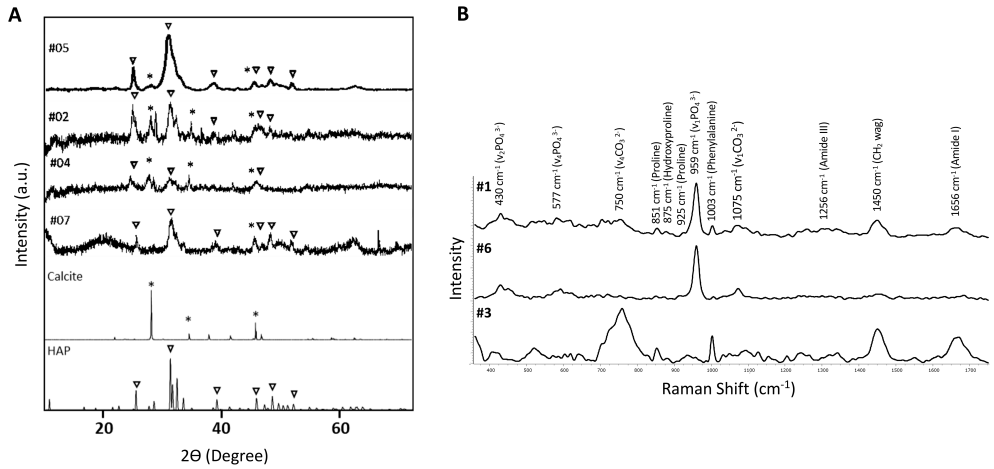

FIGURE 5
X-ray diffraction pattern and Raman spectrum of calcinosis samples. **(A)** X-ray diffraction patterns of minerals in samples #2, #4, #5 and #7. Symbol ∇ corresponds to hydroxyapatite (HAP) and * to calcite. HAP (JCPDS: 96-900-1234) and calcite (JCPDS:96-901-5836) reference patterns are shown at the bottom of the figure. **(B)** RAMAN spectrum of samples #1, #3 and #6.

Fetuin A is a systemic inhibitor of calcification and is known to protect tissues from inflammation-related damage. No difference was observed between C and NC patients, in contrast with data from Belloli and coworkers who found fetuin A to be significantly lower in serum of SSc patients with calcinosis compared to those without calcinosis (10). This discrepancy could be attributed to the smaller randomly selected sample size of patients analyzed in the previous study (10). Moreover, we cannot exclude that the levels of fetuin A might differ between the two groups in the early phases of SSc. For instance, in chronic kidney disease (CKD) patients, it has been suggested that fetuin A may have a protective role being upregulated in the early stages of the disease, whereas severe or prolonged exposure to a pro-inflammatory and/or pro-calcific environment may eventually lead to low levels due to decreased production and/or increased consumption (34). As mentioned above, calcinosis usually occurs after a prolonged disease course (*i.e.*, 7-10 years after SSc diagnosis).

Indeed, several studies have highlighted an association between fetuin A levels and the severity of different pathologic conditions (*e.g.*, chronic obstructive pulmonary disease, coronary calcification; CKD; calcific aortic valve diseases, aortic stiffness) (35–37). Therefore, the inverse correlation found between fetuin A levels and the global score in the C group of patients may suggest that lower fetuin A is associated with an increased number of clinical manifestations.

Osteopontin (OPN), a highly negatively charged secreted protein, regulates several processes including apoptosis, bone metabolism, inflammatory response, fibrosis. It has been also found in sites of ectopic calcification (*e.g.*, renal stones, aortic stenosis) suggesting that it can interact with crystal surfaces, and regulate the mineral process (38). No changes were observed in the present study, although high levels of serum/plasma OPN were previously found in SSc patients or in SSc patients with interstitial lung disease, however, in these studies SSc patients were compared with healthy controls without considering the presence of calcinosis (39–41).

Osteoprotegerin (OPG) is a soluble decoy receptor for the receptor activator of nuclear factor-κB ligand (RANKL), which stimulates osteoclastic bone resorption (42). The literature on OPG levels in SSc is quite heterogeneous. For instance, one study reported higher OPG levels in lcSSc patients compared to healthy subjects (43), while Tayalan Ali and collaborators (44) found similar OPG levels in both SSc patients and healthy subjects. In the present study, OPG levels were similar in C and NC patients. To our knowledge, only one study showed higher OPG levels comparing SSc patients with and without calcinosis (45). Differences between our results and previous findings could be due to the technical procedures utilized, as suggested by the detection range, and/or by the fact that in the previous study males and females were not separated, despite the known sex-dependent variation in OPG levels (higher in females than in males) (45).

SPARC/osteonectin is a protein with collagen type I-binding domain and hydroxyapatite-binding sites (46, 47) that may be involved in both the initial and the progressive stages of the calcification process (48, 49). This molecule was previously found to be significantly increased in the dermis of lcSSc patients with calcinosis compared to those without calcinosis (50) and in the plasma/fibroblasts from SSc patients compared to healthy subjects (51, 52). The discrepancies between our findings showing no differences in the two groups of patients and those reported in the literature may be related to: i) different types of bio-samples analyzed (*i.e.*, dermis/fibroblasts/plasma *vs* serum) and/or on ii) differences in the studied population, *i.e.*, we firstly stratified SSc patients based on the presence or the absence of calcinosis, whereas in other studies, patients with or without calcinosis were selected within one SSc cutaneous subtype (*i.e.*, lcSSc).

Bone morphogenic proteins –2 and -4 (BMP-2 and BMP-4) play a crucial role in vascular calcification by promoting osteogenic activation of vascular cells (53) and have also been implicated in cutaneous calcification (54) and inflammation (55).

Currently, there are no data in the literature on BMP-2 and BMP-4 in SSc patients. Our results indicate no changes for BMP-2, but higher BMP-4 levels in the C group. BMP-4 has been extensively investigated as a promoter of calcification either in the vascular system (53) and in the skin (54). Besides its role as pro-osteogenic signaling inducer, BMP-4 is also related to inflammation and vascular damage, inducing endothelial dysfunction through oxidative stress (56). Since overexpression of BMP-4 in endothelial cells enhances vascular remodeling in pulmonary hypertension (57) it cannot be excluded that higher serum levels of BMP-4 can contribute to the vascular abnormalities frequently observed in the C group (*e.g.*, telangiectasia, PAH and arterial hypertension). This may, in turn, accelerate the need of remodeling and renewal of endothelial cells, potentially leading to a shift towards a pro-osteogenic phenotype (58) and underlines the potential role of BMP-4 in SSc calcinosis.

Under certain conditions, Ca, P and specific serum proteins (*e.g.*, fetuin A and albumin) can aggregate to form amorphous, soluble Ca–P particles (CPP1). These particles can subsequently transform into larger and crystalline calciprotein particles (CPP2) and the half-maximal time of transformation from CPP1 to CPP2 is known as T50. A lower T50 indicates a faster conversion from CPP1 to CPP2, and this has been associated, for example, with cardiovascular mortality, and with progressive aortic stiffening (59, 60). In our study, we did not observe significant differences of T50 and CPP2 between C and NC groups. However, we revealed a correlation between T50 and the global score, as well as between CCP2 and circulating inhibitors of calcification.

Since its introduction in 2012 (16), T50 test has been evaluated in both healthy subjects and in various patient cohorts (*e.g.*, diabetic, hemodialysis, Pseudoxanthoma elasticum patients) (61–65) demonstrating that lower T50 values could predict all-cause and cardiovascular mortality in hemodialyzed patients, in those with CKD or with diabetes. Furthermore, some studies have proposed the use of T50 value as a surrogate marker of arterial calcification or as biomarker of disease severity (65). Interestingly, a negative correlation between T50 and global score was found in the C group. Although further studies with a larger number of SSc patients are necessary to confirm this result, T50 could be an important biomarker in SSc, potentially useful for risk stratification and management of the SSc patients with calcinosis.

Interestingly, in the C group we found that fetuin A and albumin negatively correlated with CCP2, consistently with the inhibitory role of fetuin A (66, 67) and of albumin (68) (69, 70) in preventing the growth and the aggregation of CPP2 thereby hindering their precipitation.

In addition, in the C group, larger CPP2 radius correlated with higher serum OPG. These results are akin to those obtained in hemodialysis patients and in pre-dialysis CKD patients (69, 71). However, further studies are needed to clarify the role of OPG in influencing the size of CPP2.

Ectopic calcification is generally associated with deposition and progressive accumulation of insoluble HAP, however, several studies demonstrated that different type of crystals with a different solubility are present in mineralization sites (72). Present results highlight that calcinosis, even in the same SSc patient, is characterized by a mixture of carbonate and phosphate minerals including calcite and HAP, respectively. It is important to note that analyses were performed on calcinosis samples removed from patients and not on isolated crystals. Since X-ray analysis is not capable to detect crystals that constitute less than 5% of the sample's weight (73), we cannot exclude the additional presence of other minerals further increasing the complexity of calcinosis samples. Previous studies described SSc calcification composed solely of HAP or of B type carbonated apatite (11, 12, 74, 75). These discrepancies can be explained by the fact that calcification is a complex, non-linear process, and that mineral characteristics (*i.e.*, structure, composition and morphology) depend on several factors such as ion concentration, temperature, pH value and location (76–79). Some *in vitro* and *in vivo* studies report that a range of intermediate phosphates (*e.g.*, carbonated apatite, octacalcium phosphate) are formed before transforming into HAP (80–82), even though Thomson and collaborators suggested that hydroxyapatite is formed directly on cholesterol-containing droplets suggesting the absence of intermediate phases (83). The demonstration that different types of mineral deposits are present in SSc calcinosis may lead to the possibility of selecting the most appropriate treatment to improve the solubility of mineral components and to decrease extraosseous calcification. The differences observed among various samples could be due to changes in the composition/organization of the organic matrix. It is well known that aging, oxygen availability, mechanical stress, for example, can modify the characteristics of the extracellular matrix favoring the development/progression of pathological mineralization (84–86). Therefore, a thorough study of both the mineral and the organic components will provide a better understanding of the mechanisms leading to the development of calcinosis.

While the present study indicates some molecules that can correlate with calcinosis in SSc patients, it comes with some limitations such as: i) the relatively small number of patients in both subgroups; ii) the lack of data in the early phases of the disease; iii) the absence of male patients; iv) the difficulty to recruit patients in a fairly homogeneous phase of the disease.

Although these potential biases should be kept in mind to draw general conclusions, in summary, present data allow: i) to find statistically significant differences in the prevalence of clinical manifestations (*i.e.*, telangiectasias, pulmonary arterial hypertension, artery hypertension, gastro-oesophageal reflux disease, intestinal involvement) and laboratory tests (*i.e.*, ACA and Scl70 autoantibodies) in SSc patient with calcinosis compared to those without calcinosis; ii) to highlight higher serum levels of BMP-4 in SSc patients with calcinosis; iii) to identify fetuin A as potential circulating prognostic marker in SSc patients with calcinosis; iv) to underline a higher global score in C patient group; v) to show negative correlation between T50 and global score in patients with calcinosis; vi) to demonstrate the heterogenous composition of the mineral component in calcinosis samples.

The present data may pave the way for future studies on larger cohorts of patients. Moreover, measuring pro- and anti-calcifying molecules at the time of SSc diagnosis and during the clinical long-term follow-up in patients who will do or do not develop calcinosis might clarify their pathogenetic role.

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

The studies involving humans were approved by Local Institutional Ethical Committee (protocol no. 275/16). 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

FDL: Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. DG: Data curation, Funding acquisition, Investigation, Writing – review & editing. SB: Data curation, Investigation, Writing – review & editing. MO: Data curation, Investigation, Writing – review & editing. AS: Data curation, Investigation, Writing – review & editing. MD: Data curation, Investigation, Writing – review & editing. OS: Data curation, Investigation, Writing – review & editing. CF: Data curation, Investigation, Writing – review & editing. FB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, 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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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Advances in the genetics of myasthenia gravis: insights from cutting-edge neuroscience research

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Myasthenia gravis (MG) is an autoimmune disorder involving complex interactions between genetic and environmental factors. Genome-wide association studies (GWAS), transcriptome-wide association studies (TWAS), and other methods have identified multiple novel susceptibility loci and genes, providing crucial insights into the genetic etiology of MG. Moreover, the pivotal roles of epigenetic mechanisms, such as DNA methylation, histone modifications, and non-coding RNAs, in the pathogenesis of MG are gradually being unveiled. This review comprehensively summarizes the latest advances in MG genetic research, focusing on the discovery and validation of susceptibility genes, genetic heterogeneity and subtype-specific genetic factors, gene–environment interactions, epigenetic mechanisms, and progress in genetics-based diagnostic and prognostic biomarkers.

KEYWORDS

myasthenia gravis, genetic susceptibility, genome-wide association study, transcriptome-wide association study, epigenetic mechanisms

1 Introduction

Myasthenia gravis (MG) is an acquired autoimmune disorder characterized by skeletal muscle weakness and abnormal fatigability (1). MG is caused by autoantibodies targeting components of the postsynaptic muscle membrane at the neuromuscular junction. In most cases, antibodies against the acetylcholine receptor (AChR) can be detected, while other targets such as muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4) have been discovered in recent years (2, 3). MG can be classified based on the location of affected muscles (e.g., ocular or generalized), age at symptom onset, and autoantibody profile. These criteria are crucial for optimizing the management and treatment of MG patients (4). In patients with anti-AChR antibodies, thymic abnormalities, immunoregulatory defects, and sex hormones are thought to play important roles. Genetic susceptibility may also influence disease occurrence (5, 6).

GWAS have identified multiple MG susceptibility loci, mainly involving immune-related genes such as human leukocyte antigen (HLA) and PTPN22 (7, 8). Additionally, epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNAs, are gradually being revealed to play a role in the pathogenesis of MG (3).

2 Discovery and validation of MG susceptibility genes

In recent years, with the rapid development of molecular genetics and genomics technologies, the understanding of MG susceptibility genes has deepened. GWAS, TWAS, and other methods have been successively applied to MG genetic research, uncovering multiple novel susceptibility loci and genes, providing important clues for elucidating the genetic etiology of MG (9–12).

2.1 GWAS reveals novel MG susceptibility gene loci

A recent large-sample GWAS involving 1,873 AChR antibody-positive MG patients and 36,370 healthy controls identified 10 loci significantly associated with MG, including the previously reported PTPN22, TNFRSF11A, and HLA regions, as well as newly discovered loci such as 10p14 and 11q21 (9). Another GWAS meta-analysis covering 1,401 MG patients and 3,508 controls further confirmed the association of the TNFRSF11A gene and revealed the pathogenic role of the AGRN gene through gene functional enrichment analysis (12).

Additionally, some studies have attempted to explore the genetic heterogeneity of different MG subtypes. A retrospective cohort study conducted in North America, which included 1,032 AChR antibody-positive MG patients, found differences in genetic risk factors between early-onset MG (EOMG) and late-onset MG (LOMG) (11). A study by Korean researchers focused on ocular MG and GWAS results suggested that the PTPN22 and HLA-DQA1 loci were specifically associated with this subtype (13). These studies indicate that different clinical phenotypes of MG may have distinct genetic foundations.

While GWAS has successfully identified these genomic risk loci, understanding their functional implications requires additional approaches. Transcriptome-wide association studies (TWAS) complement GWAS findings by directly examining gene expression patterns, helping to bridge the gap between genetic variation and disease mechanisms (9, 12).

2.2 TWAS reveals the association of acetylcholine receptor subunit genes with MG

A TWAS study involving 1,873 MG patients utilized gene expression profiles from skeletal muscle, whole blood, and tibial nerve to identify expression quantitative trait loci (eQTLs) significantly associated with MG in the nicotinic cholinergic receptor α1 subunit (CHRNA1) and β1 subunit (CHRNB1) genes, respectively (14). Considering that AChR is the primary autoimmune target in MG, this result suggests that abnormal expression of genes encoding AChR subunits may be an important link in the pathogenesis of MG.

Concurrently, some studies have focused on transcriptomic changes in MG thymic tissue. By constructing a single-cell transcriptional atlas of MG-associated thymomas, researchers discovered the ectopic expression of neuromuscular junction molecules in a subset of medullary thymic epithelial cells, speculating that these abnormally expressing cell subsets may play a key role in the pathogenesis of MG by activating autoreactive T and B cells (7). Another study integrating thymoma tissue and normal thymus transcriptome data identified key transcription factors and signaling pathways such as BCL2 and CXCL13, which may be core mechanisms driving the development of MG thymomas (14). Recent single-cell transcriptomics studies have provided unprecedented insights into MG pathogenesis. Zhong et al. characterized the peripheral immune landscape in myasthenic crisis using single-cell RNA sequencing, revealing distinct immune cell states associated with disease severity (15). Additionally, Liu et al. identified novel therapeutic targets through single-cell analysis of immune cell subsets in pediatric MG (16), while Tian et al. demonstrated how B cell lineage reconstitution underlies CAR-T cell therapeutic efficacy in refractory MG patients (17) (Figure 1).

2.3 Functional annotation and bioinformatics analysis of MG-related genes

Pathway enrichment analysis shows that risk genes discovered by MG GWAS are mainly involved in immune-related signaling pathways such as T cell receptor, tumor necrosis factor, and interleukin-17 (18–20). Protein–protein interaction network analysis reveals the important role of transcription factors such as MYC and STAT1 in regulating core MG pathogenic genes (20) (Figure 2).

Furthermore, a study integrating GWAS, TWAS, Mendelian randomization, and colocalization analysis identified 4 genes, including CTSH and CD226, and 3 proteins significantly associated with MG, validating their pathogenicity and suggesting that CTSH expression in Th2 cells is closely related to MG risk (14). A transcription factor-miRNA-gene feed-forward loop network constructed using bioinformatics algorithms predicts potential biomarkers and novel drug targets for MG from the perspective of epigenetic regulation (21).

Despite significant progress in the study of MG susceptibility genes, many challenges remain. The contribution of discovered genetic variants to the disease is limited and insufficient to fully elucidate the genetic risk of MG. The pathogenic roles of rare variants and structural variants warrant further investigation. There is an urgent need for large-sample, multi-center studies across different races and regions. Integrating multi-omics data to construct more precise genetic risk prediction models is an emerging trend.

3 Research on MG genetic heterogeneity and subtype-specific genetic factors

Different MG subtypes exhibit significant clinical heterogeneity. EOMG, LOMG, thymoma-associated MG, and anti-MuSK antibody-positive MG each have distinct characteristics, suggesting that their pathogenesis may involve different genetic mechanisms. In recent years, researchers have explored various MG subtypes from a genetic perspective, discovering some subtype-specific genetic variants and risk loci, providing important clues for unveiling the genetic basis of MG heterogeneity.

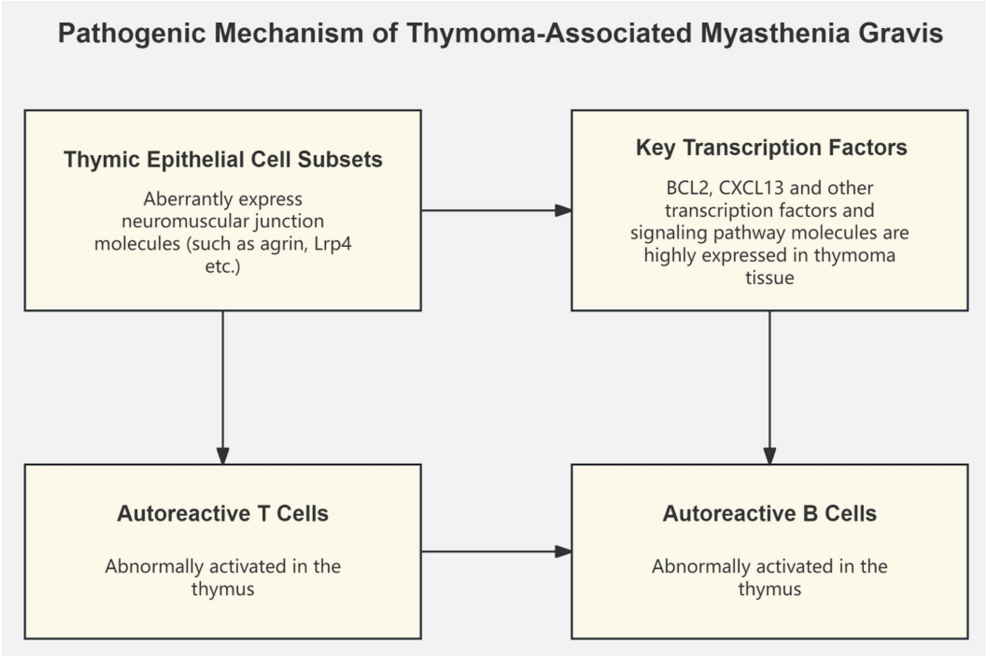

FIGURE 1
Pathogenic mechanism of thymoma-associated myasthenia gravis.

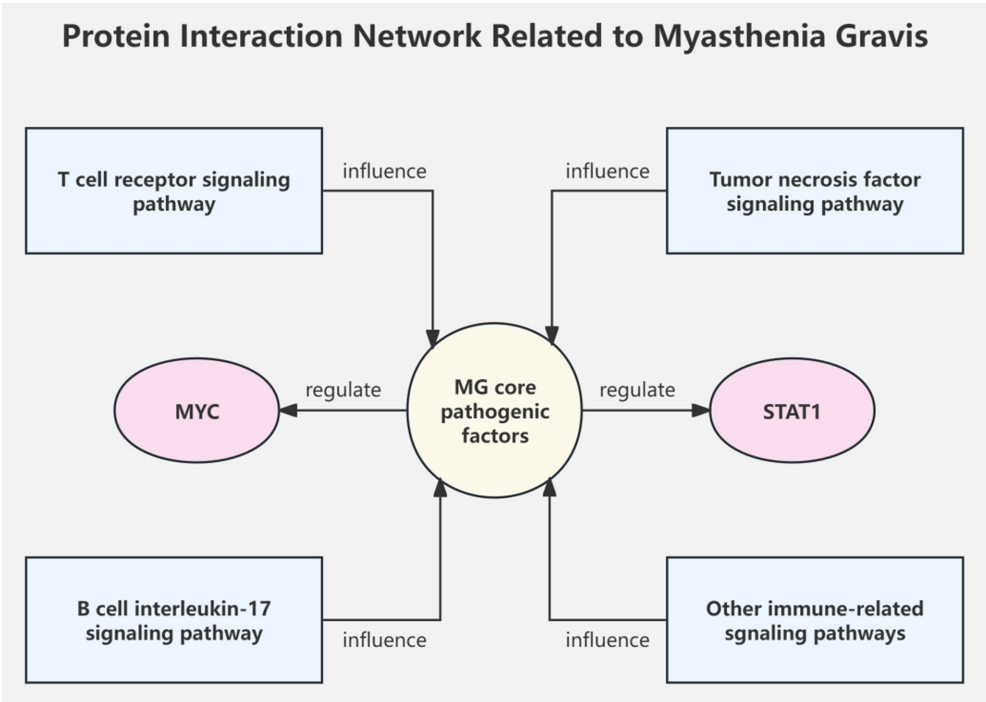

FIGURE 2
Protein interaction network related to myasthenia gravis.

3.1 Differences in genetic risk factors between early-onset and late-onset myasthenia gravis

EOMG and LOMG exhibit significant differences in age of onset, clinical features, autoantibody profiles, and other aspects, suggesting that their genetic backgrounds may not be consistent. A next-generation sequencing study analyzed the human leukocyte antigen (HLA) genes of EOMG and LOMG patients in Italian, Norwegian, and Swedish populations, finding that the HLA-B*08:01 allele was the primary risk factor for EOMG patients, while the HLA-DRB1*15:01 allele was mainly associated with LOMG patients,

indicating clear differences in immunogenetic susceptibility between the two subtypes (22). A study in the Spanish population found that HLA-DQB1*03:01 was a risk factor for EOMG, especially in female AChR antibody-positive patients with thymic hyperplasia (23).

In addition to the HLA region, the roles of some non-HLA gene loci also differ between EOMG and LOMG. PTPN22 and TNFAIP3 gene polymorphisms are mainly associated with EOMG, while ZFAT gene variants may be specific risk factors for LOMG (24). Moreover, epigenetic factors such as long non-coding RNAs and CircRNAs may also play different regulatory roles in the pathogenic mechanisms of the two subtypes (13). These studies demonstrate that EOMG and LOMG have distinct genetic susceptibilities, supporting their consideration as independent subtypes in MG genetic research. Further exploration of the differences in their pathogenic mechanisms will facilitate more precise subtype diagnosis and treatment.

Juvenile MG is an MG subtype with an early age of onset (usually <18 years) and mainly affects extraocular muscles. Some studies suggest that juvenile MG may have unique genetic susceptibility factors. A study involving 54 Chinese juvenile MG patients found that 17 (31.5%) carried TTN gene mutations, significantly higher than the control group, suggesting that TTN mutations may be potential therapeutic targets for juvenile MG (25). A cohort study in Turkey found that adolescent females were more prone to developing generalized juvenile MG with a more severe clinical course, requiring more aggressive treatment strategies, including thymectomy, which may be related to factors such as changes in hormone levels during puberty (26).

3.2 Specific genetic alterations in thymoma-associated MG

Thymoma is the most common tumor complication of MG, with approximately 10–20% of MG patients having concurrent thymoma, and 30–40% of thymoma patients developing MG symptoms, suggesting a close pathogenic link between the two (27). The latest research has discovered that the thymic tissue of thymoma-associated MG (TAMG) patients contains a unique subset of medullary thymic epithelial cells that ectopically express neuromuscular junction molecules such as agrin and Lrp4, which may be involved in the development of MG by activating autoreactive T and B cells (28). Additionally, dysregulated expression of apoptosis-related genes such as p53 and Bcl-2, and overexpression of chemokines such as CXCL13 and CCL21 in the thymic microenvironment of TAMG patients may lead to autoimmune tolerance defects, serving as core links in the pathogenesis of TAMG (8).

Genetic studies have found that TAMG patients possess a unique HLA allele profile, such as HLA-A*25, HLA-B*40:01, and HLA-DRB1*16, which may confer genetic susceptibility to thymoma and MG (29). The roles of some non-HLA genes, including PTPN22 and CTLA4, in the pathogenesis of TAMG are also gradually being revealed (8, 29). Furthermore, a small proportion of TAMG patients may have concurrent immunodeficiency, manifesting as recurrent infections, hypogammaglobulinemia, and other symptoms. These patients often carry specific HLA-DRB1 and HLA-DQB1 alleles and have a poorer prognosis, requiring attention from clinicians (30).

3.3 Genetic characteristics of other MG subtypes

Anti-MuSK antibody-positive MG (MuSK-MG) accounts for 30–50% of AChR antibody-negative MG cases, with clinical manifestations mainly involving facial, shoulder, and bulbar muscles. This subtype responds poorly to cholinesterase inhibitors but responds well to B cell depletion therapies such as rituximab, suggesting that it has a unique pathogenic mechanism (24). Research has found that more than 90% of MuSK-MG patients are mediated by IgG4 subclass antibodies, which is markedly different from conventional MG (mainly IgG1 and IgG3). Further analysis revealed that the total serum IgG4 level was elevated in MuSK-MG patients but had no significant correlation with the MuSK antibody titer, suggesting that IgG4 antibodies may have an antigen-nonspecific mechanism of action in the pathogenesis of MuSK-MG (31). The latest research has also discovered that MuSK-MG is associated with a specific HLA-DR14-DQ5 haplotype, which may lead to the development of MuSK-MG by influencing the activation of autoreactive T cells and IgG4 class switching (4, 32).

3.4 Genetic characteristics of the ocular MG subtype

Ocular MG is a localized form of MG characterized by ptosis and diplopia, with a relatively mild natural course. Genetic studies have found that ocular MG is associated with HLA-DQ and PTPN22 loci, but the overall genetic burden is lighter than that of generalized MG (4). Additionally, a small proportion of severe generalized MG patients are resistant to various conventional immunotherapies and are referred to as refractory MG. The latest research has found that the proportion of follicular helper T cells (Tfh) is increased in refractory MG patients, and serum CXCL13 levels are significantly elevated, suggesting that Tfh cell-mediated humoral immune abnormalities may be an important cause of the development and treatment resistance of this subtype (33).

4 Exploration of gene–environment interactions and epigenetic mechanisms in MG

4.1 Gene–environment interaction patterns in MG

Research on DJ-1 protein provides a new perspective for elucidating the gene–environment interaction mechanisms of MG. DJ-1 is a multifunctional protein that plays an important role in neurodegenerative diseases. Studies have found that DJ-1 gene mutations, abnormal expression, and post-translational modifications can promote the occurrence of various neurodegenerative diseases through mechanisms such as mitochondrial dysfunction, oxidative stress, and autophagy defects (34). Since DJ-1 also plays a key role in neuromuscular junction protection, it is speculated that its abnormalities may mediate the development of MG through similar mechanisms. This suggests

that certain MG-related genes may have the dual characteristics of genetic susceptibility and environmental response, serving as a "bridge" for gene–environment interactions.

4.2 Involvement of abnormal DNA methylation modifications in MG pathogenesis

DNA methylation is an important epigenetic modification that participates in disease development by influencing gene transcriptional activity. In recent years, multiple studies have found extensive DNA methylation abnormalities in the peripheral blood and thymic tissue of MG patients, suggesting its important role in the pathogenesis of MG. These epigenetic modifications specifically affect neuromuscular junction function through several critical mechanisms:

1 DNA methylation changes in the promoter regions of acetylcholine receptor subunit genes directly influence receptor density at the neuromuscular junction. For instance, hypomethylation of the CHRNA1 promoter leads to increased expression of AChR α-subunits (28), directly affecting synaptic transmission efficiency.
2 Histone modifications, particularly H3K27ac and H3K4me3 marks, regulate the accessibility of key genes involved in neuromuscular transmission. Studies have shown that alterations in these modifications affect the expression of proteins crucial for synaptic maintenance and function (28, 35).
3 The interplay between these epigenetic mechanisms and neuromuscular junction proteins creates a regulatory network that maintains synaptic homeostasis. Disruption of this network through aberrant epigenetic modifications can lead to compromised neuromuscular transmission and MG symptoms (36).

Researchers used the Illumina 850 K methylation chip to compare the DNA methylation profiles of 8 MG patients and 4 healthy controls, finding significantly reduced methylation levels of genes such as calcium/calmodulin-dependent protein kinase 1D (CAMK1D) and cAMP response element-binding protein 5 (CREB5) in the MG group. Further experiments showed that hypomethylation of the promoter regions of CAMK1D and CREB5 was associated with their upregulated expression, suggesting that these two genes may play an important role in the pathogenesis of MG through DNA methylation abnormalities (28). This study was the first to reveal the DNA methylation landscape of MG at the whole-genome level, providing important clues for subsequent in-depth research.

The NLRP3 inflammasome is closely related to various autoimmune diseases. Studies have found that polymorphisms in the gene encoding NLRP3 are significantly associated with MG, and carriers of the rs3806265 C allele have a significantly increased risk of developing MG (35). Research indicates that thymoma-associated MG (TAMG) has unique molecular genetic characteristics. However, there were no significant differences in the methylation levels of repair and tumor suppressor genes such as DNA methyltransferase (MGMT) and cyclin-dependent kinase inhibitor 2A (CDKN2A) between TAMG tissues and non-myasthenia gravis thymoma tissues, suggesting that methylation abnormalities of these genes may not be key events in the pathogenesis of TAMG (36).

4.3 Regulation of MG development by histone modifications and non-coding RNAs

In addition to DNA methylation, histone modifications and non-coding RNAs are also important epigenetic regulatory mechanisms of gene expression. The complement regulatory protein CD59 can inhibit the formation of the membrane attack complex and plays an important protective role in neuromuscular junction immune damage. Studies have found that CD59 expression is significantly increased in the skeletal muscle tissue of MG patients and is closely related to clinical severity. Further analysis showed that the upregulation of CD59 mRNA and protein levels may be a compensatory protection mechanism against complement-mediated membrane damage (37). This provides a new entry point for the treatment of MG, namely enhancing CD59 expression to inhibit excessive complement activation and reduce neuromuscular junction damage.

Non-coding RNAs are a hotspot in epigenetic research. Circular RNAs (circRNAs) have characteristics such as high stability and tissue-specific expression, showing great application potential in the diagnosis and treatment of nervous system diseases. Studies have found that hsa-circRNA5333-4 is significantly upregulated in the peripheral blood of MG patients and is closely related to MG scale scores, making it a promising new marker for monitoring MG disease progression and treatment efficacy (38). Additionally, the roles of miRNAs such as miRNA-320a, let-7, and miR-150-5p in the pathogenesis of MG have been successively discovered (39, 40). These studies have laid the foundation for elucidating the non-coding RNA regulatory network of MG.

In summary, various epigenetic mechanisms such as DNA methylation, histone modifications, and non-coding RNAs play key roles in the pathogenesis of MG and are closely related to genetic factors and environmental exposures. With the rapid development of epigenomics, RNA-omics, and other disciplines, the understanding of the gene–environment-epigenetic interaction patterns in MG will continue to deepen, providing new breakthroughs for MG etiological research.

Author contributions

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Cell therapy for scleroderma: progress in mesenchymal stem cells and CAR-T treatment

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Cell therapy is an emerging strategy for precision treatment of scleroderma. This review systematically summarizes the research progress of mesenchymal stem cell (MSC) and chimeric antigen receptor T cell (CAR-T) therapies in scleroderma and discusses the challenges and future directions for development. MSCs possess multiple functions, including immunomodulation, anti-fibrosis, and promotion of vascular regeneration, all of which can improve multiple pathological processes associated with scleroderma. Studies have demonstrated that MSCs can alleviate skin fibrosis by inhibiting CCL2 production and reducing the recruitment of pathological macrophages; their paracrine effects can exert extensive regulatory functions. CAR-T cell therapy ca specifically target and eliminate autoreactive immune cells, exhibiting enhanced specificity and personalized potential. Different cell therapies may have complementary and synergistic effects in treating scleroderma, such as MSCs exerting their effects through paracrine mechanisms while CAR-T cells specifically eliminate pathological cells. Furthermore, cell-free therapies derived from MSCs, such as extracellular vesicles or exosomes, may help circumvent the limitations of MSC therapy. Although cell therapy has opened new avenues for the precision treatment of scleroderma, it still faces numerous challenges. In the future, it is essential to strengthen integration of basic and clinical research, establish standardized protocols for cell preparation and quality control, develop personalized treatment plans, and rationally combine cell therapy with existing treatment methods to maximize its advantages and improve patient prognosis and quality of life.

KEYWORDS

scleroderma, cell therapy, mesenchymal stem cells, mesenchymal stem cell exosomes, chimeric antigen receptor T cells

1 Introduction

Scleroderma is a chronic autoimmune disease characterized by widespread microvascular damage, endothelial cell injury, immune activation, and fibrosis of the skin and internal organs (1). Patients typically present with skin hardening, thickening, facial changes, and digital ulcers. In severe cases, vital organs such as the lungs, kidneys, and heart may be involved, threatening life (2). Although the exact cause of scleroderma remains unclear, it is widely believed that its pathogenesis is closely related to dysfunction in the vascular and immune system, with sustained immune activation and uncontrolled fibrosis playing key roles in disease progression (3). This suggests that when exploring treatment strategies for scleroderma, we should focus on suppressing abnormal immune responses and fibrotic processes.

Treatment of scleroderma still faces many challenges. For localized scleroderma, mainly local and physical therapies utilized, but their efficacy is limited. In the case of diffuse scleroderma, immunosuppressants such as glucocorticoids and cyclophosphamide can control the disease to some extent; however, their effectiveness is often inadequate, and the efficacy associated side effects can be significant, making it difficult to halt disease progression fundamentally. The effects of vasodilators and antifibrotic agents are also unsatisfactory. As such, traditional treatments struggle to meet the clinical needs of scleroderma patients, highlighting the urgent need for the development of new strategies to improve prognosis. This highlights the important research significance of cell therapy in the field of scleroderma.

Stem cells have become ideal tools for treating various diseases due to their self-renewal and multi-lineage differentiation potential. In recent years, stem cell therapy has shown good prospects in clinical studies of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (4), providing inspiration for its application in scleroderma treatment. As a class of abundantly sourced and easily isolated adult stem cells, mesenchymal stem cells (MSCs) have garnered significant attention for their low immunogenicity, immunomodulation, and tissue repair properties (5, 6). Previous studies have shown that MSCs can exert anti-scleroderma effects through the paracrine secretion of cytokines, extracellular vesicles, and other products at multiple levels, including regulating immune responses, improving fibrosis, and promoting damaged tissue repair (7). This suggests that MSCs may become a new cellular tool for scleroderma treatment. In addition, chimeric antigen receptor T cell (CAR-T) therapy, an emerging immune cell technology that has made breakthroughs in B-cell malignancy in recent years, may also be applied to autoimmune disease treatment by genetically engineering T cells to specifically target and kill cells (8). Given the immune dysregulation characteristics of scleroderma, whether CAR-T therapy to provide new breakthroughs in its treatment warrants in-depth exploration.

2 Research progress of mesenchymal stem cell therapy for scleroderma

2.1 Biological characteristics and mechanisms of action of mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a type of adult stem cell characterized by their self-renewal and multi-lineage differentiation potential. Initially discovered in bone marrow, subsequent studies showed their widespread presence in adipose tissue, umbilical cord, and various other tissues (6, 9–11). Morphologically, MSCs are spindle-shaped or fibroblast-like, expressing specific surface markers such as CD105, CD73, and CD90, but not express hematopoietic and endothelial cell markers like CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR (6, 9, 10). MSCs can differentiate into osteoblasts, chondrocytes, adipocytes, and other cell lineages, thereby playing a key role in tissue repair and regeneration (11).

In recent years, numerous studies have revealed the unique advantages of MSCs in regulating immune responses, inhibiting fibrosis, and promoting angiogenesis, making them as a focus in autoimmune disease treatment (12–16). Mechanistic studies indicate that MSCs can secrete various immunosuppressive factors such as IL-10, HGF, and PGE2 to inhibit the proliferation and activation of immune cells like T cells, B cells, NK cells, and dendritic cells. This process induces inducing immune tolerance and thus alleviating autoimmune damage (15, 17, 18). Moreover, MSCs can downregulate the expression of pro-fibrotic factors such as TGF-β and CTGF to inhibit fibroblast proliferation and collagen deposition, thereby reducing tissue fibrosis (12, 19, 20). Simultaneously, MSCs secrete angiogenic factors like VEGF and HGF to stimulate endothelial cell proliferation and angiogenesis, thereby improving tissue ischemia and damage (13, 18). Given the synergistic effects of MSCs in immunomodulation, anti-fibrosis, and tissue repair, they may become an ideal cell therapy approach to intervene in complex pathological processes such as scleroderma.

Although the exact mechanisms by which MSCs exert their therapeutic effects are not fully elucidated, studies have suggested their paracrine effects may be key. Extracellular vesicles, especially exosomes, secreted by MSCs can selectively enrich and deliver various bioactive substances such as, microRNAs and growth factors to damaged tissues and effector cells, regulating gene expression and conveying the therapeutic effects of MSCs (21, 22). This discovery provides new ideas for revealing the mechanisms of action of MSCs and optimizing MSC treatment strategies. Future studies should explore the component characteristics and functional regulatory networks of MSC exosomes at both the molecular and cellular levels to clarify their therapeutic mechanisms in diseases like scleroderma, thereby providing a theoretical guidance and material basis for cell therapy (Figure 1).

2.2 Therapeutic effects of mesenchymal stem cells in scleroderma animal models

2.2.1 Comparison of efficacy of mesenchymal stem cells from different sources in scleroderma animal models

To explore the application prospects of MSCs in scleroderma treatment, several studies have employed mouse models induced by bleomycin or hypochlorous acid to investigate the therapeutic effects of MSCs from bone marrow, adipose tissue, umbilical cord, and other tissue sources (11, 23–28). Zhang et al. compared the efficacy of single tail vein injection of autologous or allogeneic bone marrow MSCs derived from the mouse in a bleomycin-induced scleroderma mouse model. They found that both sources of MSCs effectively alleviated inflammatory infiltration and collagen deposition in skin and lung tissues, reduced the imbalance of Th1/Th2 cell cytokines in the lungs, and have similar efficacy (26). Abdel et al. compared the therapeutic effects of allogeneic bone marrow MSCs and adipose MSCs on a hypochlorous acid-induced scleroderma mouse model. The results showed that both types of MSCs improved skin and lung fibrosis; however, adipose-derived MSCs were superior to bone marrow-derived MSCs in reducing inflammatory cytokines and autoantibody levels (17). In addition, Liang et al. confirmed that human umbilical cord MSCs could dose-dependently inhibit skin thickening, inflammation, and collagen deposition in bleomycin-induced scleroderma mice, with high-dose (1 × 10^6 cells/mouse) being more effective than medium and low doses (2.5 × 10^5 and 5 × 10^5 cells/mouse) (28).

However, the aforementioned studies mainly used intravenous administration, and it is unclear whether MSCs can effectively home

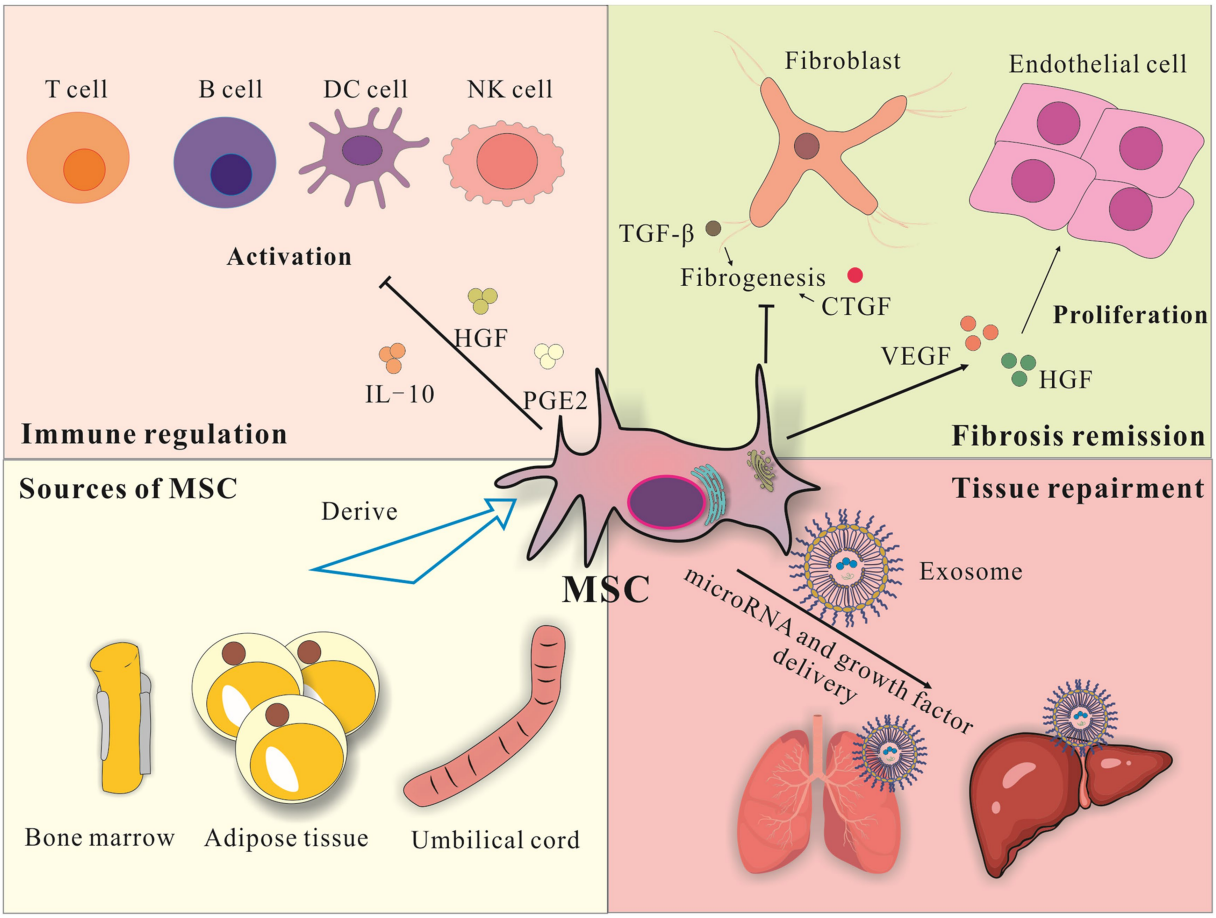

FIGURE 1
Illustration of Application of CAR-T Therapy treatment. Mesenchymal stem cells can be derived from bone marrow, fat and umbilical cord. These stem cells can themselves home to diseased tissues, or release vesicles or exosomes carrying various signaling substances such as proteins, nucleic acids and micro-RNAs through paracrine action to act on diseased tissues. The main affected organs in systemic sclerosis include the skin, lungs, kidneys, heart and blood vessels. Stem cells themselves or the exosomes secreted by them can act on the diseased tissues of systemic sclerosis and improve the inflammation and fibrosis of these tissues.

to damaged tissues. Considering that scleroderma lesions mainly involve local tissues such as the skin and lungs, future studies should explore more precise cell delivery strategies such as local administration, to enhance the enrichment and therapeutic efficacy of MSCs in lesion tissues. In addition, scleroderma animal models differ considerably from clinical phenotypes, and caution is needed when extrapolating the above results to the clinic. Translational research based on humanized animal models and clinical samples to provide reliable evidence for formulating optimal MSC treatment regimens.

2.2.2 Mechanism exploration of mesenchymal stem cell exosomes exerting effects through MicroRNAs and signaling pathways

The paracrine effects of MSCs are an important mechanism through which they exert their therapeutic effects. Among them, extracellular vesicles, especially exosomes, secreted by MSCs, have received much attention for their potential role in scleroderma treatment. Studies have shown that MSC-derived exosomes can selectively enrich and deliver various bioactive substances such as microRNAs and growth factors, to regulate gene expression and signal transduction in recipient cells, conveying the therapeutic effects of MSCs (21, 22). Yamashita et al. isolated and purified human MSC exosomes and administered them in bleomycin-induced scleroderma mice. The results demonstrated that exosomes significantly alleviated fibrosis in the skin and visceral organs and inhibited fibroblast activation and macrophage infiltration. Further mechanistic studies revealed that MSC exosomes were enriched in anti-fibrotic microRNAs such as miR-196a, which could target and downregulate the expression of fibrotic genes like Col1a1, Col1a2, and αSMA in fibroblasts (22). Similarly, Zhang et al. also confirmed that MSC exosomes alleviated skin hardening and lung fibrosis in scleroderma mice by carrying miR-29a, which inhibits the fibrosis-related TGF-β/Smad signaling pathway (21).

These studies reveal, at the molecular level, that MSC exosomes sever as important carriers of MSCs' therapeutic effects, exert anti-fibrotic effects by selectively enriching and delivering specific bioactive substances, such as microRNAs, to regulate fibroblast phenotype and collagen metabolism. This provides new ideas for elucidating the mechanisms of action of MSCs and optimizing MSC treatment strategies. Given the complex composition and diverse structures and functions of MSC exosomes, future studies should explore their component characteristics and functional regulatory networks to

establish standardized systems for exosome preparation and quality control, laying the foundation for advancing the clinical application of MSC exosomes. In addition, the mechanisms by which exosomes achieve tissue-specific enrichment and targeted delivery still unclear, limiting their therapeutic efficacy. In-depth exploration of the biodistribution patterns and targeting mechanisms of exosomes and optimization of administration routes and dosing regimens will help improve the therapeutic effects and clinical translation potential of exosomes. In summary, as a new cellular therapy medium, MSC exosomes may overcome the bottlenecks faced by MSC treatment of scleroderma, but transforming them into a mature treatment modality still requires extensive in-depth research.

2.3 Current status of clinical research on mesenchymal stem cell therapy for scleroderma

Given the therapeutic potential demonstrated by MSCs in scleroderma animal models, several preclinical studies have conducted preliminary explorations in scleroderma patients, using autologous or allogeneic MSC infusion to assess their safety and efficacy (29–36). The majority of participants were patients with diffuse scleroderma characterized by extensive skin and visceral involvement. They were given single or multiple intravenous infusions of bone marrow or umbilical cord-derived MSCs at a dose of (1, 2) × 10^6/kg, with efficacy assessed at 3–12 months of follow-up. The main observation indicators included skin hardening improvement (modified Rodnan skin score, mRSS), vascular lesion manifestations (digital ulcers, Raynaud's phenomenon), lung function indicators (forced vital capacity FVC, lung diffusion function DLCO), autoantibody titers (14, 20, 32, 33). Safety evaluation mainly observed the occurrence of infusion-related adverse reactions and complications, such as infections. Most studies found that 4–12 weeks post-MSC infusion, the degree of skin hardening in the face, hands, and trunk was significantly reduced, skin luster and elasticity improved, and mRSS scores decreased by more than 30% from baseline (6, 14, 37, 38). At the same time, MSC therapy was found to promote the healing of refractory digital ulcers, relieve pain, and improve patients' quality of life (14, 18). For patients with concomitant pulmonary hypertension, right heart function improved, and pulmonary artery pressure decreased after MSC treatment (6). In addition, MSCs could also alleviate visceral involvement such as gastrointestinal motility disorders (18). These studies indicate that intravenous infusion of MSCs can comprehensively improve skin, vascular, and visceral lesions in scleroderma patients, with preliminary but encouraging efficacy.

Existing research indicates that MSC therapy is well-tolerated, with only a few patients experiencing transient mild infusion reactions, such as fever and rash, and no reports of serious adverse events (20, 33). In terms of efficacy, most studies have observed significant reductions in skin hardening and mRSS scores after MSC treatment, with effects lasting for several months (29, 32, 33, 36). Some patients with concomitant interstitial lung disease also showed improvements in lung function indicators and CT imaging (31, 33). In addition, serum levels of inflammatory cytokines, such as IL-6 and TNF-α decreased after MSC treatment, as did titers of autoantibodies such as anti-Scl-70 and anti-centromere antibodies (20, 36). The above studies preliminarily confirmed the safety and potential efficacy of MSC therapy for refractory scleroderma patients, but small sample sizes, lacked control groups, and efficacy evaluation indicators and follow-up times were not uniform enough. On the other hand, existing studies are mostly single-center, open-label clinical trials lacking uniform inclusion criteria, administration regimens, and efficacy judgment standards, making objective comparisons and meta-analyses difficult. In the future, evidence-based guidelines for scleroderma cell therapy should be developed to standardize clinical trial design, unify efficacy judgment and adverse event reporting standards, and improve the comparability and evidence level of different study results. At the same time, stratified research and subgroup analyzes should be conducted for patients with disease stages and clinical manifestations to clarify the optimal indications and timing of MSC therapy and achieve precision treatment. Currently, clinical research on MSC therapy for scleroderma is still in its infancy, and many key scientific questions await systematic in-depth exploration, such as the *in vivo* homing distribution and pharmacokinetic characteristics of MSCs, optimal administration routes, dosage regimens, and treatment courses, and optimization of combination therapy regimens. Large-sample, multicenter prospective cohort studies are urgently needed to accumulate high-quality evidence-based medicine evidence.

Although clinical research on MSC therapy for scleroderma has made positive progress, transforming it into a mature treatment strategy still faces many challenges. The primary issue is the standardization of MSC preparation and quality control. Uniform protocols must be established for MSC sourcing, isolation, purification, and *in vitro* expansion to preferentially select low-immunogenicity allogeneic sources, such as umbilical cord tissue, and to ensure the stability and comparability of preparation quality. Secondly, the allogeneic MSC therapy has long-term immunogenicity and tumorigenicity risks still require vigilance (20, 39). Furthermore, whether intravenous infusion can deliver MSCs to lesion tissues remains questionable, and optimal administration routes and dosage regimens need further optimization (5). In addition, to objectively evaluate efficacy, a unified evaluation indicator system needs to be established, taking into account improvements in patient symptoms, signs, and histopathology at multiple levels (39).

3 Application of adipose-derived mesenchymal stem cells and tissue engineering in scleroderma treatment

Adipose tissue serves as an important source of MSCs, and ADSCs have the advantages of convenient material acquisition, minimal trauma, and high yield. Compared to bone marrow-derived MSCs, ADSCs have stronger proliferative capacity, differentiation potential, and immunomodulatory effects, and they are less age-dependent (11, 40, 41). In addition to osteogenic, chondrogenic, and adipogenic lineages, ADSCs can also differentiate into endothelial cells, smooth muscle cells, and other cell types. Furthermore, they secrete angiogenic factors such as VEGF and HGF, which play an important role in tissue repair (11, 42).

For autoimmune diseases such as scleroderma, ADSCs offer distinct advantages in immunomodulation and anti-fibrosis (11, 40, 43). ADSCs derived from scleroderma patients express more pluripotency genes such as IDO-1 and SOX2, have stronger ability to secrete cytokines such

as IL-6 and HGF, and have more significant inhibitory effects on T cells (41, 44). Animal experiments have confirmed that the transplantation of allogeneic or autologous ADSCs can effectively improve skin and lung fibrosis in hypochlorous acid-induced mice, with efficacy comparable to bone marrow MSCs, but ADSCs have greater advantages in reducing inflammatory cytokines such as TNF-α and IL-1β and increasing the MMP-1/TIMP-1 ratio (40). In addition, extracellular vesicles secreted by ADSCs have regulatory effects on fibroblasts and can reverse their pro-fibrotic phenotype (44). Therefore, ADSCs may become an ideal choice for cell therapy in scleroderma.

Given the therapeutic effects of ADSCs in scleroderma animal models, adipose tissue and ADSC transplantation strategies have begun to be explored clinically (11, 45, 46). For facial skin hardening, autologous fat transplantation is a minimally invasive and individualized treatment method that can not only improve skin texture and contour but also locally deliver ADSCs to exert immunomodulatory effects and promote microcirculation reconstruction (11, 45). In the case of digital ulcers, local multiple injections of autologous adipose tissue extract can significantly promote wound healing and improve pain and function (42, 45). A prospective study conducted 6 months of autologous fat transplantation treatment for 13 patients with localized scleroderma and digital ulcers showed that all ulcers healed, pain was relieved, quality of life improved, and no recurrence was observed at 1-year follow-up (45). For diffuse scleroderma with interstitial lung disease, bronchial injection of autologous adipose tissue extract can safely and effectively alleviate pulmonary inflammation and delay the progression of pulmonary fibrosis (11). However, current studies have small sample sizes, lack control groups and uniform efficacy evaluation standards, and still require large-scale randomized controlled trials to further verify their long-term efficacy and safety.

4 Research progress of chimeric antigen receptor T cell (CAR-T) therapy for scleroderma

4.1 Basic principles of CAR-T cell therapy and its application in autoimmune diseases

In recent years, chimeric antigen receptor T cell (CAR-T) therapy, as a major breakthrough in the field of cancer immunotherapy, has brought new hope to numerous patients with refractory malignancies. CAR-T cells are T lymphocytes that have been genetically engineered to specifically recognize and kill tumor cells (45). Unlike conventional T cell receptors (TCRs), CARs can directly recognize tumor cell surface antigens in a non-MHC-restricted manner and release perforin and granzyme B to induce apoptosis in tumor cells. This mechanism demonstrates enhanced killing efficiency and broader application prospects (47, 48). CD19-targeted CAR-T cells have made breakthrough progress in the treatment of B-cell malignancies, with multiple clinical studies showing that CD19 CAR-T cells can induce complete remission in up to 80–90% of patients with refractory or relapsed B-cell leukemia and lymphoma (49). These encouraging efficacy results provide new ideas and directions for CAR-T cell therapy in autoimmune diseases.

Given the key role of B cells in the pathogenesis of autoimmune diseases, CD19 CAR-T cells may effectively control disease progression by specifically clearing autoreactive B cells, blocking autoantibody production, and reestablishing immune tolerance (50, 51). Compared to the B-cell- depleting drug rituximab currently approved for autoimmune diseases, CAR-T cell therapy has several advantages: first, CAR-T cells have higher killing efficiency and can clear deep-tissue B cells to maintain long-term efficacy; second, CAR-T cells have *in vivo* proliferative capacity and can exert sustained antitumor effects following a single infusion; third, CAR-T cells also have multiple immunomodulatory effects such as regulating T cell subset balance and inducing regulatory T cells (Tregs) (4, 45, 47). Therefore, CD19 CAR-T cells may become an innovative treatment option for autoimmune diseases, particularly those that are mediated by B cells.

CAR-T therapy in the field of autoimmune diseases is still in its infancy, but it has shown good application prospects in refractory rheumatic immune diseases such as systemic sclerosis (SSc). As an acquired autoimmune disease characterized by extensive skin and visceral fibrosis, SSc seriously affects patient survival and quality of life, and conventional immunosuppressive therapy has poor efficacy. Research indicates that chimeric antigen receptor technology provides new ways to overcome the limitations of conventional SSc treatment. CAR-T cells designed to target B cell subsets and SSc-specific antigens may play unique advantages in blocking SSc autoimmune responses and reversing fibrotic processes. This article will focus on discussing the research progress of CAR-T cell therapy in SSc and related rheumatic immune diseases, analyzing the opportunities and challenges it faces, and looking forward to its future development direction.

4.2 Current status of CD19-CAR-T cell therapy for systemic sclerosis and autoimmune disease

The results of multiple preclinical and early clinical studies support the feasibility and potential benefits of using CD19 CAR-T cells for the treatment of autoimmune diseases, such as SSc. Ingelfinger et al. first reported a case of a refractory SSc patient who received HLA-mismatched donor-derived CD19 CAR-T cell infusion after conventional immunosuppressive therapy had failed. The patient's skin hardening and lung function improved significantly, and no serious adverse reactions occurred, suggesting that CD19 CAR-T cells are both safe and effective for SSc (52). A further multicenter phase I clinical trial enrolled 15 autoimmune disease patients, including SSc, systemic lupus erythematosus (SLE), and idiopathic inflammatory myopathy (IIM). Following a single CD19 CAR-T infusion treatment, the European Scleroderma Study Group (EUSTAR) activity scores of all SSc patients and disease indexes of SLE and IIM patients decreased significantly, with only a few mild cytokine release syndrome (CRS) occurrences, confirming the broad-spectrum anti-autoimmune effects and safety of this therapy. In addition to conventionally sourced CAR-T cells, healthy donor CAR-T cells can also be gene-edited and directly applied to patients as an "off-the-shelf" treatment product. The CRISPR/Cas9 was used to knock out the endogenous TCR and HLA-I genes of CAR-T cells to obtain universal CD19 CAR-T cells, which were infused into 1 patient with refractory necrotizing myopathy and 2 patients with diffuse SSc. At 6 months of follow-up, disease activity and organ function improved significantly, and no rejection reactions or severe CRS occurred, suggesting the safety and efficacy of gene-edited allogeneic CAR-T cells in SSc treatment (53). As research deepens, multiple

centers at home and abroad are conducting prospective randomized controlled clinical trials to obtain more high-quality evidence-based medicine to support the application and promotion of CD19 CAR-T in the treatment of SSc and other rheumatic immune diseases (1, 54).

Existing research has preliminarily confirmed the feasibility and potential benefits of CD19 CAR-T cell therapy for autoimmune diseases such as SSc and inflammatory myopathy, but its clinical application still faces many challenges. For example, CAR-T therapy is expensive, and the economic burden on patients is heavy. Additionally, the long-term efficacy and safety are still unclear, and there may be risks of disease recurrence and secondary infection complications; its drug dosage, infusion frequency, combination therapy regimen. Furthermore, the monitoring and management processes for adverse reactions still need further improvement. Therefore, large-sample, multicenter prospective randomized controlled studies are still needed in the future to comprehensively evaluate the benefits and risks of CAR-T therapy in the long-term management of SSc and related rheumatic immune diseases. These studies should aim to establish standardized treatment regimens and adverse reaction prevention and management of adverse reactions, ultimately transforming this emerging technology into a routine clinical treatment to benefit a larger patient population.

4.3 Exploration of CAR-T cell therapy optimization strategies for scleroderma

Although CD19 CAR-T treatment has shown feasibility and potential efficacy in SSc, how to further improve CAR-T cell function, reduce adverse reactions, prolong the duration of efficacy, and develop it as a routine treatment for SSc are still key issues that need to be urgently addressed (55, 56).

The optimized design of next-generation CAR structures may improve the anti-tumor activity and persistence of CAR-T cells. Third-generation CARs introduce two or more co-stimulatory domains that can more effectively activate T cells, promote their proliferation and cytokine secretion, and enhance their cytotoxic capabilities and *in vivo* persistence. Hunder et al. used this strategy to treat refractory SSc and achieved more significant efficacy than second-generation CAR-T cells (1, 55). In the future, introducing pro-T cell cytokine genes, such as IL-12 and IL-15, may further enhance the effector function and *in vivo* expansion capacity of CAR-T cells (56).

Selecting SSc-specific antigens such as Scl-70 and centromere as new therapeutic targets may improve efficacy while reducing off-target toxicity. The CAAR-T strategy, by integrating autoantigens into the CAR, can specifically clear autoreactive B cells and may be applied to SSc patients with characteristic autoantibody positivity (47, 56).

Optimizing administration regimens, such as reducing fludarabine dosage in pretreatment and local administration, may lower the incidence and severity of CRS and neurotoxicity while ensuring CAR-T expansion (50, 57). For SSc with concomitant interstitial lung disease, bronchoscopic airway administration of CAR-T cells may enhance pulmonary T cell infiltration, directly inhibit pulmonary inflammation and fibrosis, and reduce systemic adverse reactions (56).

CAR-T cell and ADSC transplantation have brought new hope for the treatment of scleroderma. Fully leveraging the immunomodulatory and anti-fibrotic properties of both cell types and optimizing them through genetic engineering and tissue engineering techniques, may maximize the precision and effectiveness of cell therapy and extend the duration of its efficacy. In-depth research on scleroderma-specific cell therapy strategies, expansion of clinical research sample sizes, conduction of randomized controlled trials, and establishment of standardized efficacy evaluation and adverse reaction management systems are essential for advancing cell therapy into a routine precision treatment for scleroderma.

5 Challenges and countermeasures of cell therapy in scleroderma

5.1 Limitations and optimization strategies of key aspects such as cell preparation, administration routes, and efficacy evaluation

Cell preparation is key to ensuring therapeutic effects. Currently, the heterogeneity of mesenchymal stem cells (MSCs) is still a major challenge. MSCs derived from different sources exhibit significant differences in functions, such as immunomodulation (9, 11). Establishing a standardized quality control system and optimizing cell isolation, purification, and *in vitro* expansion are important ways to improve the efficacy of MSCs. In addition, the issue of cell viability loss during cryopreservation urgently needs to be addressed (58). Developing new cryoprotectants and advanced programmed freezing technologies may help maximize the retention of cell viability.

The choice of administration route directly affects therapeutic efficacy. Although intravenous infusion is simple to perform, the homing efficiency of cells *in vivo* remains low. While local injection can improve targeted delivery efficiency, it may not achieve systemic therapeutic effects. Developing new cell delivery systems, such as constructing injectable hydrogels in combination with biomaterials, may overcome the limitations of local administration. In addition, using cell-derived extracellular vesicles and other cell-free products for treatment can, to some extent avoid the safety risks of live cell infusion.

The lack of specific indicators for evaluating efficacy evaluation is another key issue. Currently, it mainly relies on clinical scores, such as mRSS and lacks biomarkers that can accurately reflect disease progression (59). Actively developing new molecular markers and imaging evaluation methods will yield more objective and accurate evidence for assessing the efficacy e of cell therapy. Discovering specific biomarkers through omics analysis and dynamically monitoring treatment responses using functional imaging are crucial directions for future development.

5.2 Combining cell therapy with conventional treatment methods to improve efficacy

The pathology of scleroderma is complex, and single treatment methods often fail to achieve satisfactory efficacy. Combining cell therapy with conventional treatment methods such as drugs may achieve a synergistic effect. For example, combining MSCs with immunosuppressants such as glucocorticoids may promote tissue repair while simultaneously controlling inflammation (5). Exploring the combined use of MSCs with vasoactive drugs may more effectively improve microvascular lesions (37). It is worth

noting that biologics, such as anti-CD20 monoclonal antibodies, have shown good effects in scleroderma treatment (60). Combining these targeted drugs with cell therapy may achieve more precise immunomodulation. However, the safety and optimal timing for the administration of combination therapy regimens still need to be verified by rigorous clinical trials.

5.3 Functionally enhancing therapeutic cells targeting scleroderma pathogenesis to improve efficacy

Targeted modification of therapeutic cells for the specific pathological processes of scleroderma is a key strategy to improve efficacy. Using genetic engineering to make MSCs overexpress anti-fibrotic factors, such as HGF and KGF can significantly enhance their ability to inhibit fibrosis (61, 62). In addition, targeted regulation of signaling pathways, such as TGF-β can improve the therapeutic potential of MSCs within the scleroderma microenvironment (63). For CAR-T cell therapy, designing new CAR molecules, such as targeting specific antigens other than B cells may further expand its application in treating scleroderma (4, 55). However, the clinical safety and long-term effects of cell modification strategies still need to be carefully evaluated.

Using extracellular vesicles secreted by MSCs and other cells for treatment is another recently emerging research hotspot. Compared to live cells, cell-free products such as extracellular vesicles are more stable *in vivo* and may reduce safety risks, such as immunogenicity (64). Optimizing cell culture systems to selectively enrich specific miRNAs and other active components within extracellular vesicles could further enhance their immunomodulatory and anti-fibrotic functions. This strategy may improve the controllability of MSC therapy while retaining its therapeutic advantages.

5.4 Strengthening the evidence level of cell therapy clinical trials to support standardized clinical application

Several studies have preliminarily confirmed the efficacy and safety of cell therapy in scleroderma, but the current evidence level

TABLE 1 CAR targeting various targets or MSC in different organs and systems affected by scleroderma.

Target/Action object	Effects	Related studies
CD19 for CAR-T	• Improves skin sclerosis and lung function • Reduces disease activity scores	• Infusion of CD19 CAR-T cells from HLA-mismatched donors improved skin sclerosis and lung function in refractory SSc patients • Phase I multicenter trials showed significant reduction in disease activity scores after a single infusion of CD19 CAR-T cells in SSc patients • Genetically edited allogeneic CD19 CAR-T therapy improved organ function in refractory necrotizing myopathy and diffuse SSc patients
Scl70, centromere antigens (under exploration) for CAR-T	• Enhances efficacy and reduces off-target toxicity • Specifically eliminates autoreactive B cells • Promising for SSc patients with characteristic autoantibodies	• Research on CAAR-T strategies
MSC Therapy (Skin)	• Reduces inflammatory infiltration and collagen deposition • Improves sclerosis, elasticity, and appearance • Promotes healing of refractory finger ulcers and relieves pain and dysfunction • Autologous fat grafting improves texture and contour, promotes microcirculation reconstruction	• Various MSCs reduced skin-related lesions in SSc mouse models • Clinical studies showed reduced skin sclerosis after MSC infusion • Studies on autologous fat grafting for treating facial skin sclerosis and finger ulcers
MSC Therapy (Lungs)	• Reduces imbalance of Th1/Th2 cytokines • Improves pulmonary fibrosis lesions • Alleviates inflammation and delays fibrosis progression • Improves pulmonary function and lowers pulmonary artery pressure	• Animal model studies showed improvement in pulmonary fibrosis • Research on bronchial injection of autologous fat-derived extracts for treating diffuse SSc with interstitial lung disease • Clinical studies showed partial improvement in lung function
MSC Therapy (Blood vessels)	• Promotes vascular regeneration • Improves vascular damage, such as healing finger ulcers and reducing Raynaud's phenomenon • Enhances endothelial function and improves right heart function	• Studies on MSC secretion of pro-angiogenic factors • Clinical studies showed improvements in vascular lesions after MSC therapy
MSC Therapy (Immune System)	• Suppresses proliferation and activation of immune cells • Induces immune tolerance • Reduces inflammatory cytokine levels and autoantibody titers	• Studies on MSC secretion of immunosuppressive factors • Clinical studies observed changes in inflammatory cytokines and autoantibodies after MSC therapy
MSC Therapy (Gastrointestinal Tract)	• Alleviates gastrointestinal motility disorders and other visceral involvement	• Observations from clinical studies

remains low. Most existing studies are small-sample, single-center, non-randomized controlled trials (14, 32). To achieve standardized application of cell therapy in scleroderma, high-quality prospective, multicenter randomized controlled clinical trials are still needed. At the same time, establishing standardized cell preparation and administration processes and optimizing dosage and administration frequency, is crucial for reducing the heterogeneity of clinical trial results (5, 65). Developing individualized cell therapy regimens tailored to different scleroderma subtypes and disease severities will be an important future direction.

In addition, the cost-effectiveness of cell therapy deserves attention. Currently, expenses associated with cell therapy are substantial, which may hinder patient accessibility (59). Actively exploring new technologies to improve cell preparation efficiency and reduce costs is crucial for promoting the widespread adoption of cell therapy (Table 1).

6 Summary and outlook

Scleroderma is a complex autoimmune disease involving multiple pathological processes, such as immune dysregulation, vascular damage, and tissue fibrosis. Although various treatment methods are currently available, they still fail to meet the clinical needs of scleroderma patients. With the development of precision medicine, cell therapy, as an emerging strategy, provides new possibilities for the precise treatment of scleroderma.

Mesenchymal stem cells (MSCs) and chimeric antigen receptor T cells (CAR-T) are two prominent representatives in the field of cell therapy. MSCs have multiple functions, including immunomodulation, anti-fibrosis, and promoting vascular regeneration, which may concurrently enhance various pathological processes of scleroderma. On the other hand, CAR-T cell therapy provides a new strategy for the targeted clearance of specific autoreactive immune cells, exhibiting stronger specificity and personalized potential.

In summary, cell therapy has opened new avenues for the precise treatment of scleroderma. In the future, it is essential to strengthen the integration of basic research and clinical application, establish standardized cell preparation and quality control, develop personalized treatment plans, and rationally combine cell therapy with existing treatment methods. These steps will maximize its advantages and improve the prognosis and quality of life of patients with scleroderma. It is believed that through the joint efforts of researchers and clinicians, cell therapy will undoubtedly promote the transformation of scleroderma treatment models and benefit a greater number of patients.

Author contributions

LC: Writing – review & editing. RH: Writing – review & editing. CH: Writing – review & editing. GN: Writing – review & editing. YM: Writing – review & editing. LY: Writing – original draft. KL: Writing – original draft. AC: 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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The authors declare that no Gen AI was used in the creation of this manuscript.

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Gut dysbiosis is associated with difficult-to-treat rheumatoid arthritis

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Background: Difficult-to-treat rheumatoid arthritis (D2T RA) refers to a subset of patients who fail to achieve adequate disease control after the use of two or more biological or targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) with different mechanisms of action, while maintaining active inflammatory disease. This presents a therapeutic challenge and highlights the need to explore contributing factors such as the potential role of the gut microbiota. Therefore, the aim of this study was to analyze the gut microbiota and inflammation in patients with D2T RA in comparison to patients with easy-to-treat RA (E2T RA).

Objective: To analyze the gut microbiota and inflammation in patients with D2T RA.

Methods: We performed an observational study of a prospective cohort between 2007 and 2011 and analyzed the gut microbiota. In 2022, we identified 2 extreme patient phenotypes: (1) D2T RA, which was defined as failure of ≥2 biological or targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) (with different mechanisms of action) plus signs of active disease; and (2) easy-to-treat RA (E2T RA), i.e., stable disease managed with a single treatment. The gut microbiota was analyzed using 16S rRNA gene sequencing; bioinformatics analysis was performed using QIIME2, and its functionality was inferred through PICRUSt. We recorded data on clinical findings, inflammation, and cytokines. A Cox multivariate analysis was performed to identify factors related to D2T RA.

Results: The study population comprised 39 patients: 13 (33%) with D2T RA and 26 (66%) with E2T RA. The families *Lachnospiraceae* and *Pasteurellaceae*, and their genera *Coprococcus* and *Haemophilus* were more abundant in E2T RA patients, while the genus *Megasphaera* was more abundant in D2T RA patients. The *Firmicutes*/*Bacteroidetes* ratio decreased in D2T RA patients. The metabolic profile of the gut microbiota was characterized by differences in Degradation/Utilization/Assimilation pathway and the Biosynthesis pathway. The factors associated with D2T RA were inflammatory activity according to DAS28-ESR (HR, 2.649; p = 0.013), prednisone (HR, 3.794; p = 0.008), and the *Firmicutes*/*Bacteroidetes* ratio (HR, 0.288; p = 0.033).

Conclusion: The composition of the gut microbiota of patients with D2T RA differed from that of E2T RA patients, as did the metabolic pathways.

KEYWORDS

rheumatoid arthritis, gut microbiota, disease modifying antirheumatic drugs, *Firmicutes/Bacteroidetes* ratio, inflammation

Introduction

Rheumatoid arthritis (RA) is an immune-mediated chronic inflammatory disease characterized by involvement of the joints and other tissues. It causes pain, stiffness, and loss of mobility. Although there is no cure for RA, affected patients can be treated with a combination of conventional synthetic disease-modifying antirheumatic drugs (scDMARDs), biological DMARDs (bDMARDs), and targeted synthetic DMARDs (tsDMARDs) (1). Despite this therapeutic arsenal, between 15 and 30% of patients with RA have disease that is difficult to treat (D2T RA) and do not achieve remission or low disease activity (2).

According to the "*European Alliance of Associations for Rheumatology*" (EULAR) (3), patients with RA are considered D2T if at least 2 biological or targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) (with different mechanisms of action) have failed after previous failure of a csDMARD (unless contraindicated). Furthermore, the patient must have signs of active/progressive disease in which management of signs and/or symptoms is problematic for the rheumatologist and/or doctor.

Various studies have attempted to identify factors associated with a poor response to biologics in RA (4–7). Some of these factors include, once poor adherence and inadequate dosing have been excluded, higher autoantibody levels (6), a greater number of comorbid conditions (4, 6, 7), and more marked inflammatory activity at initiation of therapy (5–7). However, other studies have shown that patients with marked inflammatory activity can respond favorably to bDMARDs (6).

While the etiology of RA is not fully clear, it has been suggested that the disease results from the interaction between genetic and environmental factors (8). In recent years, several studies have focused on the gut microbiota as a major pathogenic factor in RA (9). Many studies have suggested that the degree of dysbiosis differs between patients with RA and controls and that microbial diversity is poorer in patients with RA than in controls. Similarly, intestinal dysbiosis and specific uncommon and harmful bacterial lineages have been associated with continuous high inflammatory activity or with the presence of factors affecting the severity of RA (9–12). Data on the effect of DMARDs on the gut microbiota in RA are limited (13), and no studies to date have specifically evaluated the gut microbiota of patients with D2T. In addition, no evidence is available on whether there is an association between continuously high inflammatory activity and high levels of proinflammatory cytokines in patients with D2T RA. Therefore, the primary objective of the present study was to compare the gut microbiota, cumulative inflammatory activity, and other clinical characteristics between patients with D2T RA and patients who respond well to therapy in order to identify microbial profiles and other factors associated with D2T RA.

Abbreviations: 16S rRNA, Ribosomal 16S RNA; anti-TNF, Tumor necrosis factor inhibitors; ASVs, Amplicon sequence variants; bDMARDs, Biological disease-modifying antirheumatic drugs; DAS28-ESR, 28-joint Disease Activity Score for Rheumatoid Arthritis with erythrocyte sedimentation rate; D2T, Difficult to treat; E2T, Easy to treat; EULAR, European Alliance of Associations for Rheumatology; HAQ, Health Assessment Questionnaire; HDL, High-density lipoprotein; hsCRP, High-sensitivity C-reactive protein; IQR, Interquartile range; LDL, Low-density lipoprotein; LDA, Linear discriminant analysis; LEfSe, Linear discriminant analysis Effect Size; PERMANOVA, Permutational multivariate analysis of variance; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin; QIIME2, Quantitative Insights into Microbial Ecology software; RA, Rheumatoid arthritis; scDMARDs, Conventional synthetic disease-modifying antirheumatic drugs; SCFA, Short-chain fatty acid; SD, Standard deviation; STAMP, Statistical Analysis of Metagenomics Profiles; tsDMARDs, Targeted synthetic disease-modifying antirheumatic drugs.

Methods

Design, data source, and scope

The data for this controlled cross-sectional study came from a prospective cohort of incident cases recruited between 2007 and 2011 in the Department of Rheumatology, Hospital Universitario Regional de Málaga, Málaga, Spain (9). All the patients were aged ≥18 years, fulfilled the 2010 criteria of the American College of Rheumatology/European League Against Rheumatism for RA (14), and had been diagnosed and treated for the first time during the 12 months after onset of their disease. All participants provided their written informed consent before inclusion. The study was conducted according to the principles of the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of Málaga (Project identification code 4/2016, P19).

Participants and study protocol

Since the creation of the prospective cohort (2007–2011), all patients have been followed up at the outpatient clinic every 3–6 months by a rheumatologist using a systematic clinical data collection protocol. The data collected included inflammatory activity and physical function throughout follow-up.

During 2016–2018, all the patients in the cohort consented to a relevant modification to the protocol aimed at new, broader study objectives that enabled, among other options, the present study. Peripheral venous blood samples were collected after overnight fast, and fecal samples were refrigerated immediately and transported to the laboratory, where they were stored at −80°C for subsequent analysis. The date of this visit was the index date.

At the last visit in 2022 (final visit in the present study), we identified 2 groups of patients with extreme RA phenotypes: (1) a

group comprising patients with difficult-to-treat RA (D2T RA); and (2) a group of easy-to-treat RA (E2T RA) patients at a 2:1 ratio, matched by age, sex, and time since diagnosis.

D2T RA was defined according to the EULAR criteria for D2T RA, as follows: failure of ≥2 biological or targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) (with different mechanisms of action) after failure of a csDMARD (unless contraindicated); signs suggestive of active/progressive disease; and management of signs and/or symptoms perceived as problematic by the rheumatologist and/or the patient (3). E2T RA was defined as treatment throughout follow-up with a single csDMARD and neither active nor progressive disease. Following the EULAR recommendations (3), active/progressive disease was defined as ≥1 of the following: at least moderate disease activity (according to validated composite measures including 28-joint Disease Activity Score for Rheumatoid Arthritis with erythrocyte sedimentation rate [DAS28-ESR] >3.2), signs and/or symptoms suggestive of active disease, inability to taper glucocorticoid treatment (below 7.5 mg/day prednisone or equivalent), rapid radiographic progression, and symptoms that diminish quality of life.

Outcome measures

On the index date, we collected demographic, clinical, and treatment-related data, including comorbid conditions associated with traditional cardiovascular risk factors (smoking, obesity, arterial hypertension, diabetes mellitus, history of cardiovascular disease, and sedentary lifestyle).

Similarly, on the index date, we evaluated inflammatory activity at the visit and calculated the cumulative activity. Activity was estimated as an arithmetic mean of all the values collected regularly since diagnosis (time-averaged disease activity). Inflammatory activity was measured using the DAS28-ESR (range, 0–9.4) (15). A DAS28-ESR value >3.2 was considered high and ≤3.2 was considered low. The erythrocyte sedimentation rate (ESR; mm/h) was measured. High-sensitivity C-reactive protein (hsCRP; mg/L) was measured for all participants using nephelometry (MMAGE-Immunochemistry Systems, Beckman Coulter, Brea, CA, United States). Physical function on the index date was assessed (average value) using the Health Assessment Questionnaire (HAQ).

We recorded the presence of autoantibodies and their titers, as follows: rheumatoid factor (positive if >10 IU/mL) and anti–citrullinated peptide antibody (positive if >20 IU/mL), as well as the presence or absence of radiologic erosions. We recorded all csDMARDs, such as methotrexate, leflunomide, and sulfasalazine, and bDMARDs, such as tumor necrosis factor inhibitors (anti-TNF), tocilizumab, abatacept, and rituximab. We also included tsDMARDs, such as the Janus kinase inhibitors (JAKi) tofacitinib and baricitinib, and glucocorticoids. Adherence to the Mediterranean diet was evaluated using a validated questionnaire in which adherence was defined as a score of >9 out of 14 (16).

Plasma levels of interleukins, human growth factors, and lipoproteins

Inflammatory mediators such as TNF-α, IL-1β, and IL-6 in plasma were quantified using enzyme-linked immunosorbent assay (ELISA) Quantiglo kits (R&D Systems Inc., Minneapolis, United States) according to the manufacturer's instructions. Plasma levels of insulin-like growth factor I were analyzed using ELISA (Mediagnost GmbH., Tuebingen, Germany). Malondialdehyde-oxidized low-density lipoprotein (LDL) was also measured in plasma using an ELISA kit (Biomedica GmbH., Vienna, Austria).

Gut microbiota analysis

DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of DNA were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States). Ribosomal 16S RNA (16S rRNA) gene sequences were amplified from DNA using the Ion 16S Metagenomics Kit (Thermo Fisher Scientific, Waltham, MA, United States). The kit includes 2 primer sets (V2-4-8 and V3–6, 7–9) that selectively amplify the corresponding hypervariable regions of the 16S region in bacteria. Libraries were built with the Ion Plus Fragment Library kit (Thermo Fisher Scientific). Barcodes were added to each sample using the Ion Xpress™ Barcode Adapters kit (Thermo Fisher Scientific). Emulsion PCR and sequencing of the amplicon libraries were performed on an Ion 530 chip (Ion 530™ Chip Kit) using the Ion Chef System and Ion Torrent S5™ system (Ion 510™/520™/530™ Kit-Chef, Thermo Fisher Scientific), respectively, according to the manufacturer's instructions.

Bioinformatic analysis

Base calling and run demultiplexing were performed using Torrent Suite™ Server software (Thermo Fisher Scientific), version 5.4.0, with default parameters for targeted sequencing of 16S (bead loading ≤30, key signal ≤30, and usable sequences ≤30). Quality sequences were further translated into amplicon sequence variants (ASVs) using DADA2 with adapted parameters for Ion Torrent data within the open-source Quantitative Insights into Microbial Ecology software (QIIME2, version 2022.2) (17). Taxonomic assignment was performed through clustering with VSEARCH and the reference base Greengenes version 13_8 at 97% identity. Before analysis, samples with fewer than 1,500 sequences, features with a count sum of less than 10 across all samples, those presented only in one sample, mitochondrial features, and features unidentified at the phylum level were removed in the pre-processing step. Alpha and beta diversities were evaluated using the core-metrics-phylogenetic plugin in QIIME2 after rarefaction to the minimum number of sequences per sample. Alpha diversity was assessed through different indexes, including Pielou-evenness, Faith-PD, observed-features, and Shannon index. Statistically significant differences in alpha diversity between groups were determined by using the Kruskal-Wallis test with a significance level of $p = 0.05$. The beta diversity was assessed using non-phylogenetic Bray–Curtis dissimilarity index. The structure of the gut microbial community of the different groups was examined using PCoA plots for Bray-Curtis distances. The significance of variations among groups was determined using permutational multivariate analysis of variance (PERMANOVA) with a p-value of 0.05. Linear discriminant analysis (LDA) Effect Size (LEfSe) was used

to identify potential biomarker taxa. LEfSe was performed on the online Galaxy web application[1] (18), where data were normalized using counts per million (CPM). The nonparametric factorial Kruskal–Wallis sum rank test was used to detect differential abundant features (at genera, family, class, order, and phylum level) within the two groups. As a last step in the LEfSe analysis, LDA was used to determine the effect size of each differentially abundant feature. The differences in microbial relative abundance were considered significant when the LDA < 2 and $p < 0.05$ (19). The cladogram from the LEfSe method indicates the phylogenetic distribution representing differentially abundant taxonomic groups. The size of each node represents its relative abundance. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin (PICRUSt2) (20) was used to predict metagenome function within QIIME2 with the DADA2 output. MetaCyc pathways (21) were normalized within QIIME2 and further analyzed using the open-source software STAMP (Statistical Analysis of Metagenomics Profiles) with Welch's *t* test option (22).

Statistical analysis

A descriptive analysis of the main outcome measures was performed. Values are expressed as frequencies and percentages or as mean (standard deviation [SD]) or median (interquartile range [IQR]), as applicable. Normality was assessed using the Kolmogorov–Smirnov test. We compared clinical and laboratory characteristics and inflammatory activity between patients with D2T RA and patients with E2T RA using the Pearson χ^2 test or the *t* test, as applicable. A multivariate analysis was performed, as was a Cox regression analysis, to identify factors associated with D2T RA adjusted for disease duration. The variables entered into the models were those that proved to be significant in the bivariate analysis and of clinical interest. Statistical significance was set at $p < 0.05$. The statistical analyzes were performed using IBM SPSS Statistics for Windows, Version 28 (IBM Corp., Armonk, NY, United States).

Results

We included 39 patients with RA, of whom 13 (33%) were D2T RA and 26 (66%) were E2T RA. Most were women (82%), with a mean (SD) age of 55.1 (11.6) years on the index date. In line with the definitions, at the end of follow-up (final visit), all D2T RA patients had been treated with at least 2 different lines of biologic therapy, whereas E2T RA patients had only received treatment with methotrexate, except for 1 patient who was treated with leflunomide owing to intolerance to methotrexate at 10 mg/wk.

Epidemiological and clinical characteristics and comorbid conditions

Table 1 shows the clinical and epidemiological characteristics of D2T RA patients and E2T RA patients on the index date. Data from both groups were consistent for most of the clinical-epidemiological characteristics and comorbid conditions, except that erosions were more frequent in D2T RA patients than in E2T RA patients ($p = 0.021$). There were no significant differences in the studied comorbidities related to cardiovascular risk, osteoporosis, fibromyalgia, or depression between the two groups. As for treatment, most D2T RA patients were receiving treatment with an anti-TNF agent on the index date (69.2%) or an IL-6 inhibitor (23.1%) (Table 1).

However, at the final visit (see Table 2), most of the 13 patients with D2T RA were receiving rituximab (30.8%) or tofacitinib (23.1%) after a mean (SD) of 2.6 (1.3) switches of biologics, with a mean (SD) retention period of 95.8 (56.3) months. In patients with D2T RA, the main reason for switching biological or targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) was loss of efficacy (18/34 treatments used [52%]), followed by insufficient response (11/34 [32%]) and nonserious adverse events (5/34 [14%]).

In contrast, as expected, the 26 E2T RA patients maintained the same csDMARDs throughout follow-up. Similarly, a larger number of patients with D2T RA were receiving glucocorticoids on the index date ($p = 0.018$) and at a higher median dose than the E2T RA patients ($p = 0.040$).

Study of inflammatory factors and cytokines

Of the 39 patients included, 28 (71.8%) were in remission or with low disease activity at the index date according to their DAS28-ESR values, and 30 (76.9) had maintained an average DAS28-ESR value indicating low activity. D2T RA patients had higher average DAS28-ESR values at the cut-off than the E2T RA patients (Table 3). The same was true of physical functioning according to the HAQ. However, laboratory values were generally similar for both groups, except for some notable differences, such as homocysteine ($p = 0.010$) and CRP ($p = 0.029$), which were superior in D2T RA.

Regarding proinflammatory cytokines, D2T RA patients had higher levels of IL-6 ($p = 0.031$), and numerically higher levels of TNF-α ($p = 0.085$). In the case of lipoproteins and human growth factors, the values remained similar in both groups. Additionally, D2T RA patients were similar to those with E2T in their adherence to the Mediterranean diet (69.2% vs. 73.0%; $p = 0.901$).

Comparison of gut microbiota between groups

There were no differences in any of the alpha diversity measured indexes (Pielou's evenness, $p = 0.121$; Faith's phylogenetic diversity, $p = 0.121$; observed features, $p = 0.318$; and Shannon's diversity, $p = 0.101$) between E2T RA and D2T RA patients (Figure 1A). While microbiota populations tended to differ based on beta diversity (Bray–Curtis dissimilarity; PERMANOVA, $p = 0.089$; Figure 1B).

At the phylum level, the dominant bacterial phyla were *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, while *Actinobacteria*, *Synergistetes*, *Lentisphaerae*, and *Verrucomicrobia* accounted for smaller proportions, between 1 and 10%, in both RA groups (Figure 2A). Analysis of the abundance of *Bacteroidetes* and *Firmicutes* did not reveal significant differences between E2T RA and D2T RA

1 http://huttenhower.sph.Harvard.edu/galaxy/

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TABLE 1 Clinical and epidemiological data on the index date.

Variable	D2T RA *n* = 13	E2T RA *n* = 26	*p*-value
Epidemiological characteristics			
Age, years, mean (SD)	52.9 (12.4)	56.7 (11.0)	0.323
Female sex; *n* (%)	11 (84.6)	21 (80.8)	0.768
White race, *n* (%)	13 (100.0)	26 (100.0)	1.000
Smoking			0.598
Never smoked, *n* (%)	5 (38.5)	13 (50.0)	
Ex-smoker, *n* (%)	5 (38.5)	6 (23.1)	
Active smoker, *n* (%)	3 (23.1)	7 (26.9)	
Anthropometric data			
BMI (kg/m^2), mean (SD)	27.9 (4.4)	26.8 (4.0)	0.466
Abdominal circumference, mean (SD)	91.0 (10.4)	88.0 (12.7)	0.466
Hip circumference, mean (SD)	104.8 (11.1)	102.7 (8.8)	0.533
Waist-hip index, mean (SD)	0.86 (0.06)	0.85 (0.09)	0.652
Comorbid conditions			
Arterial hypertension, *n* (%)	4 (30.8)	7 (26.9)	0.801
Diabetes mellitus, *n* (%)	1 (3.8)	0 (0.0)	0.474
Dyslipidemia, *n* (%)	4 (30.8)	5 (19.2)	0.420
Obesity WHO (BMI ≥ 30), *n* (%)	5 (38.5)	6 (23.1)	0.314
Cardiovascular disease, *n* (%)	2 (15.4)	4 (15.4)	1.000
Osteoporosis, *n* (%)	2 (15.4)	3 (11.5)	0.735
Fibromyalgia, *n* (%)	0 (0.0)	2 (7.7)	0.305
Depression, *n* (%)	1 (7.7)	0 (0.0)	0.333
Anxiety-depressive syndrome, *n* (%)	2 (15.4)	2 (7.7)	0.455
Clinical characteristics			
Time since diagnosis of RA, months, median (IQR)	101.3 (85.9–142-0)	100.7 (77.5–132.4)	0.618
Diagnostic delay, months, median (IQR)	13.8 (6.3–24.0)	10.3 (4.6–22.6)	0.627
Erosions, *n* (%)	11 (84.6)	12 (46.2)	0.021
RF > 10, *n* (%)	11 (84.6)	23 (88.5)	0.735
ACPA > 20 U/mL, *n* (%)	12 (92.3)	21 (80.8)	0.401
Elevated ACPA > 340 U/mL, *n* (%)	6 (46.2)	7 (26.9)	0.199
Treatment			
Synthetic DMARDs, *n* (%)	11 (84.6)	26 (100.0)	0.040
Methotrexate, *n* (%)	6 (46.2)	25 (96.2)	<0.001
Leflunomide, *n* (%)	3 (23.1)	1 (3.8)	0.062
Sulfasalazine, *n* (%)	1 (7.7)	0 (0.0)	0.152
Hydroxychloroquine, *n* (%)	1 (7.7)	0 (0.0)	0.152
Biologic DMARDs, *n* (%)	13 (100.0)	0 (0.0)	<0.001
Anti-TNF, *n* (%)	9 (69.2)	0 (0.0)	<0.001
Anti–IL-6, *n* (%)	3 (23.1)	0 (0.0)	0.011
Tofacitinib, *n* (%)	1 (7.7)	0 (0.0)	0.151
Glucocorticoids, *n* (%)	4 (30.8)	1 (3.8)	0.018
Dose of glucocorticoid, median (IQR)	0.0 (0.0–5.0)	0.0 (0.0–0.0)	0.040

RA, rheumatoid arthritis; SD, standard deviation; BMI, body mass index; WHO, World Health Organization; IQR, interquartile range; RF, rheumatoid factor; ACPA, anti–citrullinated peptide antibody; DMARD, disease-modifying antirheumatic drug.

TABLE 2 Treatments at the index date (2016–2018) and the final visit (2022) in D2T RA and E2T RA patients.

Biologic	Time point*	D2T RA *n* = 13	E2T RA *n* = 26	*p*-value
csDMARD, *n* (%)	Index-date	11 (84.6)	26 (100.0)	0.040
	2022	10 (76.9)	26 (100.0)	0.011
Methotrexate, *n* (%)	Index-date	6 (46.2)	25 (96.2)	<0.001
	2022	5 (38.5)	25 (96.2)	<0.001
Leflunomide, *n* (%)	Index-date	3 (23.1)	1 (3.8)	0.062
	2022	3 (23.1)	1 (3.8)	0.062
Sulfasalazine, *n* (%)	Index-date	1 (7.7)	0 (0.0)	0.152
	2022	1 (7.7)	0 (0.0)	0.152
Hydroxychloroquine, *n* (%)	Index-date	1 (7.7)	0 (0.0)	0.152
	2022	1 (7.7)	0 (0.0)	0.152
bDMARD, *n* (%)	Index-date	13 (100.0)	0 (0.0)	<0.001
	2022	13 (100.0)	0 (0.0)	<0.001
Anti-TNF, *n* (%)	Index-date	9 (69.2)	0 (0.0)	<0.001
	2022	2 (15.4)	0 (0.0)	0.040
Anti–IL-6, *n* (%)	Index-date	3 (23.1)	0 (0.0)	0.011
	2022	2 (15.4)	0 (0.0)	0.040
Rituximab, *n* (%)	Index-date	0 (0.0)	0 (0.0)	–
	2022	4 (30.8)	0 (0.0)	0.001
Tofacitinib, *n* (%)	Index-date	1 (7.7)	0 (0.0)	0.151
	2022	3 (23.1)	0 (0.0)	0.011
Baricitinib, *n* (%)	Index-date	0 (0.0)	0 (0.0)	–
	2022	1 (7.7)	0 (0.0)	0.151
Abatacept, *n* (%)	Index-date	0 (0.0)	0 (0.0)	–
	2022	1 (7.7)	0 (0.0)	0.151

RA, rheumatoid arthritis; D2T, difficult-to-treat; E2T, easy-to-treat; DMARD, disease-modifying antirheumatic drug; csDMARD, conventional synthetic DMARD; bDMARD, biologic DMARD. *Treatments recorded at two specific time points: the index date (2016–2018, when blood and stool samples were collected) and the final visit (2022, when the extreme phenotypes were defined).

($p = 0.251$ and $p = 0.486$, respectively; Figure 2B). However, the *Firmicutes/Bacteroidetes* ratio was lower in the D2T RA patients than in the E2T RA patients ($p = 0.011$; Figure 2C).

Additionally, we performed LEfSe to identify changes in the gut microbiota between E2T RA and D2T RA patients. This analysis revealed an enrichment in the phylum *Bacteroidetes* and in the genus *Megasphaera* in the D2T RA group. In contrast, the E2T RA group exhibited a greater abundance of the phylum *Proteobacteria*, the family *Pasteurellaceae* and its genus *Haemophilus,* and the family *Lachnospiraceae* and its genus *Coprococcus* (LDA > 2; $p < 0.05$; Figure 3).

Predicted metabolic profiles of gut microbiota

Metacyc pathway analysis was performed using PiCRUSt2 to increase our understanding of the role of gut microbiota in each of the groups studied. A Kruskal-Wallis analysis showed that 19 pathways differed between the groups ($p < 0.05$). The main pathways affected belonged to Degradation/Utilization/Assimilation pathways and Biosynthesis. In D2T RA patients, increased values were reported for the thiamine diphosphate salvage II pathway (PWY-6897) and the carbohydrate degradation pathway (PWY-6507). Increased values were also reported for E2T RA patients in the remaining pathways, some of which are involved in peptidoglycan biosynthesis and generation of precursor metabolites and energy (PWY0-1586 and REDCITCYC, respectively), and most of which are involved in degradation, specifically, aromatic compound (PWY-6185, PWY-5417, PWY-5431, PWY-7002, PWY5F9-12), toluene degradation (PWY-5178 and PWY-5181), fatty acid and lipid degradation (PWY-6946 and LIPASYN-PWY), carbohydrate degradation (PWY-7644), secondary metabolite degradation (PWY-6507), and nucleoside and nucleotide degradation (PWY-6353) (Figure 4).

Factors associated with D2T RA patients

Table 4 shows the results of the Cox multivariate analysis (DV: D2T RA), in which 39 patients with RA were included over a mean (SD) follow-up of 103.8 (37.8) months. A total of 13/39 patients had D2T RA. The multivariate analysis showed that the *Firmicutes/Bacteroidetes* ratio was associated with a reduced risk of D2T RA (HR,

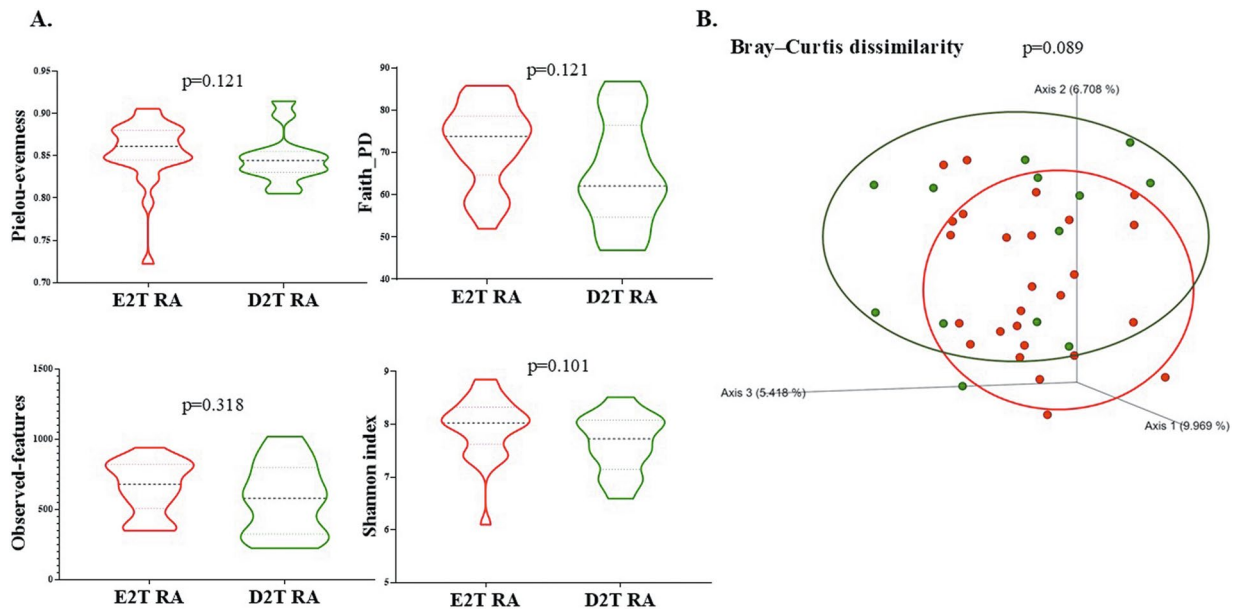

FIGURE 1
Diversity of gut microbiota between E2T RA and D2T RA patients. **(A)** Alpha diversity indexes: Pielou-evenness, Faith-PD, Observed features, and the Shannon index were compared between the 2 groups. Values are presented as mean ± SD. **(B)** Principal coordinates analysis (PCoA) corresponding to the Bray–Curtis dissimilarity index (beta diversity). The statistical analysis used permutational multivariate analysis of variance (PERMANOVA, $p < 0.05$). Green dots indicate the D2T RA patients; red dots the E2T RA patients. E2T RA, easy-to-treat rheumatoid arthritis; D2T RA, difficult-to-treat rheumatoid arthritis.

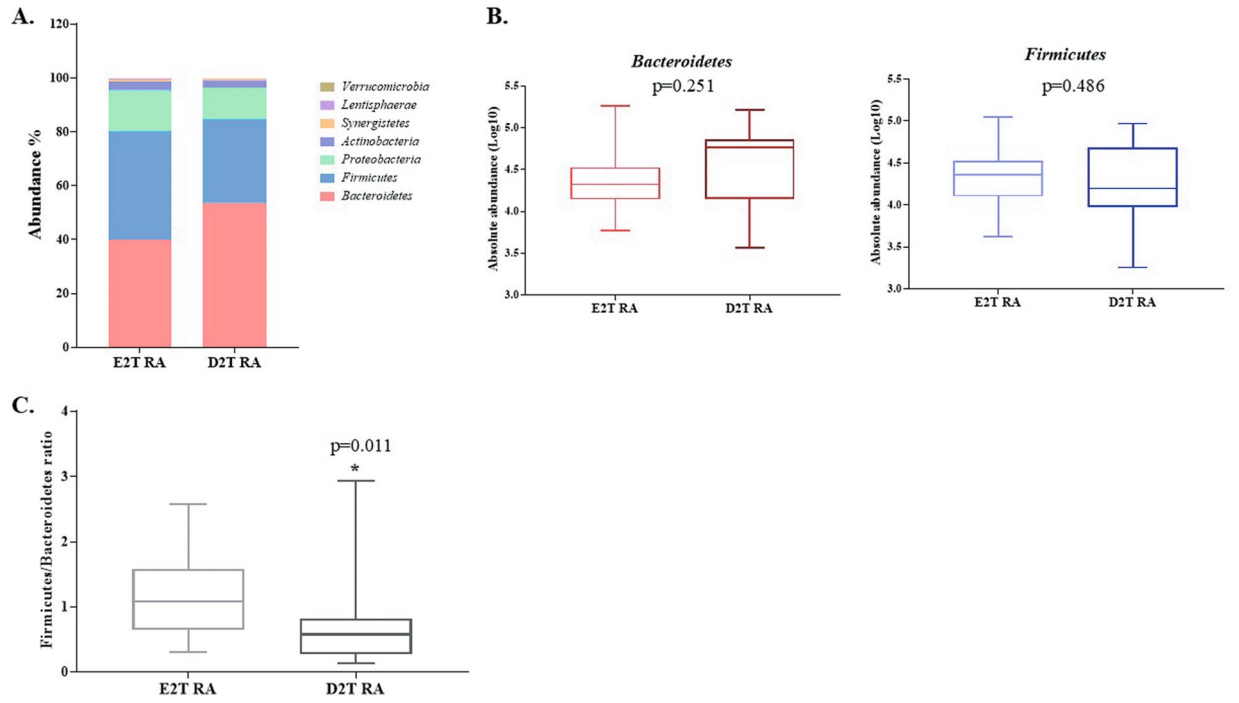

FIGURE 2
Gut microbiota analysis at the phylum level in E2T RA and D2T RA patients. **(A)** The distribution of gut microbiota at the phylum level in both RA groups. **(B)** The absolute abundance in Log10 of the phyla *Bacteroidetes* and *Firmicutes* in both RA groups. **(C)** The *Firmicutes/Bacteroidetes* ratio. * Indicates significant differences between groups ($p < 0.05$). E2T RA, easy-to-treat rheumatoid arthritis; D2T RA, difficult-to-treat rheumatoid arthritis.

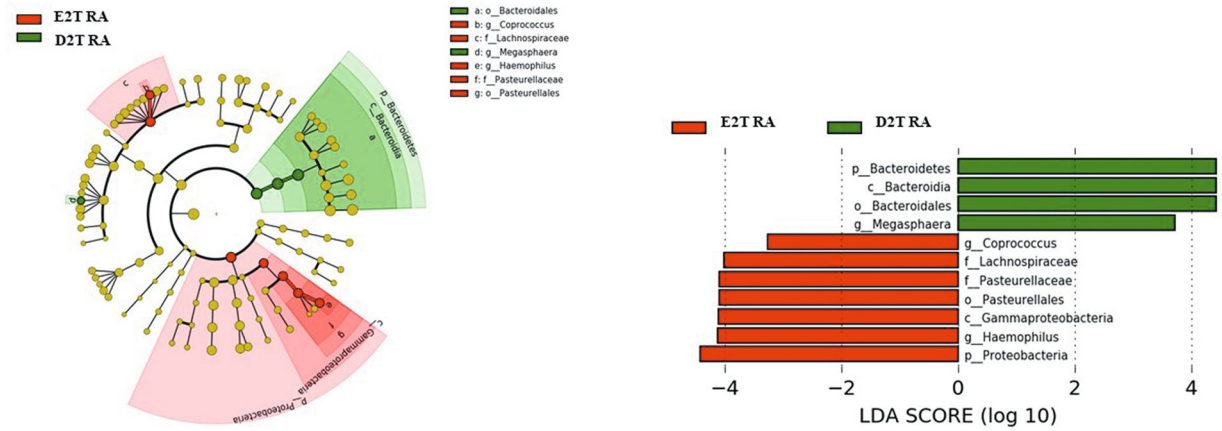

FIGURE 3
(A) Cladogram and **(B)** linear discriminant analysis (LDA) scores were used to determine differences in the abundance of microbes in E2T RA and D2T RA patients. LDA > 2; $p < 0.05$. E2T RA, easy-to-treat rheumatoid arthritis; D2T RA, difficult-to-treat rheumatoid arthritis.

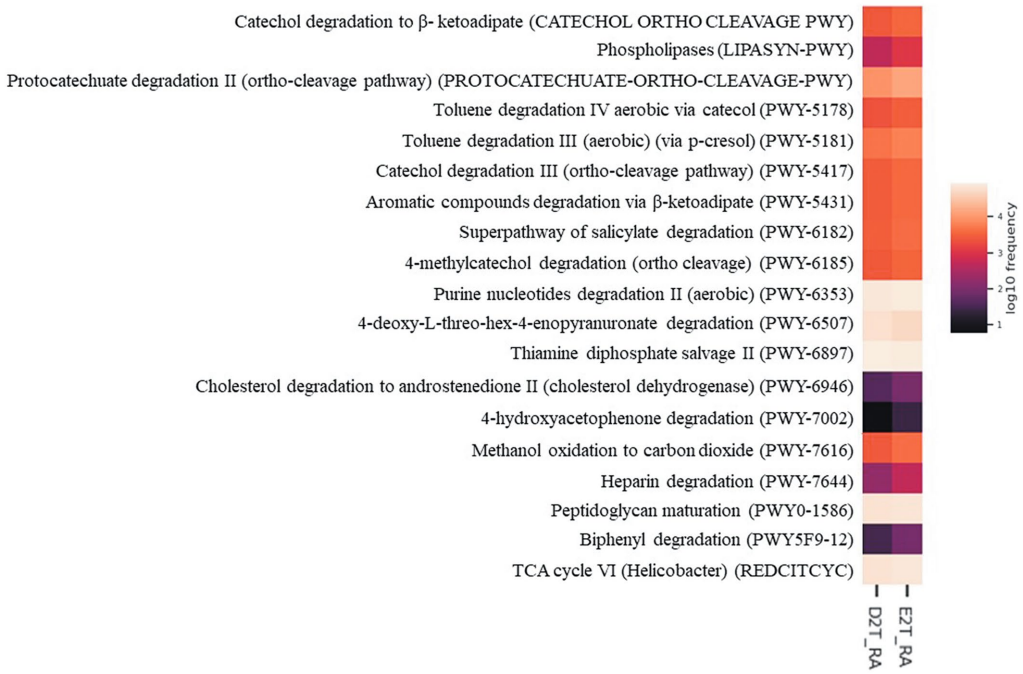

FIGURE 4
Predictive metabolic pathways by PICRUSt2. Heatmap of differentially abundant Metacyc pathways identified in the study groups (E2T RA and D2T RA patients). The values of color in the heatmap represent the normalized relative abundance of Metacyc pathways. Kruskal-Wallis test, $p < 0.05$. E2T RA, easy-to-treat rheumatoid arthritis; D2T RA, difficult-to-treat rheumatoid arthritis.

0.288; 95% CI, 0.092–0.907; $p = 0.033$), whereas the variables associated with a greater probability of D2T RA were greater average inflammatory activity according to the DAS28-ESR (HR, 2.649; 95% CI, 1.225–5.732; $p = 0.013$) and treatment with prednisone on the index date (HR, 3.794; 95% CI, 1.098–10.990; $p = 0.008$). Thus, for each 0.1-point increase in the *Firmicutes*/*Bacteroidetes* ratio, the risk of D2T RA decreased by approximately 71%.

Discussion

Patients with D2T RA continue to display symptoms after several treatment cycles, thus generating a considerable burden in clinical practice (4). Various factors are thought to affect the persistence of signs and symptoms in affected patients, which is rarely caused only by resistance to therapy (2). Today, however,

TABLE 3 Inflammatory factors and cytokines in D2T RA and E2T RA.

Variable	D2T RA *n* = 13	E2T RA *n* = 26	*p*-value
Inflammatory activity			
DAS28 average value, mean (SD)	3.3 (1.1)	2.4 (0.6)	0.003
Remission-low activity, *n* (%)	6 (46.2)	22 (84.6)	0.012
Moderate-high activity, *n* (%)	7 (53.8)	4 (15.4)	0.012
DAS28 at index-date, mean (SD)	3.0 (0.4)	2.5 (0.5)	0.016
Remission-low activity, *n* (%)	7 (53.8)	23 (88.5)	0.018
Moderate-high activity, *n* (%)	6 (46.2)	3 (11.5)	0.018
HAQ average value, mean (SD)	1.0 (0.7)	0.5 (0.5)	0.026
HAQ at index date, mean (SD)	1.1 (0.3)	0.8 (1.1)	0.516
Laboratory parameters			
ESR, mm/h, median (IQR)	16.0 (5.57–23.0)	12.0 (8.7–17.2)	0.603
hsCRP, mg/L, mean (SD)	5.6 (3.4)	3.7 (1.9)	0.029
Hemoglobin, g/dL, median (IQR)	12.8 (11.5–13.7)	13.5 (12.4–14.1)	0.066
Leukocytes, 10^9/L, median (IQR)	5960.0 (4695.0–7380.0)	6375.0 (5150.0–6897.5)	0.848
Platelets, 10^9/L, mean (SD)	291769.2 (92585.2)	240500.0 (53234.7)	0.083
Creatinine, mg/dL, mean (SD)	0.7 (0.1)	0.7 (0.1)	0.443
Total cholesterol, (mg/dL), mean (SD)	197.5 (28.8)	199.0 (45.3)	0.914
LDL cholesterol, mg/dL, median (IQR)	113.0 (90.0–135.0)	109.8 (92.9–126.7)	0.988
HDL cholesterol, mg/dL, median (IQR)	60.0 (53.5–75.0)	59.5 (54.0–81.5)	0.980
Triglycerides, mg/dL, median (IQR)	86.0 (66.0–112.0)	72.5 (64.0–89.6)	0.384
Homocysteine, mg/L, median (IQR)	18.1 (13.7–20.9)	12.0 (10.5–13.7)	0.010
Interleukins, lipoproteins, and human growth factors			
IL-6, pg./mL, median (IQR)	13.8 (9.9–60.2)	6.6 (4.1–10.6)	0.031
IL-1β, pg./mL, median (IQR)	4.2 (4.1–4.3)	4.3 (4.1–4.4)	0.473
TNF-α, pg./mL, median (IQR)	8.6 (3.7–267.5)	4.6 (3.6–6.1)	0.085
IGF-1, pg./mL, median (IQR)	138.5 (71.2–316.5)	177.9 (108.5–217.2)	0.872
Oxidized LDL (IU/mL), median (IQR)	1.0 (0.5–3.7)	3.3 (0.8–5.7)	0.343

RA, rheumatoid arthritis; DAS28, 28-joint Disease Activity Score; SD, standard deviation; D2T, difficult-to-treat; E2T, easy-to-treat; HAQ, Health Assessment Questionnaire; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; IGF-1, insulin-like growth factor-1; ESR, erythrocyte sedimentation rate; IL-6, interleukin 6; LDL, low-density lipoprotein; TNF, tumor necrosis factor.

TABLE 4 Cox regression model of factors for RA patients with refractory disease.

Dependent variable	Predictor	HR	95% CI for B	*p*-value
D2T RA*				
	Average DAS28-ESR	2.649	1.225–5.732	0.013
	Prednisone	3.794	1.098–10.990	0.008
	Firmicutes/*Bacteroidetes* ratio	0.288	0.092–0.907	0.033

*Refractory disease: failure of ≥ 2 lines of biologic therapy.
Variables included in the equation: sex, age, erosions, average DAS28-ESR, C-reactive protein, prednisone, body mass index, Mets, Firmicutes/Bacteroidetes ratio.

there is little evidence indicating the particular characteristics, mechanisms, and factors associated with D2T RA, thus further hampering patient management (2). In an attempt to address this unmet need, the present study compares the gut microbiota profile, cumulative disease activity, and other severity-related factors between patients with D2T RA and patients with E2T RA in order to identify the intestinal microbiota profile and other factors associated with this major problem. Non-responder patients do not achieve adequate control with any treatment, while D2T RA patients have failed to respond to two or more biologics or targeted

synthetic DMARDs, highlighting their clinical profile and the need for tailored therapeutic strategies.

The present study revealed that although gut microbiota populations are characterized by similar features, their abundance differed between the 2 groups. Differences were recorded in the abundance of various features, such as the families *Lachnospiraceae* and *Pasteurellaceae* and their respective genera *Coprococcus* and *Haemophilus*, which were more abundant in E2T RA patients than in D2T RA patients, in fact *Haemophilus* is implicated in RA (23). On the other hand, an increase in the abundance of *Coprococcus* has previously been observed in RA patients after therapy with methotrexate (24) and with sulfasalazine (25). Moreover, the *Lachnospiraceae* family is a major producer of butyrate (a short-chain fatty acid [SCFA]) which has beneficial effects on RA (26). In addition, a recent study (25) showed a rise in the number of SCFA-producing genera after patients with RA were treated with TNF inhibitors. These observations suggest that alterations in gut microbiota could contribute to the therapeutic effects of bDMARDs. Furthermore, this study found that the genus *Megasphaera* was more abundant in D2T RA patients. Indeed, this genus has been shown to be positively related to RA with greater abundances (27, 28).

Regarding the phyla *Firmicutes* and *Bacteroidetes*, LEfSe revealed enrichment of the phylum *Bacteroidetes* in the D2T RA group. In this line, we found that the *Firmicutes*/*Bacteroidetes* ratio was lower in D2T RA patients than in E2T RA patients. This ratio, although controversial, has been shown to be associated with different diseases; for instance, it is decreased in RA patients (29). Moreover, our multivariate analysis showed that the phylum *Bacteroidetes* was associated with a greater risk of E2T RA, whereas the phylum *Firmicutes* acted as a protective factor. The phylum *Bacteroidetes* was found to be less abundant in treated patients with established RA than in healthy controls (30, 31). In fact, as shown by Zhang et al. (32), levels of the genus *Bacteroides* were further reduced after treatment with DMARDs, especially methotrexate, which reverses the perturbations of the microbiota typically associated with RA (33, 34). In fact, several studies demonstrated that abundance of *Firmicutes* was increased while *Bacteroidetes* was decreased after treatment with methotrexate (32, 35). These changes in levels of *Bacteroidetes* could be associated with the stage of development of RA and with the response to treatment. Thus, the absence of response in patients with D2T RA could be associated with the increase we observed for the phylum *Bacteroidetes*. Moreover, in situations that alter intestinal acidity or composition, values for the phylum *Firmicutes* would decrease, thus leading to an increase in values for acetate- and propionate-producing *Bacteroidetes* (36, 37). The butyrate deficiency following the decrease in *Firmicutes* leads to a deficiency in mucin and an increase in intestinal permeability, which in turn induces a chronic inflammatory state (38). Therefore, this could be one of the factors affecting the inadequate response to treatment in patients with RA in our study.

According to the microbial metabolism approach, which was based on the inference of the metabolic pathways taken by the gut microbiota using PICRUSt2, no notable differences were found between the 2 groups studied. However, the main pathways implicated indicate that degradation is a major contributor to the results, including degradation of aromatic compounds (e.g., catechol and toluene, carbohydrates, and fatty acids), which provide energy, and that degradation was more pronounced in E2T RA patients than in D2T RA patients. Likewise, purine metabolism was increased in the E2T RA patients compared to D2T RA patients. The purine pathway plays an important role in intestinal permeability; specifically, purines help maintain a healthy energy balance and contribute to the restoration of the gut barrier (39). The findings reported may provide insight into how gut microbiota composition and metabolic activity differ between patients who respond and do not respond to treatment. This in turn could prove useful for developing targeted therapies and improving our understanding of disease mechanisms.

We also observed that patients with D2T RA had a higher average DAS28-ESR, poorer physical function according to HAQ, and higher values for inflammatory factors such as CRP and IL-6 than patients with E2T RA. Regarding these factors, the multivariate analysis showed a significant association between the average DAS28-ESR value and D2T RA. Several studies have reported that patients with D2T RA are characterized by greater inflammatory activity according to indices such as DAS28, both at initiation of biologics (40, 41) and during switches in biologic therapy over time (42). This finding could be explained by the low rates of response to treatment in patients with greater inflammatory activity (43, 44) and by the difficulty encountered when attempting to reduce very high DAS28 values until remission is achieved. Other factors that could affect higher disease activity indexes in these patients include pain and a more negative global evaluation owing to chronic disease with structural damage that has not been easily controlled. The latter observation could arise from the association between treatment with glucocorticoids and D2T RA revealed by our multivariate analysis. Reducing doses of glucocorticoids to below 5–10 mg/d has proven difficult in patients with D2T RA (4), probably because of the greater inflammatory activity. Moreover, it is worth noting that the concomitant use of glucocorticoids has been associated with severe adverse reactions and interruption of some biologics such as anti-TNF agents and anti–IL-6 agents, mainly owing to infection (45, 46).

Our study has both strengths and limitations. First, we performed a cross-sectional analysis of microbiota and inflammatory cytokines based on a single determination. However, the patients belonged to a prospective RA cohort in which all inflammation- and treatment-related data were collected longitudinally following a predetermined protocol. Second, the sample of patients with D2T RA is small, potentially limiting the possibility of detecting differences between the groups. In order to mitigate this problem, we selected 2 comparators with extreme phenotypes and twice the number of E2T RA patients per case of D2T RA. This approach enabled us to demonstrate significant differences in the main hypotheses proposed. Furthermore, while the definition of D2T RA has varied over time, we used the definition recommended by EULAR (3), which serves as a basis for most published studies. While we acknowledge that including a healthy control group would strengthen our findings, our study primarily focused on comparing D2T and E2T RA patients. Likewise, the higher proportion of female participants in our cohort may introduce confounding factors, such as hormonal influences on disease activity. Additionally, although we did not

assess dietary components in detail, we observed that a high percentage of patients in both groups adhered to the Mediterranean diet. Finally, it is important to remember that other factors may affect D2T RA and have not been the object of this study, for example, metabolic differences between conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and biological or targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs) or adherence to treatment. Nevertheless, we identified and described, for the first time, the association between microbiota-related factors and D2T RA by combining these findings with other clinical characteristics.

Conclusion

This study found that the gut microbiota profile differs between D2T RA and E2T RA patients. Specifically, patients with D2T RA were characterized by enrichment of the phylum *Bacteroidetes* and the genus *Megasphaera*, whereas in patients with E2T RA, the phylum *Proteobacteria*, the family *Pasteurellaceae* and its genus *Haemophilus*, and the family *Lachnospiraceae* and its genus *Coprococcus* were more abundant. The *Firmicutes*/*Bacteroidetes* ratio was lower in patients with D2T RA. In addition, an increase in this ratio was seen to be an independent factor for reduced risk of D2T RA, suggesting that gut dysbiosis plays a role in nonresponse to treatment. Moreover, the above-mentioned metabolic pathway analysis revealed differences in the pathways involved in degradation of aromatic compounds, carbohydrates, and fatty acids between D2T RA and E2T RA patients. Greater inflammatory activity and use of prednisone were associated with D2T RA. The identification of new factors associated with D2T RA is a relevant finding that enhances our knowledge of patients with this disease, which is currently a severe problem with high social and health care costs. A more individualized approach including these factors can improve outcomes and reduce the risk of adverse effects of medication.

Data availability statement

The datasets presented in this article are not readily available because according to the data regulations and ethical considerations, the datasets generated and analyzed during our study cannot be made public due to the fact participants only provided their consent to the original team of investigators for the use of their data, and this information may compromise their consent to participate in the study. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Research Ethics Committee of Hospital Regional Universitario de Málaga (HRUM) (Project identification code 4/2016, P19). 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

PR-L: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. NM-V: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. IM-I: Investigation, Writing – review & editing. JL-M: Investigation, Writing – review & editing. AM: Investigation, Writing – review & editing. SM-A: Investigation, Writing – review & editing. RR-R: Investigation, Writing – review & editing. LC-G: Investigation, Writing – review & editing. FT: Conceptualization, Supervision, Writing – review & editing. AF-N: Conceptualization, 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.

Publisher's note

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Efferocytosis and inflammation: a bibliometric and systematic analysis

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Objective: To visualize and analyze the trends and hotspots of efferocytosis and inflammation via bibliometric methods.

Methods: Relevant articles and reviews from 2006 to 2023 were retrieved from the Web of Science Core Collection. The data were processed with CiteSpace, and some graphs were generated with Microsoft Excel (version 2016), VOSviewer, Scimago Graphica, Bibliometrix and R Studio.

Results: A total of 1,003 papers were included, revealing a significant upward trend in efferocytosis and inflammation research. The United States (456, 45.46%), China (164, 16.35%) and the United Kingdom (99, 9.87%) were the three countries with the highest numbers of publications. Harvard University (84, 6.74%) contributes the most out of the top 5 institutions. Among the researchers in this field, Serhan CN was the author with the highest number of articles in the field (35, 3.49%), and deCathelineau AM first named "efferocytosis" in 2003. Keyword analysis identified "activation," "tam receptors," "docosahexaenoic acid" "systemic lupus erythematosus," "myocardial infarction" and "alveolar macrophages" as core topics, indicating a concentrated trend in the mechanism of physiological state and inflammatory diseases such as autoimmune, cardiovascular, and pulmonary diseases. The latest surge words "inflammation resolution" and "cancer" in the keyword heatmap indicate future research directions.

Conclusion: Research on the association between efferocytosis and inflammation has been a promising field. Key areas of focus include the crucial role of efferocytosis on tissue homeostasis and the pathogenesis of nontumorous inflammatory diseases. Future research will likely continue to explore these frontiers, with an emphasis on understanding efferocytosis in the context of chronic diseases and cancer, as well as developing novel therapeutic strategies.

KEYWORDS

efferocytosis, inflammation, bibliometrics, molecular mechanism, mesenchymal stem cells, nontumorous inflammatory diseases, cancer

1 Introduction

Billions of cells die every day in the human body (1). Efferocytosis, the process by which dying or dead cells are cleared by phagocytes, is crucial for maintaining tissue homeostasis and preventing inflammation. Professional phagocytes, such as macrophages and dendritic cells, are well-equipped with specific receptors and signaling pathways that facilitate the recognition and engulfment of apoptotic cells. Non-professional phagocytes, including epithelial cells and fibroblasts, can also participate in efferocytosis, though their mechanisms are less specialized (72). Efferocytosis is a cooperative process between phagocytes and apoptotic cells, regulated by signaling molecules known as "find-me" and "eat-me" signals. Phagocytes express receptors that recognize apoptotic ligands and interact with the cytoskeleton to bind to them, inducing phagosomes-lysosome fusion to degrade apoptotic cells (2). As apoptotic cells are phagocytosed, macrophages inhibit the production of inflammatory factors and mediate the repair process (3).

When efferocytosis is impaired, many apoptotic cells cannot be removed promptly and can accumulate in the body. This process is followed by secondary necrosis, rupture of cell membranes and the release of cellular contents, such as damage-associated molecular patterns (DAMPs) (4). Release of cellular contents triggers inflammation and the immune response, and leads to chronic inflammatory diseases and autoimmune disorders (4), such as atherosclerosis (5), obstructive pulmonary disease (6), rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, and inflammatory bowel disease (7).

Bibliometrics is used to explore emerging trends in a specialized field (8), and the most commonly used bibliometric tools for its visualization are CiteSpace and VOSviewer, both of which are widely used in fields such as medicine, biology, and immunology (9, 10). Only few articles has revealed research trends in the field of efferocytosis (11, 12), but no bibliometric study has systematically characterized the relationship between efferocytosis and inflammation. Our study highlights the research hotspots and academic trends in this field for researcher with emphasis on the role of efferocytosis in the pathogenesis of inflammatory diseases and autoimmune diseases. We hope that these findings will provide new insights for future drug development and disease treatment.

2 Methods

2.1 Data collection

The data were obtained from the Web of Science Core Collection (WoSCC), with the following search formula: TS = (efferocytosis) AND TS = (inflammatory OR inflammation OR inflammations); the type was limited to treatises and reviews; the language was limited to English; and the search was conducted on December 03, 2023. The search process is shown in Figure 1. A total of 1,003 papers were included, of which 740 were treatises and 263 were reviews.

2.2 Data analysis

The retrieved literature data were imported into CiteSpace (version 6.2.R6) for further analysis (13). The exact parameters were set as follows: method (LLR), time slicing (January 2006–December 2023), year/slice(1), term source (title, abstract, author keywords, and keyword plus), and node type (keyword).

We evaluated the number of publications, major countries, research institutions, authors and keywords in the field of efferocytosis and inflammation research. We analyzed highly cited articles, and conducted co-occurrence analysis, clustering analysis and burst visualization of keywords. The keyword co-occurrence network consisted of nodes and connecting lines. The larger the nodes are, the more articles there are in that research direction, and the thicker the connecting lines are, the closer the association. Keyword clusters are network groups composed of keywords with similar research topics, reflecting the evolution of topics in the field over a certain time interval. The keyword timeline cluster graph introduces time into the network, presenting the historical trajectory and time span of the keyword evolution in each cluster. The results of the keyword bursts indicate a sharp increase in the intensity of a research direction over time and are used to identify research hotspots. Graphs were also generated via Microsoft Excel (version 2016), Scimago Graphica, Bibliometrix and R Studio.

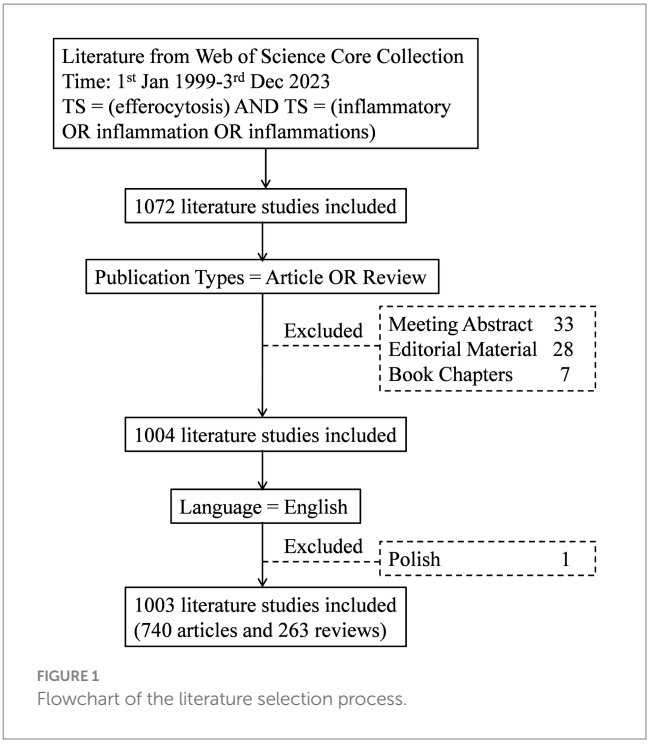

FIGURE 1
Flowchart of the literature selection process.

3 Results

3.1 The global growth trend of publications

The number of publications is an essential indicator of the development trend of the research field. A total of 1,003 papers cited 45,109 publications, with an average of 39.86 citations and an h-index of 100 citations. Figure 2 shows that annual publications in the field rose from 4 in 2006 to 143 in 2022, and annual citations grew from 6 in 2006 to 7,496 in 2022. These studies spanned 74 research areas:

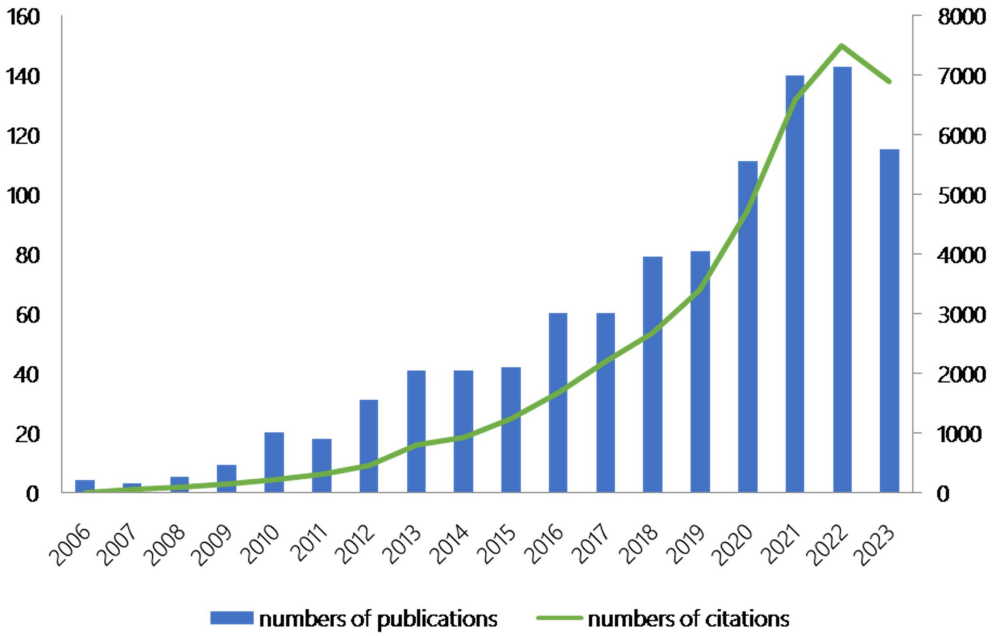

FIGURE 2
Publications and citations over time (2006–2023).

"Immunology" (267, 26.62%) and "Cell Biology" (210, 20.94%) were published most frequently. Other popular research areas included "Biochemistry Molecular Biology" (156, 15.55%), "Pharmacology Pharmacy" (91, 9.07%) and "Medicine Research Experimental" (83, 8.28%).

3.2 Analysis of country contributions, institutions and authors

The United States ranked first in terms of the number of publications (456, 45.46%), followed by China (164, 16.35%) and the United Kingdom (99, 9.87%). Moreover, the majority of research collaborations centered between North America and Europe and between North America and East Asia (Supplementary Figure S1).

According to Supplementary Table S1, Harvard University was the institution that published the most papers (84, 6.74%). The 3 authors with the greatest number of publications were Serhan CN (35, 3.49%), Tabas I (29, 2.89%) and Dalli J (26, 2.59%). Among them, Serhan CN and Dalli J are more collaborative (14–16).

3.3 Analysis of journals and cocited journals

As shown in Supplementary Table S2, the largest number of publications (83, 8.28%) were from Frontiers in Immunology (7.3, Q1, from 2022), with a total of 2,545 citations. There were 14 articles published in Circulation Research (20.1, Q1, from 2022), with 1,287 citations. The favorite categories are Molecular/Biology/Immunology/Genetics journals (Supplementary Figure S2).

3.4 Analysis of research topics and frontiers

3.4.1 Cluster network of the top 10 cited references and cocited references

Table 1 lists the top 10 most highly cited articles in the field of efferocytosis and inflammation. These highly cited articles suggest that scholars are interested in the association between efferocytosis and inflammation, with an emphasis on cell biological mechanisms and the associations with cardiovascular disease, lung disease and tissue repair.

Cluster analysis of literature cocitations provides an objective reflection of the knowledge structure in the research area. Figure 3A shows that highly cited works, such as those by Poon et al. (72), are prominently displayed, indicating their significant influence in the field. Cluster #0 is the largest category, namely lipoxin, followed by resolving (Cluster #1), phosphatidylserine (Cluster #2), high mobility group box 1 protein (Cluster #3), chronic obstructive pulmonary disease (Cluster #4), atherosclerosis (Cluster #5), resolution of inflammation (Cluster #6) and Tam receptors (Cluster #7). These findings reveal that the research hotspots are focused mostly on proinflammatory mediators of efferocytosis and their conduction pathways, which is consistent with the research hotspots. Figure 3B shows the top 25 cocited references with strong bursts, and the first cocitation was initiated in 2006. It was published in *Cell* in October 2005 and was titled "Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte." The strongest intensity of the burst was "Burying the dead—The impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease," published in *Chest* in June 2006 by Vandivier RW. Overall, 4 publications describing the burst status were published in 2023, which suggests that future research on efferocytosis and inflammation will continue to evolve.

TABLE 1 Top 10 cited references related to efferocytosis and inflammation.

Ranking	Title	Corresponding authors	Year	Journal	Citations	IF[a]	JCR-c
1	Embryonic and Adult-Derived Resident Cardiac Macrophages Are Maintained through Distinct Mechanisms at Steady State and during Inflammation	Epelman S	2014	Immunity	932	21.6	Q1
2	Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities	Back M	2019	Nature Reviews Cardiology	729	20.3	Q1
3	Apoptosis and Clearance of Apoptotic Cells	Nagata S	2018	Annual Review of Immunology	530	21.4	Q1
4	Resolution of inflammation: an integrated view	Ortega-Gomez A	2013	Embo Molecular Medicine	499	8.2	Q1
5	Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages toward a reparative phenotype	Horckmans M	2017	European Heart Journal	442	23.4	Q1
6	Macrophage Dysfunction Impairs Resolution of Inflammation in the Wounds of Diabetic Mice	Khanna S	2010	Plos One	432	4.4	Q2
7	Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators	Dalli J	2012	Blood	394	9.1	Q1
8	Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain	Serhan CN	2012	Faseb Journal	345	5.7	Q1
9	Burying the dead—The impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease	Vandivier RW	2006	Chest	331	3.9	Q1
10	Efferocytosis in Health and Disease	Doran AC	2020	Nature Reviews Immunology	329	53.1	Q1

[a]IF (Impact Factor): All of the above impact factor was from the year the article published.

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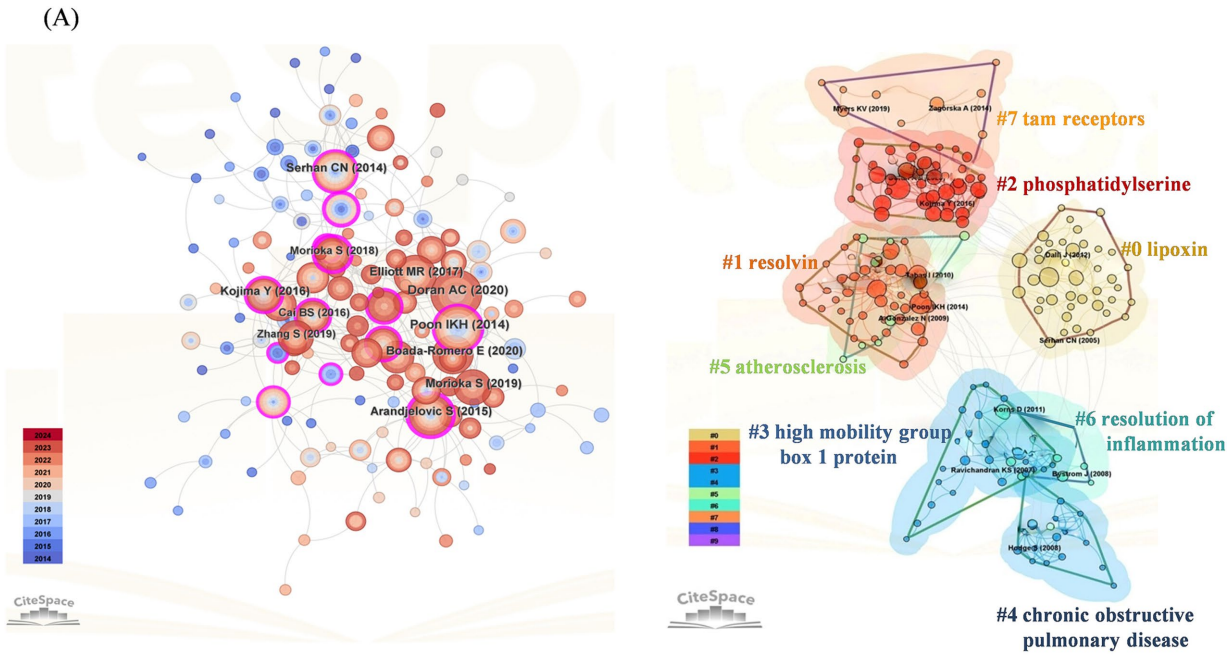


(B)

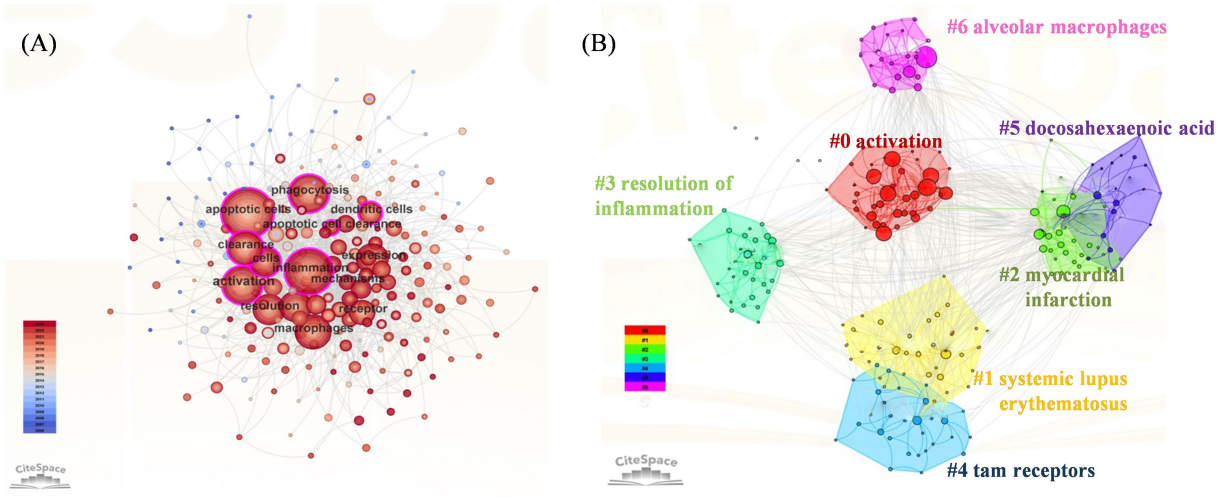
Top 25 References with the Strongest Citation Bursts

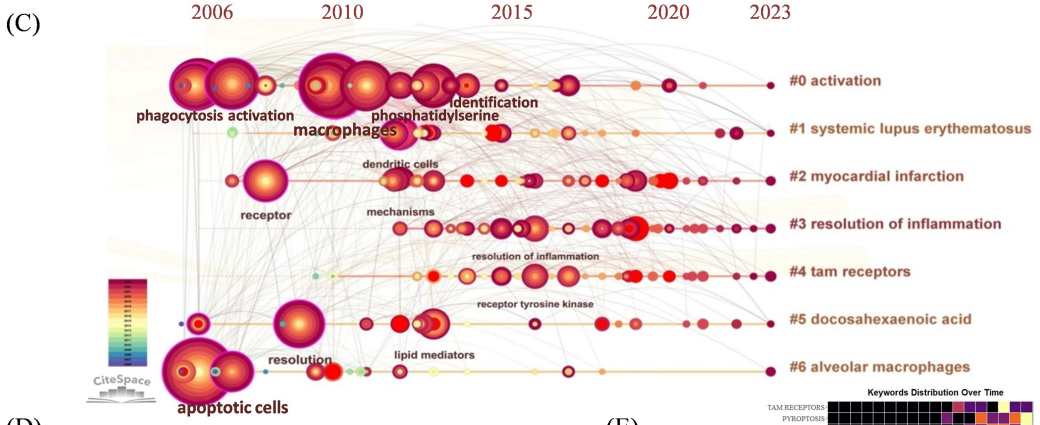
References	Year	Strength	Begin	End	2006 - 2023
Gardai SJ, 2005, CELL, V123, P321, DOI 10.1016/j.cell.2005.08.032, DOI	2005	9.74	2006	2012	
Vandivier RW, 2006, CHEST, V129, P1673, DOI 10.1378/chest.129.6.1673, DOI	2006	19.72	2007	2014	
Serhan CN, 2005, NAT IMMUNOL, V6, P1191, DOI 10.1038/ni1276, DOI	2005	11.23	2010	2013	
Ravichandran KS, 2007, NAT REV IMMUNOL, V7, P964, DOI 10.1038/nri2214, DOI	2007	10.75	2010	2012	
Bystrom J, 2008, BLOOD, V112, P4117, DOI 10.1182/blood-2007-12-129767, DOI	2008	9.57	2010	2015	
Schwab JM, 2007, NATURE, V447, P869, DOI 10.1038/nature05877, DOI	2007	9.56	2012	2014	
Tabas I, 2010, NAT REV IMMUNOL, V10, P36, DOI 10.1038/nri2675, DOI	2010	9.24	2012	2018	
Serhan CN, 2011, CHEM REV, V111, P5922, DOI 10.1021/cr100396c, DOI	2011	8.37	2012	2016	
Krishnamoorthy S, 2010, P NATL ACAD SCI USA, V107, P1660, DOI 10.1073/pnas.0907342107, DOI	2010	12.62	2013	2016	
Dalli J, 2012, BLOOD, V120, PE60, DOI 10.1182/blood-2012-04-423525, DOI	2012	12.02	2013	2016	
Chiang N, 2012, NATURE, V484, P524, DOI 10.1038/nature11042, DOI	2012	9.3	2013	2016	
Schif-Zuck S, 2011, EUR J IMMUNOL, V41, P366, DOI 10.1002/eji.201040801, DOI	2011	8.98	2013	2018	
A-Gonzalez N, 2009, IMMUNITY, V31, P245, DOI 10.1016/j.immuni.2009.06.018, DOI	2009	11.45	2014	2017	
Thorp E, 2008, ARTERIOSCL THROM VAS, V28, P1421, DOI 10.1161/ATVBAHA.108.167197, DOI	2008	9.42	2014	2016	
Poon IKH, 2014, NAT REV IMMUNOL, V14, P166, DOI 10.1038/nri3607, DOI	2014	13.12	2015	2019	
Serhan CN, 2014, NATURE, V510, P92, DOI 10.1038/nature13479, DOI	2014	9.37	2015	2019	
Korns D, 2011, FRONT IMMUNOL, V2, P0, DOI 10.3389/fimmu.2011.00057, DOI	2011	14.39	2016	2019	
Thorp E, 2011, J BIOL CHEM, V286, P33335, DOI 10.1074/jbc.M111.263020, DOI	2011	11.01	2016	2019	
Hochreiter-Hufford A, 2013, CSH PERSPECT BIOL, V5, P0, DOI 10.1101/cshperspect.a008748, DOI	2013	11.18	2017	2018	
Ortega-Gómez A, 2013, EMBO MOL MED, V5, P661, DOI 10.1002/emmm.201202382, DOI	2013	11.07	2017	2020	
Lemke G, 2013, CSH PERSPECT BIOL, V5, P0, DOI 10.1101/cshperspect.a009076, DOI	2013	9.28	2018	2020	
Morioka S, 2018, NATURE, V563, P714, DOI 10.1038/s41586-018-0735-5, DOI	2018	8.52	2020	2023	
Doran AC, 2020, NAT REV IMMUNOL, V20, P254, DOI 10.1038/s41577-019-0240-6, DOI	2020	19.63	2021	2023	
Boada-Romero E, 2020, NAT REV MOL CELL BIO, V21, P398, DOI 10.1038/s41580-020-0232-1, DOI	2020	17.32	2021	2023	
Yurdagul A, 2020, CELL METAB, V31, P518, DOI 10.1016/j.cmet.2020.01.001, DOI	2020	12.17	2021	2023	

FIGURE 3
(A) Cocited references co-occurrence network and cluster analysis. The left subpanel visualizes the network of co-cited references, with node size indicating the citation frequency and node color representing the year of publication. The right subpanel highlights the major research themes identified through cluster analysis, with 8 clusters in total and distinguished by different colors. Cluster #0 is the largest. **(B)** Top 25 references with the strongest citation bursts. The blue line indicates the time-lapse, and the red line indicates the duration of the quote burst, which shows the progression of cutting-edge hot topics.

3.4.2 Keywords co-occurrence

Co-occurrence refers to the occurrence of two or more keywords in the same article, thus, the keyword co-occurrence figure is plotted according to the frequency of keyword co-occurrence in the cited articles. As shown in Figure 4A, in addition to "efferocytosis" (105) and "inflammation" (237), the keywords with a co-occurrence frequency of more than 100 were "apoptotic cells" (261), "phagocytosis" (186), "activation" (155), "expression" (149), "clearance" (146), "macrophages" (142), "resolution" (130) and "receptor" (106).





(D)

Top 25 Keywords with the Strongest Citation Bursts

Keywords	Year	Strength	Begin	End	2006 - 2023
alveolar macrophages	2006	6.24	2006	2014	
phosphatidylserine receptor	2006	4.58	2006	2011	
in-vitro	2006	4.39	2006	2015	
clearance	2006	4.34	2006	2012	
alveolar macrophage	2007	3.71	2007	2012	
acute lung injury	2008	3.65	2008	2011	
obstructive pulmonary disease	2010	7.8	2010	2016	
neutrophils	2010	6.04	2010	2012	
apoptotic cells	2006	3.63	2010	2011	
docosahexaenoic acid	2012	8.31	2012	2016	
alternative activation	2013	4.81	2013	2015	
gene-expression	2013	3.84	2013	2014	
resolvin d1	2013	3.13	2013	2016	
lipid mediators	2013	6.2	2014	2017	
human phagocytes	2014	5.09	2014	2016	
identification	2014	3.3	2014	2016	
systemic lupus erythematosus	2015	3.72	2015	2020	
cardiovascular-disease	2015	3.22	2015	2018	
nf kappa b	2010	3.85	2018	2020	
pro resolving mediators	2018	3.62	2018	2019	
plaque necrosis	2018	3.38	2018	2020	
growth	2019	3.74	2019	2021	
nlrp3 inflammasome	2019	3.5	2019	2020	
axl	2020	3.21	2020	2021	
inflammation resolution	2020	3.13	2020	2023	

(E)

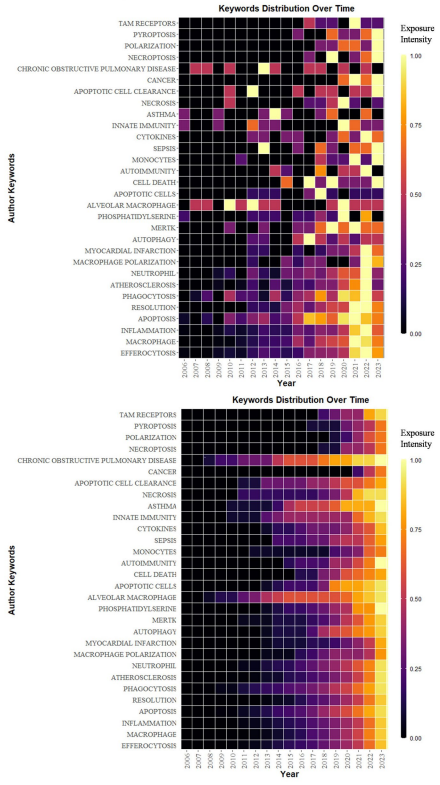


FIGURE 4
(A) Keywords co-occurrence network. The nodes represent keywords, and the larger nodes indicate more research articles. **(B)** Keyword cluster analysis. There are 7 clusters in total and are distinguished by different colors. Cluster #0 is the largest one. **(C)** Timeline view of keyword cluster from

(Continued)

FIGURE 4 (Continued)
2006 to 2023. The length of the horizontal straight line of a cluster indicates its time frame. Nodes and labels represent keywords that have been cited at least 45 times. **(D)** Top 25 keywords with the strongest citation bursts. The blue line indicates the time-lapse, and the red line indicates the duration of the quote burst, which shows the progression of cutting-edge hot topics. **(E)** Heatmap analysis of keywords. The intensity of the color in each box represents the level of attention a keyword received in a given year, with brighter colors indicating higher levels of attention. The upper heatmap illustrates the annual frequency of keyword bursts, highlighting the temporal distribution of keyword appearances. The lower heatmap depicts the cumulative keyword bursts, indicating the sequence and intensity of keywords gaining attention over the entire study period.

3.4.3 Analysis of keyword cluster timelines and keyword bursts

The results of the keyword cluster analysis are presented in Figure 4B, and a total of seven clusters were obtained. The largest cluster was Cluster #0, which was named "activation." This was followed by "systemic lupus erythematosus," "myocardial infarction," "resolution of inflammation," "tam receptors," "docosahexaenoic acid," and "alveolar macrophages." A timeline plot of the keyword clusters is shown in Figure 4C. 2000s witnessed a surge of "activation" "myocardial infarction" and "alveolar macrophage," introducing the identification of key receptors and signaling pathways involved in efferocytosis and inflammatory disease was a major advancement. After 2010, "systematic lupus erythematosus" and "resolution of inflammation" revealed that autoimmune disease and therapy has aroused researchers' attention.

We further plotted keyword citation bursts via CiteSpace (Figure 4D), "docosahexaenoic acid" had the highest intensity (8.31), and those with longer citation burst durations included "alveolar macrophages" (2006–2014), "obstructive pulmonary disease" (2010–2016), and "systemic lupus erythematosus" (2015–2020). Many researchers have investigated these issues further. The most recent keyword used to describe the outbreak was "inflammation resolution" (2020–2023). The upper heatmap of Figure 4E is designed to show the frequency of a keyword's burst during the years in detail, indicating the terms "chronic obstructive pulmonary disease," "alveolar macrophages" had the longest citation durations. While the bottom heatmap shows a cumulative keyword burst, indicating the sequence of keywords gaining attention over the entire period, showing more recent outbreaks were associated with "cancer," "pyroptosis," "necroptosis" and "polarization."

4 Discussion

4.1 General information

According to the WoSCC database, there was a trend toward an increase in the quantity of publications in this field, which occurred more rapidly from 2019 onward. This finding suggests that this area is gaining attention, which is consistent with previous findings (11).

It is noticeable that the U.S., especially the Harvard University, contributes most in the research field of efferocytosis and inflammation. The first landmark research from Harvard in the field of efferocytosis and inflammation can be traced back to the work on the role of phosphatidylserine and MerTK in apoptotic cell clearance published in 2001 by Scott et al. (17). This study was one of the first to elucidate the molecular mechanisms by which phagocytes recognize and engulf apoptotic cells, establishing a critical link between efferocytosis and immune regulation. In quick succession, Harvard's researchers noticed that mice lacking the MerTK receptor exhibited delayed clearance of apoptotic cells and developed symptoms reminiscent of systemic lupus erythematosus (SLE) (18). This work highlighted the importance of efficient apoptotic cell clearance in preventing autoimmune responses and maintaining immune tolerance. Harvard's contributions have been instrumental in defining the research landscape and guiding future investigations in this vital area of immunology.

Among the scholars in this field, Serhan CN has published the most articles and received the highest h-index, which reflects his outstanding contribution to the study of efferocytosis and inflammation. Notably, efferocytosis has been known for a long time in the academic field. However, it was not until 2003 that deCathelineau AM and colleagues named efferocytosis for the first time and elucidated the possible transmission pathway and mechanism of efferocytosis generation (19).

4.2 The role of efferocytosis in the pathogenesis of various inflammatory diseases and autoimmune diseases

A transition in research hotspots is depicted in Figures 4D,E, where the initial focus was on the role of efferocytosis in the pathophysiological processes of various inflammatory diseases (especially cardiac and pulmonary diseases), resulting in bursts of the keywords "*in vitro*," "phosphatidylserine receptor" and "apoptotic cell clearance." With the gradual improvement in the understanding of the underlying mechanisms, research hotspots have also progressively turned to treatment methods, so "inflammation resolution" has recently become a popular topic. Extensive studies of efferocytosis have been conducted in many inflammatory diseases, especially atherosclerosis, obstructive lung disease, systemic lupus erythematosus and rheumatoid arthritis.

The identification of key receptors and signaling pathways involved in efferocytosis was a major advancement in 2000s. In the process of efferocytosis, as shown in Figure 5, there are four stages: the identification of apoptotic cells (ACs), the binding of ACs, the internalization of ACs and the degradation of ACs (20). In the first phase, chemokines, known as "find me" signals, are triggered by ACs to induce the efficient mobilization of efferocytic immune cells. In the second phase, phagocytes, which are mediated by "find me" signals, accumulate abundant ACs, and phagocytosis receptors bind to "eat me" signals on the surface of ACs to initiate efferocytosis (21). Phosphatidylserine (PtdSer) is expressed on the surface of ACs and binds to the bridging molecules human growth arrest-specific protein 6 (GAS6) and milk fat globule-EGF factor 8 (MFG-E8). It is recognized by the Mer proto-oncogene tyrosine kinase (MERTK, a

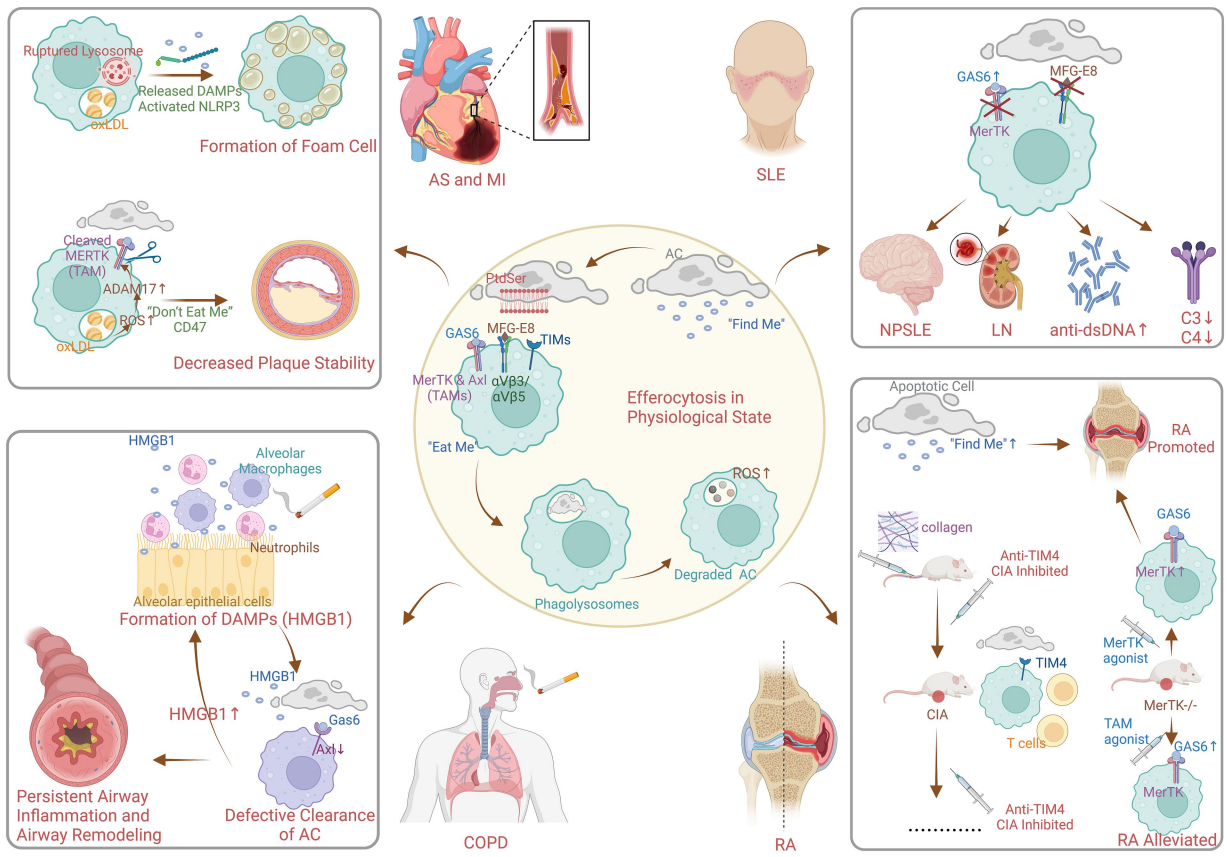


FIGURE 5
Mechanism of efferocytosis in physiological and pathophysiological state (created with BioRender.com). AC, apoptotic cell; PtdSer, phosphatedylserine; MFG-E8, milk fat globule EGF factor 8; GAS6, growth arrest specific protein 6; TIMs, T-cell immuneglobulin; MerTK, Mer protooncogene tyrosine kinase; Axl, anexelekto; TAMs, Tyro3/Axl/Mer receptor tyrosine kinases; ROS, reactive oxidative stress; DAMPs, damage-associated molecular patterns; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; oxLDL, oxidized low-density lipoprotein; AS, atherosclerosis; MI, myocardial infarction; HMGB1, high mobility group box 1 protein; COPD, chronic obstructive pulmonary disease; SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric systemic lupus erythematosus; LN, lunpus nephritis; RA, rheumatoid arthritis; CIA, collagen-induced arthritis.

TAM receptor) and the integrin receptor αVβ3/αVβ5 on the surface of efferocytes (21). It can also directly bind to PtdSer receptors, such as T-cell immunoglobulin (TIM). During the third phase, the "eat me" signal binds to the receptor on the surface of phagocytes, mediating cytoskeleton reorganization and endocytosis of ACs to form phagolysosomes (22). Finally, lysosomes fuse with and acidify phagosomes to degrade internalized ACs, the key molecule of which is reactive oxygen species (ROS) (23).

Research in the 2010s highlighted the role of defective efferocytosis in the pathogenesis of chronic inflammatory diseases. Figure 5 also shows the pathophysiological process of efferocytosis in some non-neoplastic diseases. Lipid metabolism plays an essential role in the study of efferocytosis and atherosclerosis. In addition, macrophages consume oxidized low-density lipoprotein (oxLDL), which can lead to the formation of cholesterol crystals in lysosomes. This results in instability and even rupture of lysosomes and ultimately causes the release of unesterified cholesterol into the cytoplasm, NLRP3 inflammasome activation, and foam cell formation (24–26). In addition, ROS promote the activation of the protease ADAM17 on macrophages, which, by cleaving MERTK (27), causes ACs to inappropriately express the "do not eat me' signal CD47. This leads to impaired clearance of ACs from atherosclerotic plaques, increased area of plaque lipid necrotic nuclei, and decreased plaque stability (28). The reduction in inflammation is mediated by specialized proresolving lipid mediators from omega-3 fatty acids or arachidonic acid, as well as associated proteins and signaling gas molecules; this pathway reduces inflammatory vesicle formation, alleviates oxidative stress, and enhances efferocytosis (29). Moreover, enhancing efferocytosis in atherosclerotic lesions through CD36 and MERTK receptor activation has been proposed as a strategy to reduce plaque burden and stabilize plaques, potentially decreasing the risk of cardiovascular events (3).

Existing studies on the pathogenesis of efferocytosis in obstructive pulmonary disease have focused on multiple fields. First, high mobility group box 1 protein is a typical damage-associated molecular pattern (DAMP) protein that is secreted by airway cells exposed to cigarettes and other substances, such as neutrophils, alveolar macrophages, lymphocytes, and epithelial cells (30). It binds to receptor for advanced glycosylation end products (RAGE) and Toll-like receptor 4 (TLR4) to exert its activity (31, 32), resulting in the nuclear activation and translocation of NF-κB (33). This triggers airway inflammation and plays an influential role in airway remodeling due to persistent airway

inflammation. Second, Hodge S et al. verified that dysregulation of PtdSer-recognizing receptors or bridging molecules may be responsible for defective clearance of ACs in COPD (34). However, relatively few studies on PtdSer-recognizing receptors or bridging molecules exist, and only a limited number of studies have suggested that MerTK upregulation is not sufficient to normalize macrophage efferocytosis (35, 36). In fact, animal studies have shown that another TAM receptor "Axl," expressed in mouse airway macrophages, is effectively expressed under stimulation by inflammatory factors such as type I interferon or Toll-like receptor-3 stimulation and binds to Gas6 (37).

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by overactivation of immune cells and overproduction of autoantibodies, resulting in systemic involvement of multiple organs. In SLE, efferocytosis is often defective. Inefficient clearance of apoptotic cells can lead to the accumulation of cellular debris, which can trigger an autoimmune response. This debris can be recognized as foreign by the immune system, leading to the production of autoantibodies and the formation of immune complexes (3). The efferocytosis bridging molecules described previously play an active role in the pathogenesis of SLE. Several studies have demonstrated that the serum level of the efferocytosis bridging molecule Gas6 is significantly elevated in SLE patients, which is associated with neurological involvement, plasmacytosis, renal dysfunction, high dsDNA antibody titers, and decreased levels of complement C3 and C4 (38), which is probably due to the targeting of the MerTK (17). Therapies that upregulate MerTK on macrophages have shown promise in preclinical models of SLE (18). In addition, the results of *in vitro* experiments indicated that a lack or excess of another efferocytosis bridging molecule, MFG-E8, impeded efferocytosis (39). Hanayama R. demonstrated that MFG-E8-deficient mice accumulate ACs in germinal centers and spontaneously produce autoantibodies to develop lupus-like autoimmune disease (40). Moreover, high levels of MFG-E8 have been detected in sera from human SLE patients (41).

Rheumatoid arthritis is a chronic synovial inflammatory disease that progressively contributes to cartilage and bone destruction and the risk of disability. It has been shown that efferocytosis "find me" signaling chemokines CX3CL1 (42), ATP (43), and sphingosine-1-phosphate (44) promote rather than attenuate the pathophysiological processes of RA (45–49). Interestingly, during the binding phase of efferocytosis to ACs, the induction phase of collagen-induced arthritis (CIA) is neutralized by the direct PtdSer receptor "T-cell immunoglobulin and mucin structural domain 4 (TIM4)," which exacerbates inflammation in joints. Abe Y suggested that this effect was possibly due to the effect of TIM4 on the development of T-cells (50). In contrast, treatment with anti-TIM4 administered before or after the onset of CIA significantly inhibited the development and progression of CIA by reducing proinflammatory cytokines without affecting T or B-cell responses, suggesting that anti-TIM4 treatment may be a suitable target for the treatment of RA (50). MerTK is a member of the TAM family of cytosolic indirect receptors, and MerTK$^{-/-}$ mice presented increased joint inflammation (51), which was attenuated by the overexpression of the TAM receptor agonist Gas6 and protein S (52). However, mice treated with MerTK-specific agonist antibodies also unexpectedly exhibited exacerbated joint inflammation, which was suggested by Waterborg CEJ to be related to increased numbers of efferocytosis ACs in the knee joint and elevated serum interleukin-16C levels (51). Inhibition of integrin αVβ3 attenuated joint inflammation in arthritic rabbits and rats, although this effect may be independent of the indirect efferocytosis receptor MFG-E8 (53).

In addition to systemic lupus erythematosus and rheumatoid arthritis, efferocytosis has received increasing attention in the study of autoimmune diseases involving type 1 diabetes mellitus, inflammatory bowel disease, and multiple sclerosis (MS), where the mechanism may involve defective clearance of dead cells associated with an intolerant immunogenic response and dendritic cell maturation in chronic inflammation. A study on nonobese mice that instinctively progressed to T1DM demonstrated defects in efferocytosis mechanisms underlying the development of ANA both *in vivo* and *in vitro* (54). Lipopolysaccharide-binding proteins, Toll-like receptor 4, and bacterial permeability-increasing proteins have been detected in the serum of patients with ulcerative colitis, and these complexes are recognized by CD14. CD14 is linked to ICAM3 and promotes the recognition and phagocytosis of ACs (55). MS is a degenerative disease of the central nervous system characterized by focal lesions with inflammation, oligodendrocyte death, demyelination, and axonal damage. ATP is a major neurotransmitter in the central nervous system that activates ionotropic (P2X) and metabotropic (P2Y2) receptors, which both recognize different "eat me" and "find me" signals during cytosolic drinking (43, 56). Therefore, P2X and P2Y can be considered possible targets of MS. However, how defective clearance of apoptotic nerve cells contributes to the pathogenesis of MS is unclear. The above efferocytosis molecular pathways in autoimmune diseases are poorly defined and understudied and will be the focus of future developments in the field of efferocytosis and inflammation.

4.3 Research frontiers and future prospects

Emerging surge keywords like "cancer" and "inflammation resolution" forebode research frontiers. Efferocytosis, the process by which phagocytes clear apoptotic cells, has a controversial and paradoxical role in cancer. On one hand, efferocytosis can promote anti-tumor immunity by efficiently clearing apoptotic cancer cells, thereby preventing secondary necrosis and the release of pro-inflammatory and potentially immunogenic cell contents. This clearance helps maintain tissue homeostasis and can facilitate the recruitment and activation of immune cells that target tumor cells (57). On the other hand, efferocytosis can also contribute to tumor progression by creating an immunosuppressive environment. The ingestion of apoptotic cells by macrophages and other phagocytes can lead to the release of anti-inflammatory cytokines, such as TGF-β and IL-10, which suppress effective anti-tumor immune responses and promote tumor growth and metastasis (58). This dual role complicates the development of therapeutic strategies that aim to modulate efferocytosis in cancer, as enhancing efferocytosis might inadvertently support tumor progression in certain contexts (59).

While efferocytosis presents a complex dual role in cancer, its potential in resolving inflammation highlights a promising avenue for therapeutic exploration, particularly with the use of mesenchymal stem cells (MSCs). Inflammation resolution has become a research trend in recent years. MSCs have been shown to have an important research value and broad translational application prospects. Previous

studies have shown that the efferocytosis of MSCs can be used to alleviate lung (60) and synovial (61) inflammation, improve myocardial ischemia/reperfusion (62), and enhance the therapeutic effect of sepsis (63) based on the mechanism of the shift of macrophages from a proinflammatory phenotype to an inflammation-suppressive phenotype through releasing anti-inflammatory cytokines. In turn, a therapeutic modality derived from these MSC exosomes or vesicles has been shown to alleviate lupus nephritis (64), prevent complications after vascular stent insertion (65), and ameliorate insulin resistance in type 2 diabetes mellitus patients (66). Although MSC shows inconspicuous effects in limited preclinical researches of autoimmune liver disease and Crohn's disease (67), clinical application of stem cells still facing numerous obstacles to the application of stem cell therapy at this stage because of insufficient research. Safety concerns, including the risk of tumorigenesis and immune rejection, remain significant hurdles (68). The variability in efficacy depending on stem cell source, delivery method, and timing of administration necessitates further optimization (69). Additionally, regulatory and ethical considerations complicate the approval and implementation of stem cell therapies (70). Finally, the high cost of stem cell treatments poses a barrier to widespread clinical adoption, necessitating efforts to improve cost-effectiveness and accessibility (71). Addressing these challenges through rigorous research and collaboration among scientists, clinicians, and regulatory bodies is essential for harnessing the full therapeutic potential of stem cells in efferocytosis and inflammatory diseases.

However, there is significant redundancy in the mechanisms that regulate efferocytosis. When one pathway is inhibited, others may compensate, making it challenging to develop targeted therapies. Developing therapies that modulate efferocytosis without unintended side effects is challenging. Enhancing or inhibiting efferocytosis could have unpredictable consequences depending on the disease context.

4.4 Limitations

Some limitations inherent to bibliometrics are present in our study. First, the data were extracted only from the WoSCC database, possibly missing some important findings published in other databases. Nonetheless, the WoSCC is a definitive and comprehensive database in the field of medicine. The amount of data we analyzed was large enough to reflect research in the field of efferocytosis and inflammation. Multiple databases are recommended to be searched in the future work. Moreover, VOSviewer and CiteSpace may have missed some information due to the inability to analyze the full texts of the publications, causing bias in other bibliometric studies. A more impartial software in bibliometric analysis is expected to be created and used.

5 Conclusion

Bibliometric analysis provides an objective and quantitative method for evaluating research directions toward efferocytosis and inflammation. Recent research shows that efferocytosis plays an important role in the pathogenesis of a variety of inflammatory and autoimmune diseases such as atherosclerosis, obstructive pulmonary disease, SLE and RA. Inflammation resolution by efferocytosis may be a potential method for treating not only inflammatory diseases but also cancer. However, the feasibility of targeting efferocytosis for the treatment of diseases requires further in-depth research.

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

XC: Writing – original draft. FL: Visualization, Writing – original draft. XX: Visualization, Writing – original draft. GL: Visualization, Writing – original draft. XT: Visualization, Writing – original draft. WH: Visualization, Writing – original draft. JT: Writing – review & editing. YG: 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/fmed.2025.1498503/full#supplementary-material

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