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Community series in recent advances in potential biomarkers for rheumatic diseases and in cell-based therapies in the management of inflammatory rheumatic diseases, volume II

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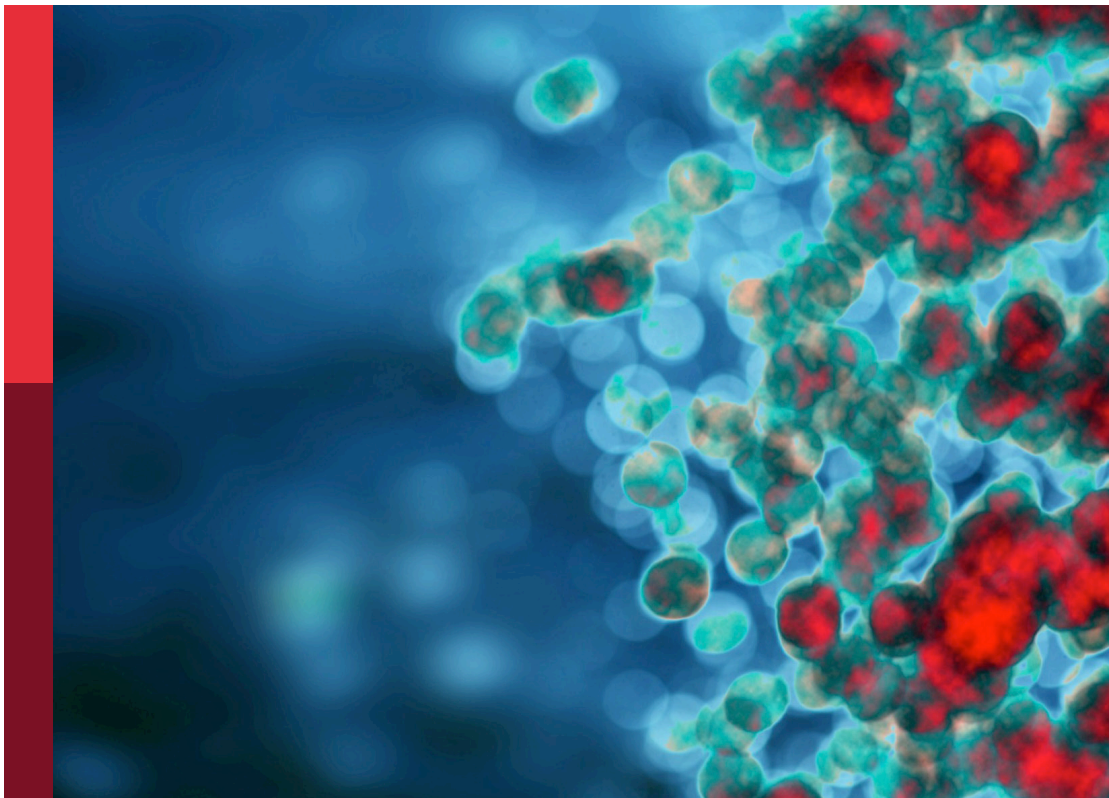

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Community series in recent advances in potential biomarkers for rheumatic diseases and in cell-based therapies in the management of inflammatory rheumatic diseases, volume II

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Dr. Saas is the author of a patent and a shareholder of Med'Inn'Pharma, related to the development of anti-inflammatory treatment. All other Topic Editors declare no competing interests with regards to the Research Topic subject.

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Editorial: Community series in recent advances in potential biomarkers for rheumatic diseases and in cell-based therapies in the management of inflammatory rheumatic diseases, volume II

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KEYWORDS

TNF inhibitors, biomarkers, cell-based therapy, inflammatory rheumatic diseases, psoriatic arthritis, rheumatoid arthritis, spondyloarthritis, systemic lupus erythematosus

Editorial on the Research Topic

Community series in recent advances in potential biomarkers for rheumatic diseases and in cell-based therapies in the management of inflammatory rheumatic diseases, volume II

This Research Topic focuses on recent advances in the identification of diagnostic, prognostic and predictive biomarkers for inflammatory rheumatic diseases (IRDs), as well as recent advances in cell-based therapies used in their treatment (1). The second volume is composed of 3 review articles and 7 original articles, and the manuscripts collected are especially dedicated to biomarkers in IRDs. They present the effects of various approaches leading to the identification and description of biomarkers, such as: (i) a review of existing biomarker recommendations and meta-analysis of existing data (e.g. of epigenetic and DNA methylation studies); (ii) application of more laboratory-based analyses, such as measurement of single protein concentrations, circulating exosomal miRNA and serum proteome profiling, detection of specific antibodies in serum and saliva; and (iii) implementation of newer biostatistical methods including machine learning. Consequently, in addition to well-known associations, several new findings in the field of IRD biomarkers are provided.

Liu et al. reviewed and formulated guidelines for biomarkers in the diagnosis and assessment of patients with axial spondyloarthritis (axSpA). They highlighted the

usefulness of currently applied HLA-B27 testing in patients with suspected axSpA and regular C-reactive protein/erythrocyte sedimentation rate monitoring as specifically recommended for axSpA evaluation (2).

Mangoni and Zinellu in their systematic review and meta-analysis focused on the pathophysiological role of neopterin known as a biomarker of inflammation and oxidative stress, suggesting that it may be useful in identifying rheumatic diseases. Neopterin is an organic compound belonging to the pteridine class of heterocyclic compounds and belongs to the chemical group known as pteridines. It is synthesized by human macrophages after interferon-gamma stimulation and serves as a marker of activation of the cellular immune system.

The role of epigenetic regulation of gene expression and function of DNA methylation was discussed by Wang et al. who argue that genome methylation analysis may be a beneficial tool to aid in the early diagnosis of Sjögren's syndrome (SjS). Interestingly, in addition to the human leukocyte antigen (HLA) locus, several regions were found to be differentially methylated in SjS patients, including genes regulated by type I interferon, the runt-related transcription factor gene (RUNX1), lymphotoxin-α (LTA) and myxovirus A resistance protein (MxA).

More experimental analyses, such the application of molecular profiling and gene expression studies for biomarker detection was also proposed. Angioni et al. performed a transcriptomic analysis of whole blood and a comparison of molecular profiles between psoriatic arthritis (PsA) patients in clinical remission after TNF inhibitor (TNFi) treatment, PsA patients with active disease and healthy controls. The results pointed the role of dysregulation of two genes involved in the processes of inflammation perpetuation and bone metabolism. Downregulation and upregulation of FOS and CCDC50, respectively, were identified as contributing to the pathophysiology of PsA as described in a study on clinical remission in PsA patients treated with TNFi.

Serum proteome profiling was used in another study by Cuesta-López et al. investigating age- and sex-matched control patients with newly diagnosed rheumatoid arthritis (RA) patients, and patients with established RA (with a disease duration of more than 25 years). An additional longitudinal study was conducted on two cohorts of RA patients treated with methotrexate or tofacitinib for 6 months. By analyzing their cardiovascular and cardiometabolic proteome (examining serum profiling of 184 proteins using the Olink technology platform), the authors were able to identify changes in serum proteins associated with cardiovascular disease in RA patients and identify candidate protein biomarkers to distinguish RA patients from healthy individuals (such as elevated levels of CTSL1, SORT1, SAA4, TNFRSF10A, ST6GAL1 and CCL18). They were also able to show how methotrexate and tofacitinib affect serum levels of these proteins, and to identify SAA4 as a potential biomarker of response to these therapies.

Serum and salivary antibody levels against *Porphyromonas gingivalis* (*P. gingivalis*, a major periodontal pathogen) were investigated by Svärd et al. in a Swedish RA patient cohort. The results indicated the local production of IgA directed against *P. gingivalis*-specific Arg-specific gingipain B (anti-RgpB) in the salivary glands, which is not accompanied by systemic antibody production. Higher levels of IgA anti-RgpB antibodies in saliva were detected in RA patients compared to healthy controls. However, despite some common features and a potential link between RA and periodontitis (e.g., elevated levels of anti-citrullinated peptide antibodies [ACPAs]) (3), anti-RgpB antibodies were not found to be associated with RA disease activity, periodontitis or serum IgG ACPAs.

Four remaining original articles addressed biomarkers related to lupus nephritis (LN) development, the disease onset, activity, and response to treatment.

The study by Li et al. aimed to assess whether serum levels of human epididymis protein 4 (HE4) can identify pathological classes of LN in adults and children with systemic lupus erythematosus (SLE). The study was conducted on three cohorts of SLE patients (those without LN as well as LN patients with adult or childhood onset) and showed an association of higher serum HE4 levels with adult onset LN. It was also observed that among patients with adult onset LN, elevated HE4 levels were more common in patients with proliferative LN and in patients with chronic class IV lesions.

It was also found that urinary L-selectin (uL-selectin) level may act as a novel biomarker of disease activity and renal histopathology in LN. Moreover, it may reflect treatment response in LN patients during follow-up, as uL-selectin concentrations decreased significantly in the complete renal remission group. This was reported by Shen et al. in a study investigating two independent cohorts, a Chinese cohort and a US cohort of SLE patients and controls.

Chen et al. analyzed and compared circulating exosomal microRNA molecules in the serum of SLE patients with or without LN. They detected significantly higher levels of exosomal hsa-miR-4796-5p and hsa-miR-7974 in LN cases compared to SLE patients without LN. The levels of these miRNAs positively correlated with proteinuria and SLE disease activity index (SLEDAI), and were significantly elevated in patients with LN compared with other autoimmune nephritis conditions such as immunoglobulin A nephropathy (IgAN) and diabetic nephropathy (DN).

Therefore, it appears that laboratory analysis of uL-selectin levels, serum He4 levels and profiling of exosomal miRNAs in patients' sera may help predict the onset and course of LN. However, the use of statistical tools such as machine learning may also be helpful in a more complex analysis, as shown by Yang et al., offering a reliable non-invasive diagnostic tool for SLE patients when renal biopsy is not possible or safe.

Yang et al. performed a retrospective analysis of clinical and laboratory data from patients diagnosed with SLE and renal involvement who underwent renal biopsy aiming to develop and validate machine learning models to predict the occurrence of proliferative lupus nephritis (PLN). Their study confirmed the efficacy of traditional indicators such as anti-double stranded DNA (dsDNA) antibodies, complement levels, serum creatinine and urinary red and white blood cells in predicting and differentiating PLN, and also demonstrated the potential value of previously controversial or underutilized indicators such as serum chloride, neutrophil percentage, serum cystatin C, hematocrit,

urine pH, routine red blood cells and immunoglobulin M in predicting PLN.

In conclusion, employment of various experimental approaches, laboratory and statistical tools provides a comprehensive perspective on the inclusion of a wider range of biomarkers in the diagnosis, prediction and treatment outcome of IRDs.

As Volume III of our Research Topic has been initiated, we invite authors to submit their manuscripts.

Author contributions

KB-K: Conceptualization, Writing – original draft, Writing – review & editing. PS: Conceptualization, Writing – review & editing. ET: Conceptualization, Writing – review & editing.

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Elevated serum levels of human epididymis protein 4 in adult patients with proliferative lupus nephritis

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Background: This study aimed to access whether serum human epididymis protein 4 (HE4) level could identify lupus nephritis (LN) pathological classes in adults and children.

Methods: The serum HE4 levels of 190 healthy subjects and 182 patients with systemic lupus erythematosus (SLE) (61 adult-onset LN [aLN], 39 childhood-onset LN [cLN], and 82 SLE without LN) were determined using Architect HE4 kits and an Abbott ARCHITECT i2000SR Immunoassay Analyzer.

Results: Serum HE4 level was significantly higher in the aLN patients (median, 85.5 pmol/L) than in the patients with cLN (44 pmol/L, $P < 0.001$) or SLE without LN (37 pmol/L, $P < 0.001$), or the healthy controls (30 pmol/L, $P < 0.001$). Multivariate analysis showed that serum HE4 level was independently associated with aLN. Stratified by LN class, serum HE4 level was significantly higher in the patients with proliferative LN (PLN) than in those with non-PLN, and this difference was found only in aLN (median, 98.3 *versus* 49.3 pmol/L, $P = 0.021$) but not in cLN. Stratified by activity (A) and chronicity (C) indices, the aLN patients with class IV (A/C) possessed significantly higher serum HE4 levels than those with class IV (A) (median, 195.5 *versus* 60.8 pmol/L, $P = 0.006$), and this difference was not seen in the class III aLN or cLN patients.

Conclusion: Serum HE4 level is elevated in patients with class IV (A/C) aLN. The role of HE4 in the pathogenesis of chronic lesions of class IV aLN needs further investigation.

KEYWORDS

serum HE4, diagnostic efficacy, proliferative lupus nephritis, pathological classes, active/chronic lesions

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that can occur during childhood or adulthood and is characterized by multisystem and multiorgan involvement (1). Lupus nephritis (LN) is one of the most common and severe manifestations, notably in African, Asian, and Hispanic populations (2, 3), affecting 40–60% of patients with SLE (4, 5), and up to 30% of patients with LN progress to end-stage renal disease (ESRD) (6–10). LN patients show 6–26-fold mortality compared with the general population, and this disease has been a major cause of death in the patients (6, 11, 12).

According to the classification system of the International Society of Nephrology/Renal Pathology Society (ISN/RPS), LN is classified into six classes (13), of which proliferative LN (PLN; class III, class IV, class III+V, and class IV+V) and membrane LN (MLN; class V) account for approximately 70% and 20% of all LN cases, respectively (6, 14). Early and accurate diagnosis of LN facilitates implementation of the optimum treatment that can prevent flares and preserve renal function (6, 15). The treatment choice in LN mainly depends on the histological class as well as on the activity and chronicity status (6). For example, current recommendations suggest intense immunosuppressive therapy for the treatment of PLN but not class II LN (5, 6, 16, 17). Therefore, early classification of LN has important implications for the therapeutic regimen and prognostic monitoring.

Currently, renal biopsy, which differentiates pathological classes and defines the severity of renal involvement, is the gold standard for the diagnosis of LN (13, 18). However, conversion between the proliferative and membranous forms of LN is frequent (19, 20), and renal biopsy is an invasive approach that may cause complications (21). Thus, this procedure is not suitable for routine monitoring of disease progression. Proteinuria is a major symptom of LN but cannot be used as a reliable LN marker since any renal impairment other than LN can cause this symptom (13, 22). Thus, markers in biofluids accessible with minimal invasiveness are needed to diagnose LN classes.

Human epididymis protein 4 (HE4), also known as whey acidic protein 4-disulfide core domain 2, is a secreted glycoprotein. Serum HE4 level is considered as a vital biomarker for ovarian cancer (23, 24) and an inflammatory biomarker which is elevated in patients with cystic fibrosis (25) and those with renal fibrosis (26). LN is characterized by renal inflammation that damages renal cells and eventually leads to renal fibrosis (27). However, LN classes differ in renal inflammation and fibrosis levels and may thus also differ in serum HE4 level. Furthermore, serum HE4 level in pediatric patients with SLE has not been investigated.

Abbreviations: SLE, systemic lupus erythematosus; LN, lupus nephritis; ESRD, end-stage renal disease; ISN, International Society of Nephrology; RPS, Renal Pathology Society; PLN, proliferative lupus nephritis; MLN, membrane lupus nephritis; HE4, human epididymis protein 4; aSLE, adult-onset systemic lupus erythematosus; IQRs, interquartile ranges; ROC, receiver operating characteristic; AUC, area under the curve; SLEDAI, systemic lupus erythematosus disease activity index.

Hence, this study aimed to assess for the correlation of serum HE4 level with adult and pediatric LN classes.

Materials and methods

Study design, patients and controls

This study is a retrospective, single-center study. Blood samples were collected from 182 patients with SLE into serum tubes (tubes without anticoagulant) during the first visit at the First Affiliated Hospital of Sun Yat-sen University. The normal control population consisted of 190 healthy adult subjects who received routine physical examination. The diagnosis of SLE was based on the American College of Rheumatology classification criteria (28), and the patient cohort included 100 SLE patients with LN (61 adult-onset LN [aLN]; 39 childhood-onset LN [cLN]) and 82 adult-onset SLE (aSLE) patients without LN. LN was diagnosed based on renal biopsy results. SLE patients complicated with myositis, primary Sjogren's syndrome, systemic sclerosis or rheumatoid arthritis were excluded. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (No. IIT-2021-778).

Renal pathology

Each biopsy contained > 10 glomeruli and was interpreted by two pathologists (HX and FW) based on 2003 ISN/RPS classification (class I, minimal mesangial LN; class II, mesangial proliferative LN; class III, focal LN; class IV, diffuse segmental or global LN; class V, membranous LN; and class VI, advanced sclerosing LN) (13). For the activity and chronicity assessment, class III and IV LN are sub-classified as LN with purely active (A), purely chronic (C), or mixed (A/C) lesions. Patients with class I or VI LN were absent in this study and thus were not analyzed.

The patients were also categorized as PLN (all the class III, IV, III+V, and IV+V patients) and non-proliferative LN (non-PLN; class II and class V patients), and the PLN patients were sub-classified into pure PLN (class III and class IV patients) and mixed PLN (class III+V and class IV+V patients).

Data collection and serum HE4 quantitation

Demographic and clinical characteristics and laboratory findings were collected on the day of renal biopsy and comprised information about age, gender, body mass index, and hematological, biochemical, and immunological test results. Serum HE4 level was measured using Architect HE4 kits and an Abbott ARCHITECT i2000SR Immunoassay Analyzer (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. Briefly, a two-step immunoassay involving the chemiluminescent microparticle immunoassay technology with flexible assay protocols (Chemiflex) was used.

Statistical analysis

Statistical analysis was performed using the SPSS software version 26 and GraphPad Prism version 9.1.0. Data were expressed as median values with interquartile ranges (IQRs) for continuous variables and as proportions for categorical variables. The Student's *t*-test, Mann-Whitney U test, and Chi-squared or Fisher's exact test were used to analyze normally distributed, non-normally distributed, and categorical data, respectively. Univariate and multivariate logistic regression models were used to assess for the association between serum laboratory findings and aLN. Receiver operating characteristic (ROC) curve was used to evaluate the sensitivity, specificity, and area under the curve (AUC). The cutoff value was determined using the optimal Youden index (sensitivity + specificity −1). Correlation was analyzed using the Spearman rank correlation test. All the *P*-values were two-sided, and a *P*-value < 0.05 was considered to indicate statistical significance.

Results

Serum HE4 level was elevated in the patients with aLN

The characteristics of the patients with LN are shown in Table 1. The highest median serum HE4 level was observed in the patients with aLN (median, 85.5 pmol/L; IQR, 49.5–314.7) compared with the levels in the patients with cLN (median, 44 pmol/L; IQR, 38–63.8) and in those with aSLE without LN (median, 37 pmol/L; IQR, 30.5–50.6) as well as in the healthy controls (median, 30 pmol/L; IQR, 26.6–34.7) (Figure 1A). ROC analysis revealed that the AUC

TABLE 1 Univariate and multivariate analyses of the variables associated with aLN.

	aLN (N = 61) % (n/N) or median (IQR)	aSLE without LN (N = 82) % (n/N) or median (IQR)	cLN (N = 39) % (n/N) or median (IQR)	Univariable *P* value (aLN *versus* aSLE without LN)	Multivariable* *P* value (aLN *versus* aSLE without LN)
HE4, pmol/L	85.5 (49.5–314.7)	37 (30.5 – 50.6)	44 (38–63.8)	< 0.001	0.031
Demographics					
Age, years	32 (25.5–42)	35 (31.8 – 48.3)	13 (11–13)	0.037	0.090
Gender, female	83.6 (51/61)	89 (73/82)	87.2 (34/39)	0.348	
Body mass index, kg/m^2	22.3 (19.7–24.5)	20.9 (19.6 – 23.1)	20.6 (17.9–22)	0.538	
Whole blood					
WBC count, ×10^9/L	6.9 (5.4–8.5)	5.4 (4.3–6.7)	6.9 (5.7–9.8)	0.001	0.225
Lymphocyte count, ×10^9/L	1.2 (0.8–1.7)	1.2 (0.9–1.7)	2 (1.5–2.5)	0.934	
Haematocrit, %	0.36 (0.3–0.39)	0.38 (0.35–0.4)	0.4 (0.36–0.42)	0.006	0.862
Platelet count, g/L	220 (180–268)	232 (189–279)	305 (258.5–337)	0.727	
Hemoglobin, g/L	115 (97.5–129)	126 (113.8–135.3)	131 (117.5–136.5)	0.011	0.601
Serum					
Serum C3 level, g/L	0.6 (0.5–0.8)	0.7 (0.5–0.8)	0.8 (0.7–1)	0.231	
Serum C4 level, g/L	0.15 (0.12–0.21)	0.14 (0.1–0.18)	0.13 (0.09–0.18)	0.092	
Serum albumin, g/L	33.8 (27.4–38)	40.7 (38.3–42.4)	42.3 (37.4–44.3)	< 0.001	0.403
Blood urea nitrogen, mmol/L	6.8 (4.8–10.8)	4 (3.3–5.1)	4.5 (3.7–6.53)	0.001	0.416
Serum creatinine, μmol/L	80 (59.5–151.5)	62 (55.3–73)	47.5 (41.3–58)	0.001	0.325
eGFR, mL/min/1.73m^2	63.9 (33.34–103.09)	93.3 (77–115.6)	137.7 (110.8–179.4)	0.028	0.466
Serum IgG, mg/dl	8.8 (5.9–12.8)	14 (11.8–17.2)	9.64 (6.3–11.7)	< 0.001	0.047
Serum IgA, mg/dl	2 (1.3–2.5)	2.6 (2–3.7)	1.72 (1.1–2.3)	< 0.001	0.843
Serum IgM, mg/dl	0.7 (0.5–1.1)	1 (0.7–1.4)	0.8 (0.5–1.1)	0.003	0.294

(Continued)

TABLE 1 Continued

	aLN (N = 61) % (n/N) or median (IQR)	aSLE without LN (N = 82) % (n/N) or median (IQR)	cLN (N = 39) % (n/N) or median (IQR)	Univariable *P* value (aLN *versus* aSLE without LN)	Multivariable* *P* value (aLN *versus* aSLE without LN)
Autoantibodies					
ANA, U/mL	93.3 (12.1–300)	244.9 (24–300)	23.2 (11.6–211.8)	0.072	
Anti-dsDNA, IU/mL	37.7 (6.2–104.8)	19.4 (5.5–134.5)	18.6 (5.1–137.3)	0.986	
Anti-Jo-1	0	1.4 (1/72)	0	1	
Anti-Sm	18.6 (11/59)	20.8 (15/72)	20 (7/35)	0.755	
Anti-Ro/SSA	54.2 (32/59)	63.9 (46/72)	37.1 (13/35)	0.264	
Anti-La/SSB	16.9 (10/59)	22.2 (16/72)	11.4 (4/35)	0.453	
Anti-RNP	23.7 (14/59)	44.4 (32/72)	28.6 (10/35)	0.015	0.853
Anti-Scl-70	0	1.4 (1/72)	1 (2.9/35)	1	
Anti-centromere B	1.7 (1/59)	2.8 (2/72)	0	0.683	

* Variables significant on univariate analysis were included in the multivariate logistic regression. aLN, adult-onset lupus nephritis; aSLE without LN, adult-onset systemic lupus erythematosus without lupus nephritis; IQR, interquartile range; HE4, human epididymis protein 4; WBC, white blood cell; C3, complement C3; C4, complement C4; ANA, anti-nuclear antibody.

for HE4 was 0.854 (95% CI 0.793–0.916, $P < 0.001$) to distinguish aLN from aSLE without LN (sensitivity, 70.5%; specificity, 85.4%; cutoff, 57.1 pmol/L) (Figure 1B). In addition, the association between the variables and aLN was assessed using univariate and multivariate analyses. Multivariate analysis showed that serum HE4 and IgG levels were significantly associated with aLN in the patients with aSLE (Table 1).

Serum HE4 level was elevated in the patients with adult-onset PLN

A total of 100 patients underwent renal biopsy and were histologically classified (Table 2). Serum HE4 level was significantly higher in the PLN patients than in the non-PLN patients, and this difference was found in the aLN (median, 98.3 *versus* 49.3 pmol/L, $P = 0.021$) but not the cLN (median, 44 *versus* 39.1 pmol/L, $P = 0.333$) patients (Figure 2A). ROC analysis showed that the optimal cut-off value to distinguish the PLN patients from the non-PLN or aSLE without LN patients was estimated to be 57.1 pmol/L, with a sensitivity and specificity of 74.1% and 84.3%, respectively, and an AUC value of 0.858 (95% CI 0.794–0.921, $P < 0.001$) (Figure 2B). Since no significant difference in serum HE4 level was found between the pure PLN (classes III and IV) and mixed PLN (classes III+V and IV+V) cases among all the aLN or cLN cases, or between the class III and class IV LN cases (Figures S1A–C), the patients demonstrating features of class III and IV LN concomitantly with features of class V LN were categorized as class

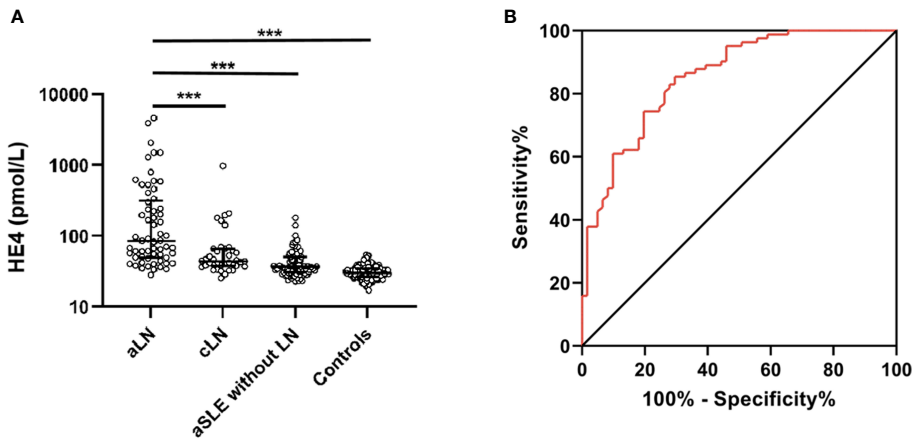

FIGURE 1
Serum HE4 level in the patients and controls. **(A)** Serum HE4 level is significantly higher in the aLN patients than in other groups. **(B)** ROC analysis of serum HE4 level in distinguishing the aLN cases from the aSLE without LN cases. HE4, human epididymis protein 4; aLN, adult-onset lupus nephritis; ROC, receiver operating characteristic; aSLE without LN, adult-onset systemic lupus erythematosus without lupus nephritis; cLN, childhood-onset lupus nephritis. ***, $P<0.001$.

TABLE 2 Frequencies of the histological classes in the patients with LN.

	LN (N = 100) % (n/N)	aLN (N = 61) % (n/N)	cLN (N = 39) % (n/N)
PLN (III/IV ± V)	89 (89/100)	88.5 (54/61)	89.7 (35/39)
Pure PLN (III, IV)	67 (67/100)	67.2 (41/61)	66.7 (26/39)
III	17 (17/100)	14.8 (9/61)	20.5 (8/39)
IV	50 (50/100)	52.5 (32/61)	46.2 (18/39)
Mix PLN (III/IV + V)	22 (22/100)	21.3 (13/61)	23.1 (9/39)
III + V	8 (8/100)	8.2 (5/61)	7.7 (3/39)
IV + V	14 (14/100)	13.1 (8/61)	15.4 (6/39)
Non-PLN (II, V)	11 (11/100)	11.5 (7/61)	10.3 (4/39)

LN, lupus nephritis; aLN, adult-onset lupus nephritis; cLN, childhood-onset lupus nephritis; IQR, interquartile range; PLN, proliferative lupus nephritis.

III and class IV LN patients, respectively, in the subsequent analyses. There was no significant difference in serum HE4 level between the class III and class IV PLN patients (Figure S1D).

The class IV aLN patients with A/C lesions had especially high serum HE4 levels

As shown in Table 3, of the 89 patients with class III/IV LN, 51 had A lesions, and 38 had a combination of A and C lesions. Overall, the patients with class III/IV (A) LN had significantly lower serum HE4 levels than those with class III/IV (A/C) LN (class III/IV [A] *versus* class III/IV [A/C]: median, 51.1 *versus* 143.5 pmol/L, $P = 0.001$). This difference was found only in the aLN cases but not in the cLN cases (Figure 3A). When stratified by LN class, no significant difference in serum HE4 level was found between the class III (A) and class III (A/C) aLN patients (Figure 3B), whereas serum HE4 level was significantly lower in the class IV (A) aLN patients than in those with class IV (A/C) aLN (class IV [A] *versus* class IV [A/C]: median, 60.8 *versus* 195.5 pmol/L, $P = 0.006$) (Figure 3C). The patients with class IV (A/C) aLN had significantly higher serum HE4 levels than those with any other aLN class (median, 195.5 *versus* 65.7 pmol/L; $P = 0.009$) (Figure 3D).

Correlations between serum HE4 level and various parameters

The patients with class IV aLN were assessed for any correlation between their serum HE4 levels and various variables. In these patients, significant positive correlations were found between serum HE4 level and blood urea nitrogen, serum creatinine, and 24-hour and random proteinuria levels, and significant negative correlations were found with serum albumin, hemoglobin, and complement C3 and C4 levels, hematocrit value, and absolute lymphocyte count (Table 4, Figures S2A–J). No significant correlation was seen

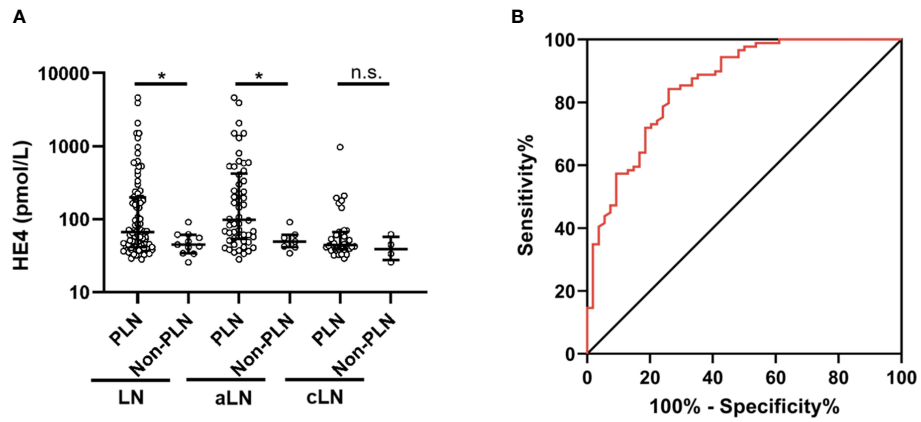

FIGURE 2
Comparison of serum HE4 levels between the PLN and non-PLN patients **(A)**, and ROC analysis of serum HE4 level in predicting PLN **(B)**. HE4, human epididymis protein 4; PLN, proliferative lupus nephritis; ROC, receiver operating characteristic; aLN, adult-onset lupus nephritis; cLN, childhood-onset lupus nephritis; aSLE without LN, adult-onset systemic lupus erythematosus without lupus nephritis. *, $P<0.05$; n.s., not statistically significant.

TABLE 3 Serum HE4 level in the PLN patients with A or A/C lesions.

	PLN (N = 89) % (n/N) or median (IQR)			aPLN (N = 54) % (n/N) or median (IQR)			cPLN (N = 35) % (n/N) or median (IQR)		
	n	HE4	*P* value	n	HE4	*P* value	n	HE4	*P* value
III/IV (A)	51	51.1 (38.5–96)	0.001	27	66.7 (44.7–243.9)	0.024	24	42.6 (37.9–56.7)	0.133
III/IV (A/C)	38	143.5 (53.4–586.2)		27	166.5 (70.7–622.5)		11	60.3 (39.2–164.1)	
III (A)	15	48.4 (36.6–297.6)	0.531	8	270.8 (81.1–387)	0.491	7	38.5 (32.6–44)	0.174
III (A/C)	10	96.4 (45–173.3)		6	96.4 (71.9–1128)		4	94.7 (41.1–158.8)	
IV (A)	36	52.1 (39.6–68.1)	0.002	19	60.8 (40.3–96)	0.006	17	45.2 (38.8–62)	0.494
IV (A/C)	28	162 (55–615)		21	195.5 (63.9–708.9)		7	60.3 (37.1–208)	

HE4, human epididymis protein 4; PLN, proliferative lupus nephritis; A, purely active lesions; A/C, active and chronic lesions; aPLN, adult-onset proliferative lupus nephritis; cPLN, childhood-onset proliferative lupus nephritis; IQR, interquartile range.

between serum HE4 level and the other parameters analyzed (Table S1).

Discussion

Previous studies have revealed that serum HE4 level is a risk factor for developing in LN among adult patients with SLE (29, 30). Given that LN includes various classes and serum HE4 level in pediatric patients with SLE has not been investigated, the present study focused on serum HE4 level in patients with different aLN or cLN classes. Here, we revealed that serum HE4 and IgG levels were independently associated with aLN. The reason why patients with aLN included in this study had lower levels of IgG compared to SLE patients without LN may be that they were more likely to have been treated with immunosuppressants that led to a reduced synthesis of

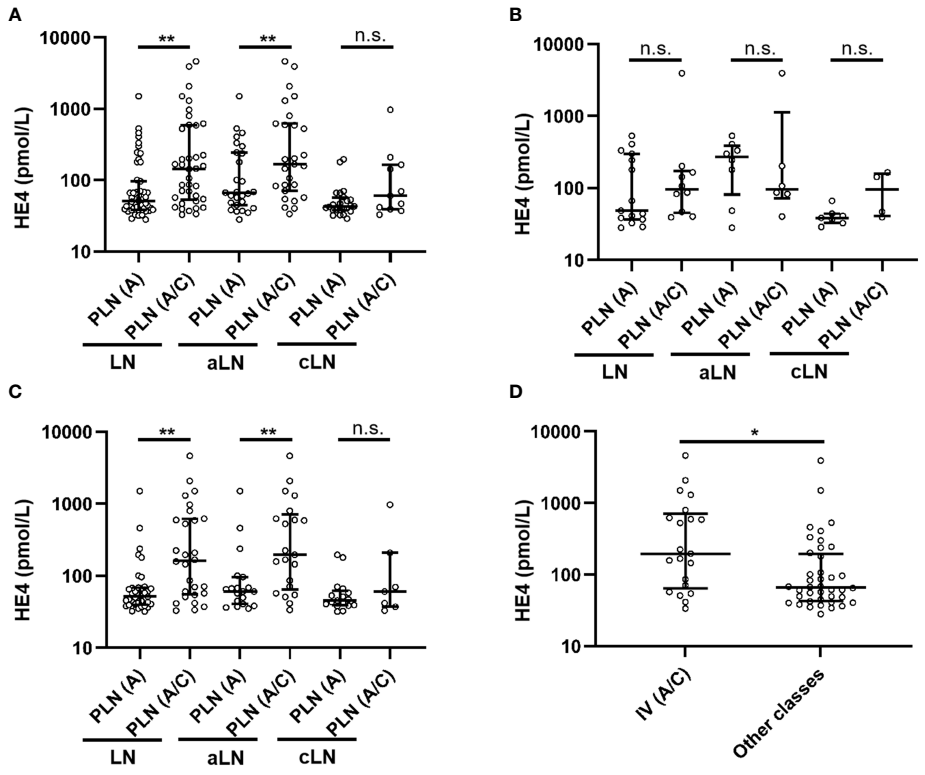

FIGURE 3
Comparison of serum HE4 levels between A and A/C lesions in the patients with class III/IV LN **(A)**, class III LN alone **(B)**, and class IV LN alone **(C)**, as well as between the aLN patients with class IV (A/C) and those with other classes **(D)**. HE4, human epididymis protein 4; A, purely active lesions; A/C, active and chronic lesions; LN, lupus nephritis; PLN, proliferative lupus nephritis; aLN, adult-onset lupus nephritis; cLN, childhood-onset lupus nephritis. *, $P<0.05$; **, $P<0.01$; n.s., not statistically significant.

TABLE 4 Bivariate correlations of serum HE4 level with the variables in the patients with class IV aLN.

	r	95% CI	*P* value
Blood urea nitrogen	0.765	0.541 – 0.888	< 0.001
Serum creatinine	0.754	0.522 – 0.882	< 0.001
24-hour proteinuria	0.513	0.126 – 0.765	0.010
Random proteinuria	0.421	0.045 – 0.692	0.026
Serum albumin	-0.667	-0.836 – -0.382	< 0.001
Hemoglobin	-0.660	-0.832 – -0.370	< 0.001
Haematocrit	-0.644	-0.824 – -0.346	< 0.001
Serum C3 level	-0.494	-0.737 – -0.136	0.008
Lymphocyte count	-0.407	-0.684 – -0.029	0.032
Serum C4 level	-0.398	-0.678 – -0.018	0.036

HE4, human epididymis protein 4; aLN, adult-onset lupus nephritis; CI, confidence interval; C3, complement C3; C4, complement C4.

IgG. Additionally, we demonstrated that serum HE4 level was increased in the PLN subgroup of aLN, especially in class IV (A/C) aLN, and observed a significant association between serum HE4 levels and renal functions measured by blood urea nitrogen (BUN) and serum creatinine levels. However, the association between serum HE4 and SLE disease activity index (SLEDAI) was not available to be analyzed due to the lack of SLEDAI data.

The pathogenic mechanism of LN is not completely understood. To date, LN has been thought to be initiated by the immune complexes and complement components in the glomeruli, and its pathogenesis involves continuing inflammation, hypoxia, metabolic abnormalities, aberrant tissue repair, and tissue fibrosis (31–33). PLN is a frequent and severe type of LN and entails a more aggressive course and deterioration of renal function and higher risk of progression to ESRD than non-PLN (6, 14, 19, 34). Patients with PLN have poor early response to treatment (within 6 months) and poor outcomes (35, 36). Effective clinical management of PLN is vital for maximal renal survival in patients with this disease and highly dependent on accurate and timely diagnosis and therapy. Thus, early laboratory parameters of (non-)response to induction treatment and of high risk of poor renal outcome may prove beneficial in determining the optimum treatment choice. Conventional parameters, such as serum creatinine level and proteinuria, are neither sensitive nor specific for differentiating LN from other glomerulopathies or distinguishing active inflammation from chronic scarring in the kidneys and do not accurately reflect histopathological changes (37). In this study, we found that serum HE4 level is increased in adult patients with PLN, indicating that serum HE4 level can be a promising non-invasive diagnostic biomarker of PLN. Though serum HE4 level was not significantly higher in childhood patients with PLN, this may be explained by the relatively small sample size of childhood patients, thus a larger sample size of childhood proliferative LN patients are needed to analyze the difference in serum HE4 levels when stratified by LN class. Overall, this finding provides a biofluid-based diagnostic method complementary to renal biopsy in PLN. Besides PLN, the relationship between kidney involvement and serum HE4 level were also found in primary Sjogren's syndrome (38) and systemic sclerosis (39).

Class III lesions were defined as proliferative glomerulonephritis affecting fewer than 50% of the glomeruli, whereas class IV was defined as proliferative glomerulonephritis affecting more than 50% of the glomeruli. In this study, we further subdivided PLN into A and A/C subgroups based on renal histopathological features. We found that patients with class IV (A/C) aLN have significantly higher serum HE4 levels than those with class IV (A) aLN, indicating that HE4 might be involved in C lesions. However, there was no significant difference in serum HE4 level between the class III (A/C) and class III (A) aLN patients. These results may be caused by the difference in the severity of the lesions between class IV and class III. Furthermore, since PLN patients with A/C lesions have significantly higher severity scores of interstitital fibrosis than those with A lesions (40), HE4 might be involved in interstitial fibrosis. A positive association between serum HE4 level and renal fibrosis has been reported (41, 42). Myofibroblasts are important mediators of renal fibrosis. LeBleu et al. have revealed that *HE4* is an upregulated gene in myofibroblasts, and it can bind to and inhibit multiple proteases, including serine proteases and matrix metalloproteinases, thereby suppressing the proteolytic degradation of type I collagen (26). Neutralization of HE4 accelerates collagen I degradation and alleviates renal fibrosis in mouse models of renal diseases (26). Our study provides an additional insight for a better understanding of the pathogenesis of C lesions in PLN. Accordingly, HE4 might be a potential therapeutic target for the treatment of PLN. Nevertheless, the role of HE4 in PLN should be investigated further in the future.

The composition of urine, containing waste products from blood that are filtered and excreted by kidneys, can reflect the state of renal function. However, we did not measure urine HE4 levels of the patients. In addition, sera from patients with other renal diseases could be collected to detect serum HE4 level. Thus, a prospective cohort study should be designed to concurrently collect serum and urine samples from patients and analyze whether urine HE4 level can predict the diagnosis of PLN.

Conclusion

Serum HE4 level is elevated in adult patients with PLN, and HE4 may play a role in the pathogenesis of chronic lesions in patients with class IV aLN.

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 human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (No. IIT-2021-778). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization: LBL, LSL, and ML. Study design: LBL, FW, and HZhang. Sample collection: LBL, HYX, YL, RL, QS, HZhu, HXX, FW, and HZhang. Data acquisition: LBL, HYX, YL, RL, QS, and HZhu. Data analysis and interpretation: LBL, HYX, HXX, LSL, ML, FW, and HZhang. Statistical analyses: LBL, and RL; funding acquisition: LBL. Original draft preparation: LBL, and HYX. Manuscript review and editing: FW, and HZhang. Supervision: HXX, ML, FW, and HZhang. All authors contributed to read and approved the final version of the manuscript. Authors take full responsibility for all aspects of the presented work.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1179986/full#supplementary-material

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Antibodies against *Porphyromonas gingivalis* in serum and saliva and their association with rheumatoid arthritis and periodontitis. Data from two rheumatoid arthritis cohorts in Sweden

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Background: Periodontitis and oral pathogenic bacteria can contribute to the development of rheumatoid arthritis (RA). A connection between serum antibodies to *Porphyromonas gingivalis* (*P. gingivalis*) and RA has been established, but data on saliva antibodies to *P. gingivalis* in RA are lacking. We evaluated antibodies to *P. gingivalis* in serum and saliva in two Swedish RA studies as well as their association with RA, periodontitis, antibodies to citrullinated proteins (ACPA), and RA disease activity.

Methods: The SARA (secretory antibodies in RA) study includes 196 patients with RA and 101 healthy controls. The Karlskrona RA study includes 132 patients with RA ≥ 61 years of age, who underwent dental examination. Serum Immunoglobulin G (IgG) and Immunoglobulin A (IgA) antibodies and saliva IgA antibodies to the *P. gingivalis*–specific Arg-specific gingipain B (RgpB) were measured in patients with RA and controls.

Results: The level of saliva IgA anti-RgpB antibodies was significantly higher among patients with RA than among healthy controls in multivariate analysis adjusted for age, gender, smoking, and IgG ACPA ($p = 0.022$). Saliva IgA anti-RgpB antibodies were associated with RA disease activity in multivariate analysis ($p = 0.036$). Anti-RgpB antibodies were not associated with periodontitis or serum IgG ACPA.

Conclusion: Patients with RA had higher levels of saliva IgA anti-RgpB antibodies than healthy controls. Saliva IgA anti-RgpB antibodies may be associated with RA

disease activity but were not associated with periodontitis or serum IgG ACPA. Our results indicate a local production of IgA anti-RgpB in the salivary glands that is not accompanied by systemic antibody production.

KEYWORDS

rheumatoid arthritis, periodontitis, porphyromonas gingivalis, anti-citrullinated antibodies (ACPAs), saliva, gingipain and periodontitis

Introduction

A mucosal association to development of rheumatoid arthritis (RA) is becoming generally accepted, and the lungs as well as the intestine and the oral cavity have been implicated as possible initiating sites (1–5). Regarding the oral cavity, the periodontis-associated pathogen *Porphyromonas gingivalis* (*P. gingivalis*) is one of the mechanisms discussed. A connection between serum antibodies to *P. gingivalis* and RA has been established (6), but data on saliva antibodies to *P. gingivalis* in RA are lacking. The presence of saliva antibodies to Arg-specific gingipain B (RgpB) in RA has not been investigated previously and may provide additional clues to explain the link between inflammation of the oral mucosa and RA.

RA and periodontitis (PD) are two chronic diseases associated with elevated levels of circulating pro-inflammatory cytokines and destruction of soft tissue and bone (7, 8). *P. gingivalis*, a Gram-negative anaerobic bacterium, is a major etiological agent of PD unique among bacteria with respect to expression of peptidylarginine deiminase (PPAD), which catalyzes the posttranslational modification of arginine residues to citrulline. *P. gingivalis* has arginine-specific (RgpA and RgpB) and lysine-specific (Kgp) cysteine proteases, gingipains, expressed on the surface of the bacterial outer membrane, which are essential for attachment, colonization, and evasion of host defense (9). *P. gingivalis* has been hypothesized to play a causative role in RA by inducing the production of antibodies to citrullinated proteins (ACPA) (10), and, recently, the ability of C-terminal citrullinated peptides generated by concerted action of Arg-specific gingipains and PPAD to breach immunotolerance was implicated as a causal link (11).

A meta-analysis by Bae and Lee reported that serum *P. gingivalis* antibody levels in the RA group were higher as compared to controls, whereas, in the ACPA-positive group, serum antibody levels were significantly higher compared to that in the ACPA-negative group (12). These results confirmed the results of an earlier meta-analysis by Bender et al., which showed higher antibody levels for serum *P. gingivalis* IgG in RA as compared to systemically healthy controls with and without PD (13).

We have previously shown in the Karlskrona RA study in Sweden that PD was associated with RA with an odds ratio (OR) of 2.5 as compared to age-matched controls from the normal population from Karlskrona city (14) and that ACPA in serum and saliva were not associated with PD (15).

In the present study of antibodies to RgpB in serum and saliva, we used two well-characterized cohorts of patients with typical RA in Sweden with long-standing disease, comorbidities, systemic conditions, and anti-rheumatic medication. We had access to paired samples of serum and saliva and also clinical PD status from the Karlskrona RA study. Our hypotheses were that patients with RA would have higher levels of antibodies to RgpB than healthy controls, that anti-RgpB antibodies would be associated with higher disease activity of RA, and that patients with RA with PD would have higher levels of antibodies to RgpB than patients with RA with no PD.

Materials and methods

The SARA study

Patients with established RA (n = 196) and healthy controls (n = 101) were included in the SARA (secretory antibodies in RA) study in Sweden, which has been described in detail previously (16). Patients with a clinical diagnosis of RA (M05 and M06, ICD-10) and a planned follow-up visit were randomly selected for inclusion. Healthy controls were recruited among blood donors from the same geographical region. Disease activity among patients with RA was measured by the disease activity score of 28 joints using erythrocyte sedimentation rate (ESR) (DAS28ESR) (www.das-score.nl). Paired serum and saliva samples were collected from both patients with RA and healthy controls. Serum samples from 195 of the 196 patients with RA and all 101 controls, as well as saliva samples from 188 of the 196 patients with RA and 100 of the 101 controls, were available for analysis of anti-RgpB antibodies.

The Karlskrona RA study

The Karlskrona RA study has been described in detail in previous studies (14, 15, 17). Briefly, between October 2013 and January 2015, all individuals with a clinical diagnosis of RA (M05 and M06, ICD-10) ≥ 61 years of age living in Karlskrona city in Southern Sweden were identified from the electronic regional database (Region Blekinge, Sweden). The patients with RA were invited per mail once to a rheumatological consultation with examination at the outpatient clinic at the Rheumatology Department. No data on previous antibiotic treatment or previous periodontal treatment were available. A total of 132 of the 242 (55%) patients with RA were recruited, and 83% of the patients with RA fulfilled the 1987 ACR classification

criteria (18) and 72% fulfilled the 2010 ACR/EULAR classification criteria (19) for RA. A total of 89% of the patients had regular dental healthcare at least once a year. Serum samples from 130 of the 132 patients with RA and saliva samples from 111 of the 132 patients with RA were available for analysis of anti-RgpB antibodies.

Definition of gingivitis and periodontitis (Karlskrona RA study)

A dental hygienist performed the clinical dental examinations. Panoramic radiographs were assessed by a periodontist (author RGP) masked to clinical dental and medical data. Gingivitis was defined as having ≥ 20% of measured sites with evidence of bleeding on probing. PD was defined as the clinical presence of bleeding on probing at > 20% of recorded tooth surfaces, presence of > 2 non-adjacent sites with a periodontal probing depth (PPD) ≥ 5 mm, presence of bone loss at ≥ 2 sites with a distance between the cementoenemel junction (CEJ) and bone level of ≥ 5 mm, or if evidence of a furcation invasion at molar teeth was found either clinically (grade II) or clearly visible on panoramic radiographs and bone loss ≥ 5 mm at ≥ 30%. The definition of PD was based on 2013 standards and included only individuals with chronic PD.

Samples and antibody analyses

Saliva sampling was performed using passive secretion, with study participants drooling for 10 min into a test tube placed on ice. Patients unable to provide 0.5 ml or more of saliva were excluded from the study, which means that patients with severe Sjögren's disease were most likely excluded. The samples were centrifuged for 5 min at 5,000 g, and the supernatant was stored at −80°C until analysis (16). Serum samples were stored at −80°C until analysis.

RgpB antibody analyses

Anti-RgpB antibodies were analyzed by an Enzyme-linked immunosorbent assay (ELISA) developed by Quirke et al. (20) and modified by Kharlamova et al. (6). In short, the recombinant RgpB antigen expressed by genetically modified *P. gingivalis* W83 strain and purified as described earlier (21) was diluted to 2.8 µg/ml in coating buffer, and 96-well Nunc high-binding plates were coated with 100 µl per well, incubated overnight at 4°C, washed four times with PBS-Tween (0.05%), and blocked with 2% Bovine serum albumin (BSA) overnight at 4°C. Saliva was diluted 1:20, serum was diluted at 1:800 (for IgG RgpB) or 1:100 (for IgA RgpB) in a Tris-HCl buffer (pH 7.6), and 100 µl per well was added in duplicates and incubated 1.5 hours at room temperature. Plates were washed four times, and secondary antibody was added: anti-IgG (Goat Anti-Human IgG, Jackson ImmunoResearch) at a dilution of 1:10,000 or anti-IgA (Polyclonal Rabbit Anti-Human IgA/HRP, DakoCytomation) at a dilution of 1:2,000. Tetramethylbenzidine (TMB) substrate (Sigma) was added (100 µl per well), and, after 25 min, the reaction was stopped by adding 0.5M H_2SO_4 (100 µl per well); absorbance was measured at 450 nm. The inter-assay assay variation was 8.2% for serum IgG anti-RgpB antibodies, 7.6% for serum IgA anti-RgpB antibodies, and 9.8% for saliva IgA anti-RgpB antibodies. A serial dilution of the same high-level serum was used on each plate to create a reference curve, to obtain arbitrary units (AU).

ACPA analyses

IgG and IgA ACPA in serum and IgA ACPA in saliva samples have been analyzed previously (16). Serum IgG antibodies to citrullinated peptides were analyzed using the second-generation anti-CCP immunoassay (Svar Life Science, Malmö, Sweden). IgA ACPA was analyzed similarly, but using an anti-human α-chain antibody as secondary antibody (22). Cutoff limit for IgG ACPA positivity was set at 25 U/ml according to the manufacturer's instructions and for IgA ACPA at 25 AU/ml, corresponding to the 99th percentile of 101 healthy blood donors.

Ethical approval

The Regional Ethical Review Boards at Uppsala and Lund Universities, Sweden, approved the studies (Uppsala 2011/159, LU 2013/323). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committees of Uppsala and Lund University in Sweden and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Statistics

Mann–Whitney U-test was used to evaluate the difference in anti-RgpB antibody levels among patients with RA versus controls and among patients with RA positive versus negative for IgG ACPA in serum. Chi-square was used for categorical variables. Spearman's correlation coefficient was used to study the correlation of the levels of anti-RgpB antibodies and DAS28ESR as a continuous variable.

To evaluate the difference in anti-RgpB antibody levels between patients with RA and healthy controls, binary logistic regression analyses using RA as the dependent variable were performed with either saliva IgA RgpB antibodies or serum IgG or IgA RgpB antibodies as the independent variables, adjusted for age, gender, smoking status, and serum IgG ACPA status (positive or negative).

To evaluate the difference in anti-RgpB antibodies between patients with RA with and without PD, binary logistic regression analyses were performed using PD as the dependent variable and either saliva IgA antibodies to RgpB or serum IgG or IgA antibodies to RgpB as the independent variable, adjusted for age, gender, disease duration, smoking status, DAS28ESR, socioeconomic status, body mass index, and serum IgG ACPA status (positive or negative).

To evaluate whether anti-RgpB antibodies were associated with disease activity, binary logistic regression analyses were performed using DAS28ESR remission (< 2.6) or DAS28ESR moderate/high

disease activity (>3.2) versus remission/low disease activity as the dependent variable and anti-RgpB antibodies (IgG and IgA in serum and IgA in saliva) as the independent variables, adjusted for age, gender, and smoking status. Simulations in the statistical program R (version 4.2.2) were used to confirm goodness of fit of data in the logistic regression analyses in the SARA study and to create Figure 1. The significance level was set at $\alpha < 0.05$. Statistical analyses were performed using IBM SPSS Statistics version 28.0.0.0 (Armonk, NY, USA).

Results

Table 1 shows the demographics, disease activity, and anti-rheumatic medication in the SARA and Karlskrona RA studies and the healthy controls in the SARA study. Figure 1 shows the levels of the different anti-RgpB antibodies in both studies. The corresponding numbers are provided in Supplementary Table 1.

Anti-RgpB antibodies in patients with RA versus healthy controls: the SARA study

The median level of saliva IgA anti-RgpB antibodies was higher among patients with RA (14 AU/ml) than among healthy controls (0 AU/ml) in the SARA study ($p < 0.001$ using Mann–Whitney U-test). In a binary logistic regression analysis adjusting for age, gender, smoking, and serum IgG ACPA, there was still a significant association between the level of IgA anti-RgpB in saliva and RA, with 1.3% increased risk for having RA per increased unit of IgA anti-RgpB [odds ratio (OR) = 1.013; 95% confidence interval (CI), 1.002–1.024; $p = 0.022$].

There were no statistically significant associations between serum anti-RgpB antibodies and RA in the univariate analyses or in the logistic regression analyses, regarding neither IgG anti-RgpB antibodies (OR = 1.001; 95% CI, 0.999–1.002; $p = 0.368$) nor IgA anti-RgpB antibodies (OR = 1.001; 95% CI, 1.000–1.003; $p = 0.111$).

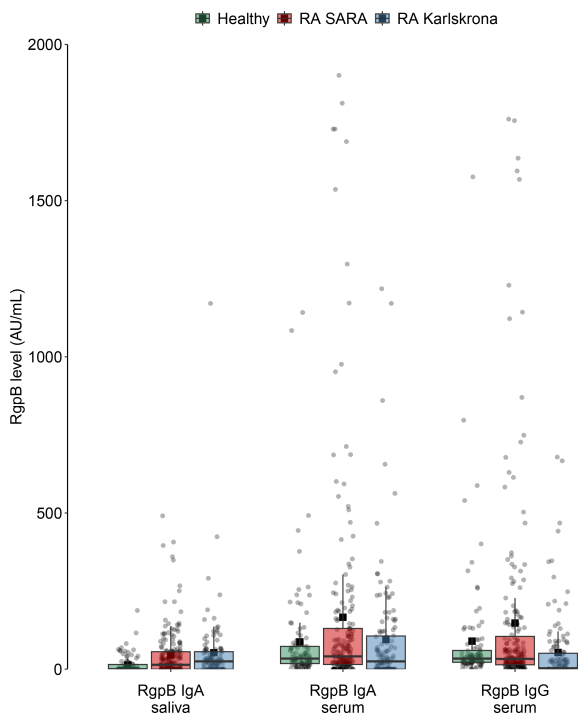

FIGURE 1
Levels of anti-RgpB antibodies among healthy controls and among patients with RA on the SARA and Karlskrona studies. All individual values are symbolized by gray dots, median value, and quartiles in the boxplots, and mean values symbolized by black squares.

Anti-RgpB antibodies and ACPA

No significant associations were seen between anti-RgpB antibody levels and ACPA status, in neither serum nor saliva, using Mann–Whitney U-test. In the SARA study, patients with RA positive for IgG ACPA did not have higher levels of IgG anti-RgpB in serum ($p = 0.773$), IgA anti-RgpB in serum ($p = 0.696$), or IgA anti-RgpB in saliva ($p = 0.253$). In the Karlskrona RA study, the corresponding values were IgG anti-RgpB in serum ($p = 0.373$), IgA anti-RgpB in serum ($p = 0.145$), and IgA anti-RgpB in saliva ($p = 0.240$).

Anti-RgpB antibodies and RA disease activity

In the SARA study, correlations between IgA anti-RgpB antibodies in both saliva and serum and disease activity parameters such as DAS28ESR, swollen, and tender joint counts were highly significant but weak in the univariate analyses, with a Spearman's rho of 0.2 to 0.3. No significant correlations were seen between IgG anti-RgpB antibodies in serum and disease activity parameters. Saliva IgA anti-RgpB antibodies were associated with DAS28ESR ($r_s = 0.295$, $p < 0.001$), swollen joint count ($r_s = 0.323$, $p < 0.001$), tender joint count ($r_s = 0.224$, $p = 0.004$), ESR ($r_s = 0.212$, $p = 0.004$), and C-reactive protein (CRP) ($r_s = 0.179$, $p = 0.014$). Serum IgA anti-RgpB antibodies were associated with DAS28ESR ($r_s = 0.228$, $p = 0.004$), swollen joint count ($r_s = 0.203$, $p = 0.008$), tender joint count ($r_s = 0.202$, $p = 0.009$), and ESR ($r_s = 0.145$, $p = 0.044$). When evaluating the association between anti-RgpB antibodies and disease activity in binary logistic regression analyses using DAS28ESR remission (DAS28ESR < 2.6) (yes/no) as the dependent variable, adjusting for age, gender, and smoking, no significant association could be seen; regarding IgA anti-RgpB antibodies in saliva, the OR was 1.003 (95% CI, 0.998–1.009; $p = 0.251$); regarding IgA anti-RgpB antibodies in serum, the OR was 1.001 (95% CI, 1.000–1.002; $p = 0.085$), and regarding IgG anti-RgpB antibodies in serum, the OR was 1.001 (95% CI, 1.000–1.002; $p = 0.233$). In the SARA study, similar non-significant results were seen when using moderate/high disease activity (≥ 3.2) versus remission/low disease activity (<3.2) as the dependent variable. In all these regression analyses, age was the one variable showing a highly significant association to DAS28ESR.

In the Karlskrona RA study, saliva IgA anti-RgpB antibodies were associated with DAS28ESR ($r_s = 0.232$, $p = 0.017$), swollen joint count ($r_s = 0.207$, $p = 0.03$), and tender joint count ($r_s = 0.192$, $p = 0.044$). Serum IgG anti-RgpB antibodies were associated with CRP ($r_s = 0.255$,

TABLE 1 Baseline characteristics and disease activity variables in the Karlskrona RA study and the SARA study.

Variable	Karlskrona RA study N = 132	SARA study	
		RA N = 196	Controls N = 101
Females %	71%	80%	53%
Age years, mean (SD)	70 (6.6)	64 (13.3)	49 (14.0)
Disease duration years, mean (SD)	14 (13.0) N = 127	12 (10.5) N = 193	–
Ever smokers %	62%	52%	36%
Never smoker %	38%	48%	64%
RF positive %	58% N = 129	74%	–
Serum IgG ACPA positive %	67%	80%	0%
VAS pain, mean (SD)	33 (28) N = 129	29 (23) N = 163	–
VAS global, mean (SD)	33 (26) N = 131	32 (23) N = 162	–
ESR, mean (SD)	19 (16) N = 129	19 (17) N = 194	–
CRP, mean (SD)	9.4 (8.8)	7.7 (14) N = 195	–
DAS28ESR, mean (SD)	3.0 (1.1) N = 127	2.8 (1.2) N = 158	–
csDMARDs %	67%	86%	–
On biologics %	22%	37%	–
On methotrexate %	57%	76%	–
On glucocorticoids %	47%	27%	–
With periodontitis %	61%		

BMI, body mass index; VAS, visual analogue scale; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28ESR, disease activity score (28 joints) calculated with ESR; ACPA, anti-citrullinated protein antibodies; RF, rheumatoid factor; DMARD, disease-modifying anti-rheumatic drug; csDMARDs, conventional synthetic DMARDs.

p = 0.003) and serum IgA anti-RgpB antibodies with CRP (r_s = 0.206, p = 0.019). In the binary logistic regression analyses, using DAS28ESR remission (DAS28ESR < 2.6) as the dependent variable, adjusting for age, gender, and smoking, no significant association could be seen; regarding IgA anti-RgpB antibodies in saliva, the OR was 0.994 (95% CI, 0.987–1.002; p = 0.147); regarding IgA anti-RgpB antibodies in serum, the OR was 0.998 (95% CI, 0.996–1.001; p = 0.200); and regarding IgG anti-RgpB antibodies in serum, the OR was 1.001 (95% CI, 0.997–1.004; p = 0.749). In the Karlskrona study, when using moderate/high disease activity (≥ 3.2) as the dependent variable, similar non-significant results were seen regarding serum IgG and IgA anti-RgpB antibodies. However, regarding saliva IgA anti-RgpB antibodies, a significant association was seen with an OR value of 1.008 (CI, 1.001–1.015; p = 0.036).

Periodontitis: Karlskrona RA study

A total of 80 of the 132 (61%) patients with RA in the Karlskrona RA study had PD. There were no significant differences in level of any of the anti-RgpB antibodies between patients with and without PD (serum IgG anti-RgpB, p = 0.106; serum IgA anti-RgpB, p = 0.054; saliva IgA anti-RgpB, p = 0.086; using Mann–Whitney U-test). In the binary logistic regression analyses, neither saliva IgA anti-RgpB antibodies (OR = 0.999; 95% CI, 0.966–1.003; p = 0.703), serum IgG (OR = 1.002; 95% CI, 0.998–1.006; p = 0.306), nor serum IgA anti-RgpB antibodies (OR = 1.001; 95% CI, 0.998–1.005; p = 0.371) were significantly associated with PD.

Discussion

This is the first study to explore saliva IgA anti-RgpB antibodies in patients with RA and healthy controls (the SARA study) and in patients with RA with and without PD (the Karlskrona RA study). The levels of saliva IgA anti-RgpB antibodies were found to be significantly higher among patients with RA than among healthy controls; among patients with RA, levels of saliva IgA anti-RgpB antibodies were associated with RA disease activity. In contrast, there was no difference in the level of serum anti-RgpB antibodies between

rheumatologically healthy controls and patients with RA, and serum RgpB antibodies were not associated with RA disease activity. The other main finding was that neither serum nor saliva anti-RgpB antibodies were associated with PD among patients with RA. Methodological limitations of this study include the cross-sectional design and rather small sample size.

In our study, anti-RgpB antibodies were not associated with ACPA positivity or levels. In the meta-analysis by Bae and Lee, a positive correlation was found between *P. gingivalis* antibody levels and ACPA levels (12). However, out of the five studies in the meta-analysis (23–27), only one small study (23), including 50 patients with RA, could demonstrate a significant association between antibodies to *P. gingivalis* and IgG ACPA and one study including 78 patients with RA (24) found an association between antibodies to *P. gingivalis* and IgM ACPA, but not IgG or IgA ACPA. Kharlamova et al. (6) performed a large study including 1,974 patients with RA and found higher levels of serum IgG anti-RgpB in ACPA-positive RA compared to ACPA-negative RA, with median levels of 231 AU/ml *vs.* 166 AU/ml. Patients with RA in the study by Kharlamova et al. had a disease duration of less than 1 year, whereas our patients had a disease duration of more than 10 years. This may indicate that immunological mechanisms involving an association between ACPA and RgpB may be of importance in early RA, and possibly even before the development of arthritis, rather than in established RA. In addition, our patient cohorts were smaller.

Anti-RgpB antibody levels were not found to associate with disease activity in the regression analyses in the SARA study, whereas an association between saliva IgA anti-RgpB antibody levels and DAS28ESR could be seen in the Karlskrona study. As this association was seen only when using DAS28ESR dichotomized in moderate/high versus remission/low disease activity as the dependent variable but not when using DAS28ESR remission yes/no as the dependent variable and was not seen at all in the SARA study, the significance of this finding is unclear. It is, however, interesting to notice that it concerns saliva antibodies only and not serum antibodies. The correlation between saliva anti-RgpB antibodies and disease activity has earlier not been investigated, but correlations have been reported between serum antibodies to *P. gingivalis* and ESR (23, 25) or CRP (28). However, those studies include DMARD naïve patients (23) and patients with higher DAS28ESR, ESR, and CRP than patients in our study, most of whom are well treated with mean DAS28ESR of 2.8 and 3.0. A large majority of patients in our study are on treatment with conventional and/or biologic DMARDs. Longitudinal studies would be better suited to clarify a possible connection between antibodies to *P. gingivalis* and disease activity in RA.

In this study, serum anti-RgpB antibodies did not differ between patients with RA and controls, whereas Kharlamova et al. (6), using the same anti-RpgB method as we did, found higher levels of IgG anti-RgpB in serum in Swedish patients with RA than in non-RA controls. In our study, patients with RA had a mean disease duration of >10 years and were well treated for their RA, whereas the study by Kharlamova et al. includes patients with early RA. The difference in results may indicate that *P. gingivalis* antibodies are of importance especially in early RA.

Our results, showing a connection between saliva but not serum anti-RgpB antibodies and RA, indicate a local production of IgA anti-RgpB in the salivary glands that is not accompanied by systemic antibody production or increased prevalence of PD. This finding supports the mucosal association hypothesis, suggesting that inflammatory events in mucosal compartments, such as the oral cavity, may be an important part of RA pathogenesis. Further understanding of these events may contribute to the development of new therapeutic or even preventive strategies, possibly by reducing oral pathogens and mucosal inflammation in RA or pre-RA individuals and more active treatment of PD. However, further work is needed to delineate possible mechanisms by which mucosal immune response to *P. gingivalis* is involved in RA development or progression.

Serum anti-RgpB antibody levels were found to be lower in the Karlskrona RA study than in the SARA study. We do not have a good explanation for this. The populations are similar, although the Karlskrona RA patients are older (all being ≥ 61 years of age), and we possibly have a small sample size problem in the Karlskrona RA study.

Earlier studies on non-RA patients have shown that serum and saliva *P. gingivalis* antibodies are associated with PD (29, 30) or with PPD and clinical attachment levels (31). In the present study on patients with RA, such an association was not found. One reason for this different finding could be that we used RgpB as antigen when analyzing antibodies to *P. gingivalis* and not lipopolysaccharide as in previous studies. In addition, in the present study, patients were older, and they were well treated with anti-rheumatic medications as well as having medications for their comorbidities, which may influence antibody production. In the study by Pudakalkatti and Baheti, patients with any systemic diseases were excluded, which may have an impact on the differences in results (31). It is further not known whether individuals classified as having PD had an ongoing infection with *P. gingivalis* at the time of sampling. Although *P. gingivalis* has been identified as a key pathogen in the development of PD (32), it must be recognized that this anaerobic pathogen is part of a very complex biofilm that may include a large variety of pathogens linking periodontal infection to systemic disease (33). Recent evidence suggests that there are significant differences in the composition of subgingival microbiota between individuals with or without a diagnosis of PD that may also include other pathogens than *P. gingivalis* (34).

Our RA patient cohorts are representative of general Swedish RA patient populations. In addition, the Karlskrona RA study is a truly population-based cohort with systematic sampling, and, for these patients, we also had a clinical PD status. We included patients with typical RA with comorbid and systemic conditions and smokers, as well as healthy controls. Our RA patient populations were well-treated and stable. It should be recognized that all study participants had access to government-subsidized dental and medical care. The lack of association between anti-RgpB antibodies and disease activity or PD in this study may partly be explained by this, as all anti-inflammatory medication theoretically could inhibit antibody production.

It may seem counterintuitive that anti-RgpB antibodies were associated with RA but not with PD, because *P. gingivalis* is considered a major cause of PD. A possible explanation for this could be that not all individuals with *P. gingivalis* infection and PD develop antibodies to the bacterium and that this tendency to produce antibodies is associated with the RA disease. This would correspond to the specific disposition of patients with RA do

develop ACPA, which non-RA individuals rarely do. In addition, the small sample size in the Karlskrona RA study may be an explaining factor.

One weakness of this study is that we did not have a clinical PD status for the SARA RA patients and healthy controls. Previous studies on the association between RA and *P. gingivalis* antibodies use different in-house ELISA techniques using different antigenic parts of the bacterium. As there is no standardized unit, direct comparisons of these studies are difficult. It is currently not confirmed whether the association of *P. gingivalis* and RA is a causal one or non-causal based on genetic risk factors and environmental factors such as smoking. Our cross-sectional study design does not allow us to assess causality.

In conclusion, the levels of saliva IgA anti-RgpB antibodies, but not serum anti-RgpB antibodies, were significantly higher among patients with RA than among healthy controls. In patients with RA, saliva IgA anti-RgpB antibodies were associated with RA disease activity in the Karlskrona study. None of the anti-RgpB antibodies were associated with PD. Anti-RgpB-antibodies were not associated with serum IgG ACPA.

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 human participants were reviewed and approved by Uppsala 2011/159 and LU 2013/323. The patients/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

AS, KL, SR, GP, JB and MS contributed to the conception and design of the study and to acquisition of data. BP and JP produced and provided RgpB antigen. AS, AK, SR, GP, JB and MS contributed to analysis and interpretation of data. AS wrote the first draft of the manuscript. MS, AK, BP and JP wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1183194/full#supplementary-material

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Urine L-selectin reflects clinical and histological renal disease activity and treatment response in lupus nephritis across multi-ethnicity

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Objective: There is an urgent need for novel biomarkers in lupus nephritis (LN). We report a non-invasive urinary biomarker, L-selectin, in two independent multi-ethnic cohorts.

Methods: uL-selectin was tested cross-sectionally in a Chinese cohort (n=255) and a US cohort (n=219) of SLE patients and controls using ELISA. A longitudinal cohort includes 20 active Chinese LN patients.

Results: uL-selectin was significantly increased in active LN patients compared to active non-renal SLE, inactive LN, inactive non-renal SLE, chronic kidney disease patients, and healthy controls. uL-selectin positively correlated with global and renal disease activities and was significantly associated with histological activity index and chronicity index (CI). Low uL-selectin was an independent predictor for high CI. During follow-up, uL-selectin levels decreased significantly in the complete renal remission group.

Conclusion: uL-selectin is a novel biomarker of disease activity and renal histopathology in LN across multiple ethnicities. It also reflects treatment response in LN patients during follow up.

KEYWORDS

lupus nephritis, L-selectin, urinary biomarker, renal histopathology, treatment response

Introduction

Lupus nephritis (LN) is one of the most common and serious manifestations of Systemic lupus erythematosus (SLE), which is more prevalent in African American, Hispanic, and Asian patients. Without prompt diagnosis or proper treatment, ~5~20% of LN patients would proceed to end-stage renal disease (ESRD) within 10 years from the initial diagnosis (1).

Currently, a kidney biopsy is the gold standard for the diagnosis of LN, which guides management strategy. However, various drawbacks limit its application in practice, including its invasive nature with major complications, interobserver variability, and patients' unwillingness. Although repeat biopsy has been implicated in long term management of LN (2), serial biopsies to monitor renal disease is not always practical. Hence, biomarkers, especially urine biomarkers, have become a promising tool for diagnosing and monitoring disease, evaluating treatment response, and predicting renal flares in LN patients, due to its non-invasive nature and repeatability.

Recent advances in "omics" technologies have changed the strategy of biomarker discovery from a hypothesis-driven approach to an agonistic approach. Previous studies using affinity-based techniques such as antibody-based or aptamer-based assays have identified novel protein biomarkers in urine (3, 4). Whether these biomarkers are robust enough for clinical use is still under investigation. One of the most important investigations is to rigorously validate the screened biomarkers in large-scale studies with multi-ethnic cohorts. Among the recently reported newly discovered urinary biomarkers, L-selectin emerged as a novel urinary biomarker of LN with good potential in distinguishing active LN patients from active non-renal SLE patients in a small validation cohort (4). In the current study, we aim to systematically validate L-selectin as a urinary biomarker of disease activity and treatment response in LN patients across multiple ethnicities, cohorts and test centers, a pre-requisite for eventually using these biomarkers in clinical practice.

Materials and methods

Study subjects

Cross-sectional cohorts

The cross-sectional study included subjects of two cohorts from three centers. The primary cohort was comprised of 195 Han Chinese SLE patients from the Renji Hospital, Shanghai Jiao Tong University (SJTU) School of Medicine, China, recruited from 2017 to 2019, including 87 biopsy-proven active LN (aLN), 57 active non-renal SLE (aNR), 25 inactive LN (iLN) and 26 inactive non-renal SLE (iNR). All aLN patients in the Chinese cohort had concurrent renal biopsies performed. Additionally, 33 patients with chronic kidney diseases (CKD) and 27 age- and gender-matched healthy subjects were also recruited as disease and healthy controls, respectively. The US-based cohort included 63 SLE patients from the Johns Hopkins University (JHU) School of Medicine, Baltimore, MD, United States; 103 SLE patients and 53 healthy controls from the University of Texas Southwestern (UTSW) Medical Center's Renal Clinic, Dallas, TX, United States, among which 32 SLE patients with active LN also had concurrent renal biopsies performed. The US-based cohort consisted of 34 Caucasian subjects, 114 African American subjects and 71 Hispanic subjects (Figure S1).

SLE patients with clinical components of Systemic Lupus Erythematosus Disease Activity Index-2k (cSLEDAI-2k) ≥ 4 were defined as active SLE, whereas patients with renal SLEDAI (rSLEDAI, refers to the total score of the four kidney-related parameters in SLEDAI) ≥ 4 were classified as aLN patients. Patients with active SLE and no history of renal involvement with rSLEDAI =0 were defined as aNR patients (5). The iLN patients had a history of LN with SLEDAI < 4 and rSLEDAI $= 0$. The iNR patients had no history of renal involvement with SLEDAI < 4 and rSLEDAI $= 0$ (Tables S1-5). All SLE patients met the 1997 revised American College of Rheumatology (ACR) classification criteria for SLE or 2012 SLICC criteria for SLE (6, 7). Informed consent was obtained from all participants and the study was approved by the ethics committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, and the Institutional Review Boards of the University of Houston, JHU School of Medicine, and UTSW.

Longitudinal cohort

Among the 87 active LN patients in the Chinese cohort, 20 patients who were followed up for at least 6 months had urine samples collected and stored at the end of the follow-up, and their clinical and laboratory results were documented and treatment outcomes were also evaluated.

Disease assessment

Disease activity was assessed by SLEDAI and rSLEDAI. In the SJTU cohort, SLICC renal activity score (SLICC RAS) was also

calculated to assess renal activity in aLN patients as previously described (8).

For the longitudinal cohort, treatment response was defined as complete renal remission (CRR), partial renal remission (PRR) or no renal remission (NRR), and the definitions were described in detail in Supplementary Materials (9).

Renal histology

All renal biopsies were documented for LN classes and scored for activity index (AI), chronicity index (CI), and their component attributes by two independent experienced renal pathologists who were blinded to the design of the study, using the 2018 revision of International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification for lupus nephritis (10). For US-based cohort, only AI and CI were recorded without detailed component attributes.

Assay of urinary L-selectin

Clean-catch midstream urine was collected from each patient in a 50 mL sterile container in the morning. For biopsy-concurrent aLN patients, urine samples were procured within 5 days before kidney biopsy. Urine samples were aliquoted to avoid repeated freeze-thaw cycles and stored at − 80°C. Urinary L-selectin (uL-selectin) was assayed using a commercially available human L-selectin enzyme-linked immunosorbent assay (ELISA) kit (DY728, R&D System; ELH-LSelectin, Raybiotech; 1:20) according to the manufacturer's instruction. uL-selectin was normalized by urine creatinine using Creatinine Parameter Assay Kit (KGE005, R&D Systems).

Statistical analysis

Data were analyzed and plotted using SPSS 26, GraphPad Prism 9.0 or R (Version 4.2.0). Data were expressed as mean (SD) for continuous variables with normal distribution, median (interquartile range (IQR)) for continuous variables with non-normal distribution and counts and percentage for dichotomous variables. The Kolmogorov–Smirnov tests established the normality of data. Group comparisons were made using the Mann-Whitney U test, Kruskal–Wallis, Wilcoxon matched-paired signed rank test, Chi-Squared or Fisher exact tests as appropriate. Non-parametric Spearman's method was performed for correlation analysis. Receiver operating characteristic (ROC) curve and areas under curve (AUC) were performed as appropriate. Correlation heatmap was generated using corrplot and Hmisc packages in R.

Patients of 87 biopsy-proven LN in the Chinese cohort were dichotomized according to median renal histological activity (median of AI, 6) and median renal histological chronicity (median of CI, 3). The association between uL-selectin levels at the baseline and high AI (AI > 6) or high CI (CI > 3) was investigated using univariate and further multivariate logistic regression by controlling the effect of confounding variables, including age, gender, SLE disease duration, LN disease duration, 24h proteinuria, eGFR and SLEDAI. A two-tail P value less than 0.05 was considered significant.

Results

uL-selectin was exclusively elevated in active lupus nephritis

In the Chinese cohort, uL-selectin levels were increased exclusively in aLN patients when compared with aNR patients, iLN patients, iNR patients, HC, or CKD patients (all $p < 0.0001$, Figure 1A). uL-selectin could significantly discriminate aLN patients from other groups of patients (all $p < 0.0001$, Figure 1A). uL-selectin outperformed conventional markers including C3, C4 and anti-dsDNA antibody in discriminating aLN patients from aNR patients (Figure 1E).

The US-based cohort was comprised of 219 subjects including 121 aLN patients, 17 aNR patients, 28 iLN patients and 53 HC. Results are presented parsed by ethic/racial groups – non-Hispanic Caucasians, non-Hispanic African Americans and Hispanics. In all of three subgroups, uL-selectin levels were significantly elevated in aLN patients when compared with HC (all $p < 0.001$, Figures 1B, C). uL-selectin levels further discriminated aLN from aNR patients in the Caucasian group ($p < 0.01$, Figure 1B) and the African American group ($p < 0.0001$, Figure 1C); aLN patients had higher uL-selectin levels than iLN patients in the African American group ($p < 0.001$, Figure 1C) and the Hispanic group ($p < 0.05$, Figure 1D). uL-selectin showed a better capability than conventional biomarkers in discriminating aLN and aNR patients in the Caucasian and African American groups (Figures 1F, G).

uL-selectin correlated with disease activity and other clinical characteristics

In the Chinese cohort, there is significant correlation between uL-selectin and disease activity indices, including SLEDAI, rSLEDAI and SLICC RAS (all $p < 0.0001$, Figures 1A, H) as well as 24h proteinuria, eGFR, serum Cr, complements, ESR and hemoglobin levels (all $p<0.05$) (Table S6). Furthermore, uL-selectin levels were associated with lymphadenopathy, Raynaud's phenomenon and serous effusion in SLE patients (Table S7).

In the US-based cohort, uL-selectin was positively correlated with SLEDAI and rSLEDAI in the African American (both $p < 0.0001$) and Hispanic (both $p < 0.05$) groups. But there was no significant correlation between uL-selectin levels and disease activity in the Caucasian group (Figures 1B–D). uL-selectin levels also correlated with 24h proteinuria, anti-dsDNA titers, serum C3, C4 and ESR (Table S8).

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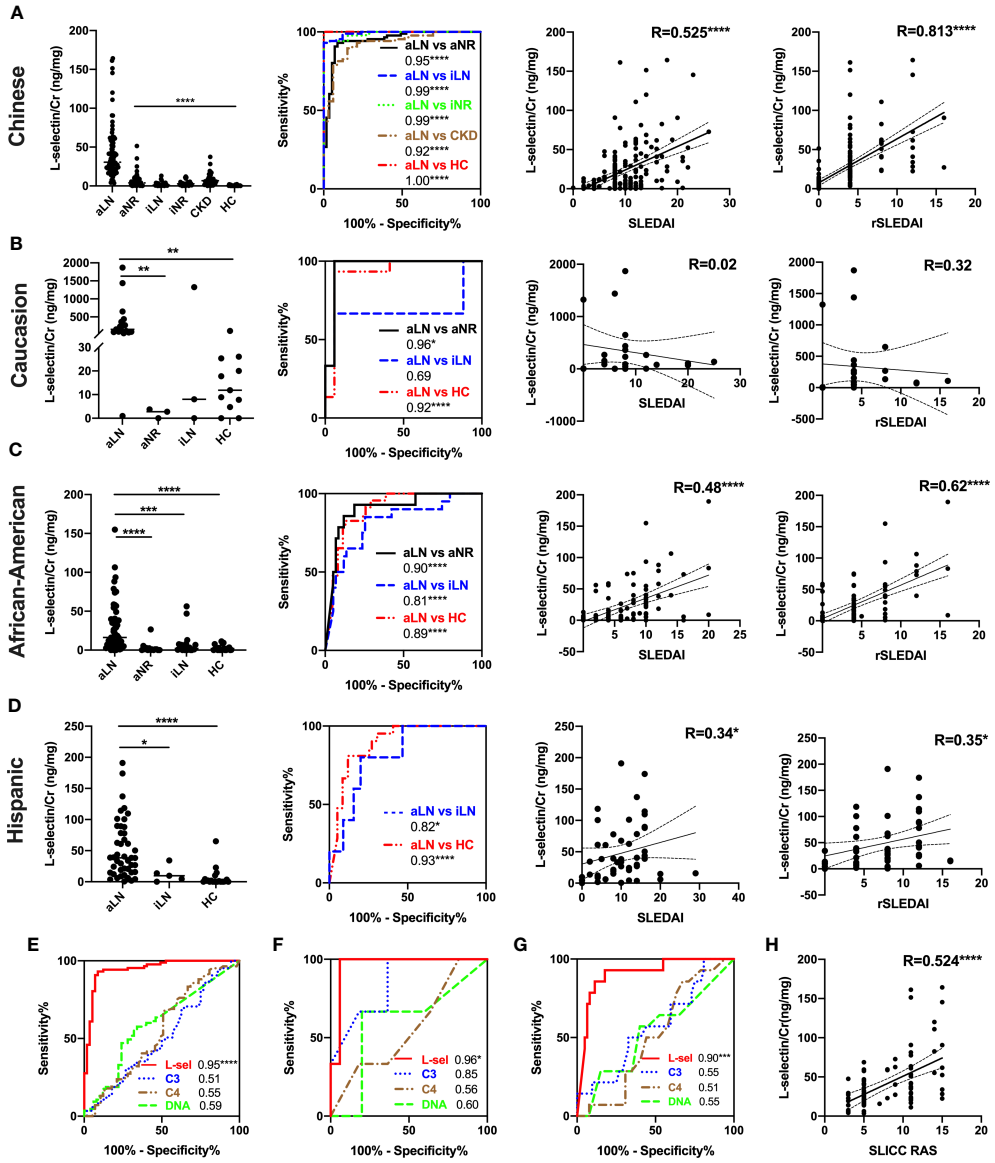

FIGURE 1
Comparison of uL-selectin levels among subject groups and their correlation with clinical indices among Asian (all were Chinese) **(A, E, H)**, non-Hispanic Caucasian **(B, F)**, non-Hispanic African American **(C, G)** and Hispanic **(D)** subjects. Kruskal-Wallis test and Dunn *post hoc* multiple comparisons test were used among subgroups across ethnicities. ROC curve analyses were performed, demonstrating the ability of uL-selectin to discriminate aLN from other subgroups **(A–D)** and its better performance in discriminating aLN from aNR than conventional biomarkers (C3, C4 and anti-dsDNA antibody) in the Asian **(E)**, Caucasian **(F)** and African American **(G)** groups. Values in the plot indicate areas under curve (AUC). uL-selectin was correlated significantly with SLEDAI, rSLEDAI in the Asian, African American, and Hispanic groups **(A, C, D)**, as well as with SLICC RAS in the Asian group **(A, H)**. With respect to the data from the African American subjects, the shown analyses have been executed after removing 2 outliers. Whereas the mean ± SD of uL-selectin level in the rest of the patients was 29 ± 31.5 (ng/mg), the mean in these 2 outliers was 3533.7 (ng/mg). Inclusion of both subjects yielded correlation coefficients of 0.54 and 0.61 with SLEDAI and rSLEDAI, respectively (not shown). aLN, active lupus nephritis; HC, healthy control; aNR, active non-renal; iLN, inactive lupus nephritis; iNR, inactive non-renal; CKD, chronic kidney disease; L-sel, urinary L-selectin adjusted by creatinine; DNA, anti-dsDNA antibody; R, Spearman's correlation coefficient; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

uL-selectin reflected concurrent renal pathological indices

In the 87 active LN patients with concurrent renal biopsies in the Chinese cohort, uL-selectin levels were elevated in each LN pathology class when compared with HC (p<0.0001). There was a trend for higher uL-selectin levels in proliferative LN (III ± V & IV ± V) patients than in non-proliferative LN (II & V) patients, but no statistical significance was observed (Figure S2). Importantly, uL-selectin positively correlated with AI (r = 0.34, p < 0.01) and negatively correlated with CI (r = -0.30, p < 0.01) in renal histopathology (Figures 2A, B). When we looked into the detailed aspects of AI and CI, uL-selectin correlated significantly with endocapillary hypercellularity, fibrinoid necrosis, wire loop deposits and interstitial inflammation of AI and with glomerulosclerosis, fibrous crescents, interstitial fibrosis and

tubular atrophy of CI (Figure 2C; Table S9). ROC analyses confirmed the potential of uL-selectin to discriminate high AI (AI>6) from low AI (AI ≤ 6) (p<0.01, Figure 2D), and to differentiate high CI (CI>3) from low CI (CI ≤ 3) (p<0.01, Figure 2E). uL-selectin enhibited a similar capability to 24h urine protein and rSLEDAI in discerning high and low levels of AI. Additionally, uL-selectin displayed an exceptional proficiency when distinguishing between high and low levels of CI. Univariate logistic regression analysis was performed to discover potential risk factors for high CI. The results showed that lower uL-selectin (p = 0.005), higher age (p = 0.047), lower eGFR (p < 0.001) and lower SLEDAI (p = 0.014) were associated with high CI (Figure 2F; Table S10, Figure S3). Multivariate logistic regression models constructed for predicting high CI revealed that the addition of uL-selectin to all the evaluated models significantly improved the model fit after adjustment for age, gender, SLE disease duration, LN duration and 24h proteinuria, and its contribution was always significant. (Figure 2F; Table S11, Figure S3).

Correlation analyses of uL-selectin with renal pathology indices were also performed in 32 biopsy-concurrent LN patients from the US-based cohort. As observed in the Chinese cohort, uL-selectin showed significant positive correlation with AI (r=0.47, p<0.01), and negative correlation with CI (not attaining significance) (Figure S4). In this cohort, uL-selectin was superior to proteinuria in distinguishing patients with high AI and those with high CI from the controls (Figure S4). To evaluate the potential influence of race, we incorporated subjects from two cohorts representing four different races to re-perform the regression analyses. The results indicated that even after adjusting for race and other crucial confounding factors such as age, gender, and proteinuria, uL-selectin remained an independent predictor of high CI in the multivariate logistic regression analyses (Table S12).

In addition, biopsy-concurrent LN patients were divided into four subgroups with combined AI and CI. uL-selectin could significantly discriminate patients with both high AI (AI>6) and low CI (CI ≤ 3) (HL subgroup) from those with both low AI (AI ≤ 6) and high CI (CI>3) (LH subgroup) in both Chinese cohort and US-based cohort, which showed a better performance than 24h urine protein in differentiating these two subgroups by ROC curve analyses (Figure 3). In the Chinese cohort, rSLEDAI also could discriminate HL group from low AI group (LH group + LL group). In addition, other conventional markers such as C3 levels and anti-dsDNA levels showed no statistical differences in the four subgroups of the two cohorts.

Changes in uL-selectin were associated with treatment response

Twenty SLE patients with active lupus nephritis in the Chinese cohort were followed up for at least 6 months. At the end of follow up, 13 patients achieved renal remission, 9 of whom achieved complete renal remission (CRR) and 4 patients achieved partial renal remission (PRR), while 7 patients had no renal remission (NRR). Importantly, uL-selectin significantly decreased in the complete renal remission group at the end of follow-up (p = 0.0039), while in the partial remission (p = 0.125) and no renal remission group (p = 0.578), uL-selectin displayed no differences (Figures 4A-C; Table S13).

Discussion

Previous studies have shown that the serum levels of L-selectin were associated with several autoimmune diseases (11, 12). A recent study using array-based proteomics had identified uL-selectin as a novel biomarker for LN with good performance in reflecting disease activity (4). As a pre-requisite for eventual clinical use, here we test the biomarker potential of uL-selectin in two different laboratories in two nations, using multi-center, multi-ethnic cohorts. These studies have successfully validated uL-selectin as a promising biomarker for disease activity, renal histological changes and treatment response in LN. In the Chinese cohort, this urinary molecule was elevated exclusively in active LN patients (compared to other SLE and CKD patients) and showed better performance than conventional markers to discriminate active LN from active non-renal SLE. It also correlated with systemic and renal disease activity in LN. Importantly, while high uL-selectin was predictive of concurrent renal pathological activity, low uL-selectin emerged as an independent predictor of high CI, and could significantly discriminate patients with AI >6 and CI ≤3 from those with AI ≤ 3 and CI>3, showing similar patterns observed in both cohorts, and performing better than proteinuria. Furthermore, a longitudinal study demonstrated the potential role of uL-selectin in monitoring disease activity and treatment response in active LN. Comparable results were also observed in the US-based cohort comprised of Caucasian, African American and Hispanic subjects. The findings of significant increase of uL-selectin levels in active LN patients compared with healthy controls and its correlation with 24h proteinuria, serum C3, C4, ESR and AI were consistent across all four ethnicities. Possible reasons for the subtle difference noted between ethnicities or cohorts (e.g., correlation with CI) may relate to the inadequate sample size in some ethnic/racial groups and potential genetic heterogeneity (13).

Additional analyses of uL-selectin levels were also performed in 33 patients with CKD (Table S14). They had higher levels of uL-selectin compared to HC. No difference of uL-selectin levels was found among different types or stages of CKD. (Figure S5). Although uL-selectin levels in CKD patients were lower than those in active LN patients and comparable to those in active non-renal SLE patients, it indeed indicates that uL-selectin could be a more general biomarker of renal involvement, and not SLE-specific, which is in line with the results by Vanarsa et al. (4).

L-selectin, also called CD62L, is a type I transmembraenne cell adhesion molecule broadly expressed on neutrophils, monocytes and most circulating leukocytes. The sticky binding of this molecule to its ligands on endothelial cells or other leukocytes triggers cell adhesion and migration from blood vessels to sites of local inflammation. The process of rolling and transendothelial migration (TEM) activates inducible-shedding of the molecule on the cells and results in the release of soluble (s) L-selectin into body fluids (14). Like other cell adhesion molecules, L-selectin expressed

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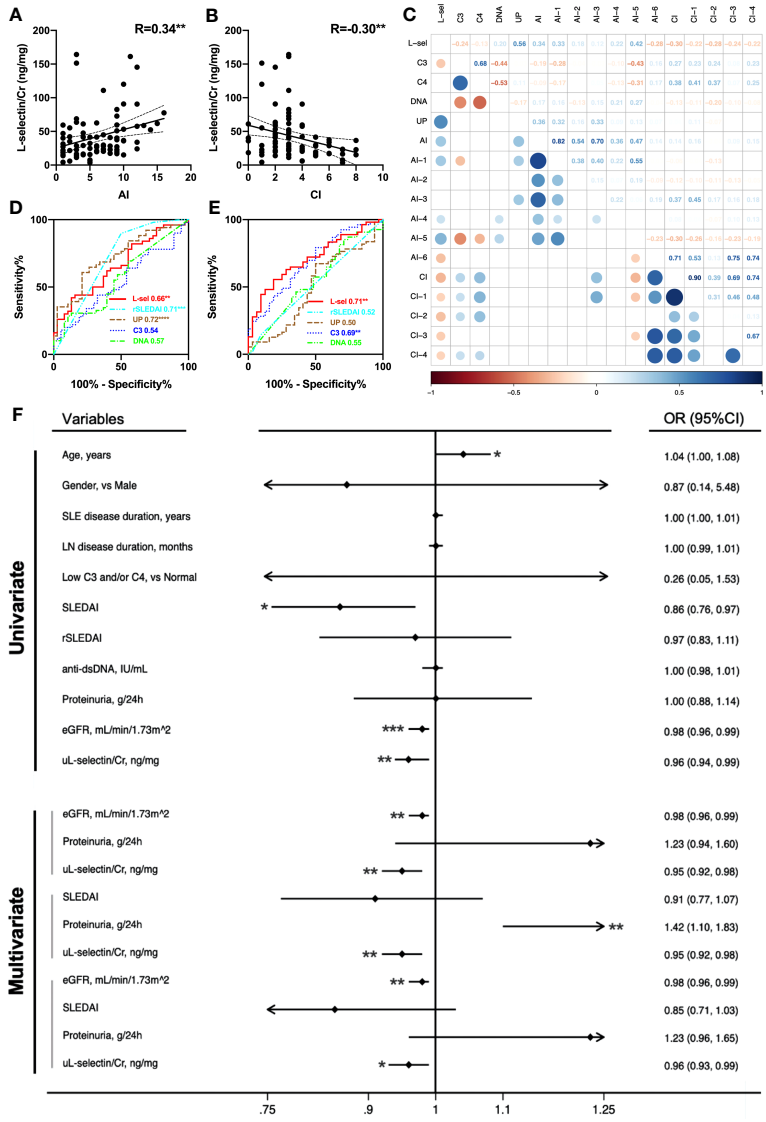

FIGURE 2
uL-selectin reflects concurrent renal pathology indices in LN. uL-selectin in LN patients correlated with AI **(A)** and CI **(B)** by Spearman correlation analysis. **(C)** Correlation matrix for comparison of uL-selectin and conventional metrics (serum C3, C4, anti-dsDNA and 24h proteinuria) with renal pathology AI, CI and their component attributes. The numbers in squares (upper right), and the colors and size of circles (lower left) all represent the corresponding Spearman's correlation coefficient. The upper right and lower left halves of the plot depict the same results across an imaginary diagonal. The circles (lower left) were removed where the corresponding P value for the correlation coefficient exceeded 0.05. **(D)** uL-selectin could discriminate high AI (AI>6) from low AI (AI ≤ 6), and **(E)** could also differentiate high CI (CI>3) from low CI (CI ≤ 3), compared with conventional disease indices. Values in the plot indicate areas under curve. **(F)** The forest plot summarizes results from univariate and multivariate logistic regression analysis for high renal pathology CI scores. Lower urinary L-selectin was associated with an increased risk of high CI (CI>3) even after adjusting for age, gender, SLE disease duration and LN disease duration. OR values in multivariate logistic regression were adjusted OR values. AI, activity index; CI, chronicity index; R, Spearman's correlation coefficient; L-sel/uL-selectin/Cr, urinary L-selectin adjusted by creatinine; DNA, anti-dsDNA antibody; UP, 24-hour urine protein quantity; AI-1, Endocapillary hypercellularity; AI-2, Neutrophils/karyorrhexis; AI-3, Cellular/fibrocellular crescents; AI-4, Fibrinoid necrosis; AI-5, Hyaline deposits; AI-6, Interstitial inflammation; CI-1, Glomerulosclerosis; CI-2, Fibrous crescents; CI-3, Interstitial fibrosis; CI-4, Tubular atrophy; eGFR, estimated glomerular filtration rate; OR, odds ratio; 95% CI, 95% confidence interval. *P<0.05, **P<0.01, ***P<0.001.

on renal-infiltrating leukocytes may play a pathogenic role in renal tissue inflammation and disease progression in LN, and this warrants further mechanistic investigation.

Although uL-selectin levels were positively correlated with AI, the association of high uL-selectin levels with high AI did not attain statistical significance by univariate or multivariate logistic regression analyses after adjusting for 24h proteinuria, indicating the weak correlation might be caused by other confounding factors such as proteinuria and suggesting the potential influence of urinary leakage in active renal injury in interpreting urine biomarker levels (Tables S15-17). However, lower uL-selectin level was independently associated with high CI, which is an independent risk factor of poor prognosis in LN (15), even after adjusting for 24h proteinuria and eGFR, with similar patterns being observed in both cohorts. It is

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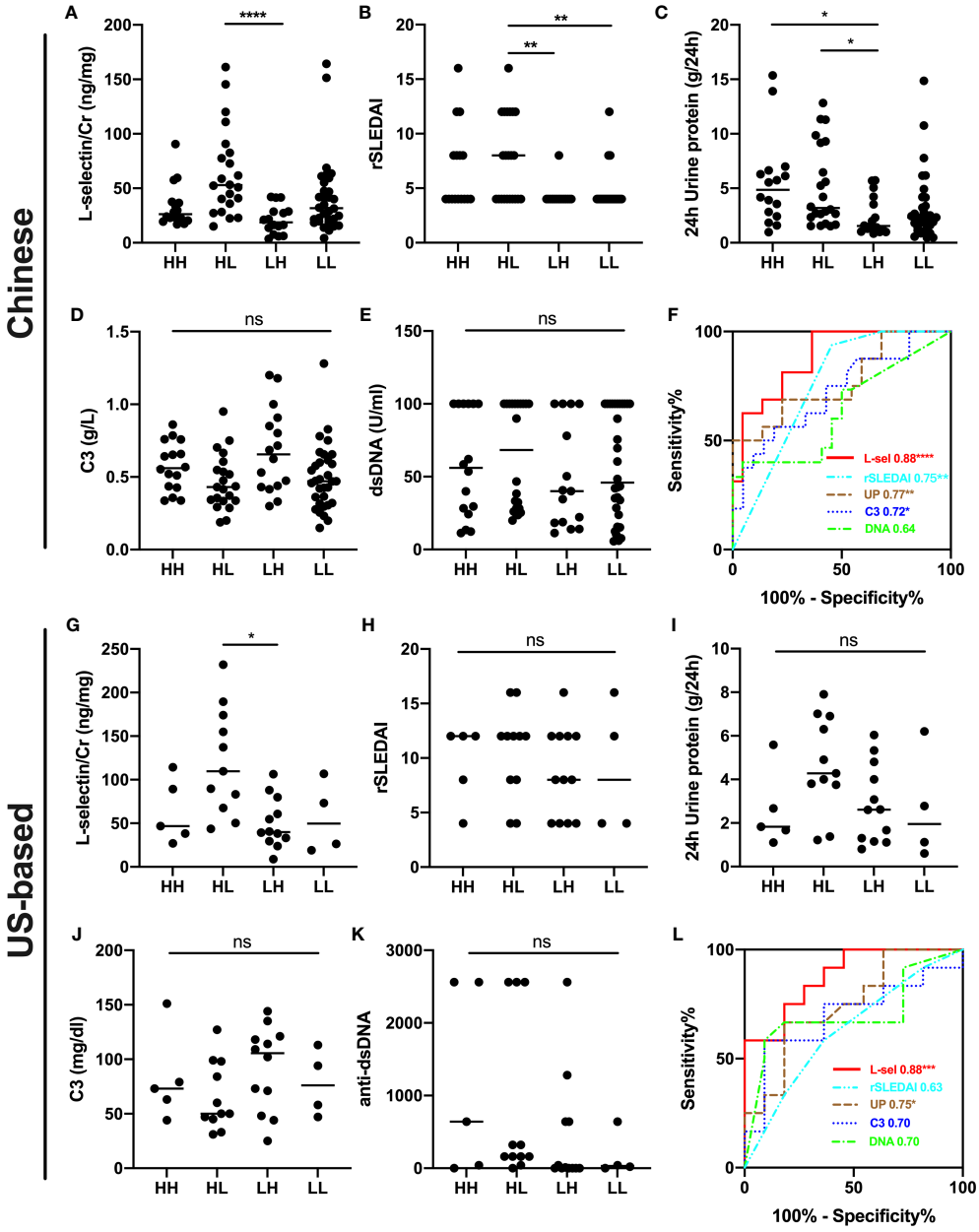

FIGURE 3
Subgroup analyses for combined AI+CI in the Chinese cohort **(A–F)** and US-based cohort **(G–L)**. uL-selectin levels **(A, G)**, rSLEDAI **(B, H),** 24h urine protein **(C, I)**, C3 levels **(D, J)** and anti-dsDNA levels **(E, K)** were shown in four subgroups of LN patients; Kruskal-Wallis test and Dunn's multiple comparisons test. **(F, L)** ROC curve analyses were performed to discriminate HL subgroup from LH subgroup of LN patients. HH both high AI (AI>6) and high CI (CI>3); HL both high AI (AI>6) and low CI (CI ≤ 3); LH both low AI (AI ≤ 6) and high CI (CI>3); LL both low AI (AI ≤ 6) and low CI (CI ≤ 3); AI activity index; CI chronicity index; ns, no significance. *P<0.05, **P<0.01, ****P <0.0001.

important to note that the relationship between uL-selectin levels and CI in LN may have important implications for predicting disease prognosis and guiding treatment decisions. However, further research may be necessary to better understand the underlying mechanisms driving this association and to determine the clinical utility of measuring uL-selectin levels in the context of LN.

Conventional markers such as proteinuria may not be informative in assessing pathological activity and chronicity in lupus nephritis. uL-selectin showed a better performance in discriminating patients with high AI and low CI from those with high CI and low AI in kidney than proteinuria, which was observed in both cohorts. The scRNAseq data suggested that urinary L-selectin is derived in large part from infiltrating B-cells, especially on activated and ISG-high B cells (4). B-cells are less involved in renal fibrosis than in active glomerulonephritis, therefore LN patients with higher activity and lower chronicity would have larger urinary L-selectin excretion compared to patients with higher chronicity and lower activity. High uL-selectin with high

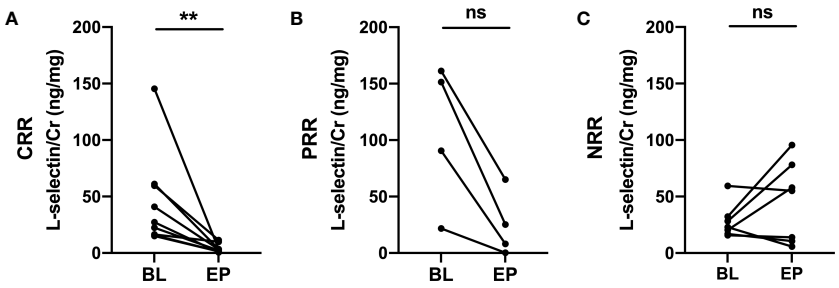

FIGURE 4
Urinary L-selectin levels in active LN patients at the endpoints of follow up compared with those at baseline. **(A)** Urinary L-selectin levels were decreased in the complete renal remission group (CRR) (n=9, P=0.0039) at the end of follow-up, while **(B)** it remained stable in the partial renal remission group (PRR) (n=4, P=0.125) and **(C)** in the no renal remission group (NRR) (n=7, P=0.578). BL, baseline; EP, endpoint; ns, no significance. Wilcoxon matched-paired signed rank test. $^{**}P \leq 0.01$.

proteinuria may reflect high AI and low CI in kidney which needs aggressive treatment, while low uL-selectin may reflect high CI and low AI which should be treated with more caution even the proteinuria level is relatively high.

Extrapolating from the results of the current study, we envision the following utilities of uL-selectin in clinical practice. Firstly, monitoring uL-selectin levels can help objectively assess clinical disease activity in LN at each follow-up visit. Secondly, close monitoring of uL-selectin levels may help predict response to treatment in terms of complete renal remission although larger prospective studies are warranted to validate this.

The limitations of this study include several important aspects that need to be considered carefully when interpreting the results. One inherent limitation is that requires consideration pertains to the restricted size of the population under investigation. This constraint becomes particularly conspicuous when focusing on specific demographic subsets, namely the Caucasian and Hispanic cohorts, as well as the longitudinal cohort. Indeed, the issue of limited population size may notably impact the generalizability of the study's findings. A broader cross-section of ethnic backgrounds within the cohort would have facilitated a more robust assessment of the relationships and trends explored in our research. Therefore, future endeavors should prioritize the inclusion of a more extensive multi-ethnic population and a prospective longitudinal cohort to further validate and strengthen the outcomes observed here. To mitigate these limitations, we employed statistical techniques to account for the constraints. We carefully performed a rigorous sample size calculation to ensure statistical power in relation to the specific objectives of this study. Despite the inherent limitations, the current sample size was determined to be adequate to detect the expected effect sizes within the context of our research questions. However, it remains imperative to acknowledge the potential impact of these limitations on the precision and applicability of our findings. Another limitation is that we cannot exclude the potential impact of medications used for clinical treatment on the levels of uL-selectin. Additionally, the absence of assessment of L-selectin levels in the blood and kidneys of LN patients limits our knowledge of the exact source of its origin in urine. Extended studies with larger sample sizes in both the cross-sectional and longitudinal cohorts, together with parallel assessment of competing biomarker candidates, are warranted before one can endorse the use of uL-selectin in routine clinical practice.

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 ethics committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; The Institutional Review Boards of the University of Houston, JHU School of Medicine, and UTSW. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/ next of kin in accordance with the national legislation and institutional requirements.

Author contributions

HD, CM, and ZZY designed the study and planned this work with YS, KV, and ZHY. YS, KV, and ZHY carried out the

experiments, acquired the data, and drafted the manuscript. HD, YS, TZ, JW, and NS performed data analysis and interpretation of the results. TZ, JC, MD, and LZ contributed to sample processing and clinical data collection. LQ, QG, RS, and MP contributed to patient recruiting and follow-up. HD, CM, and NS reviewed and revised the manuscript. The manuscript was written through the contributions of all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1200167/full#supplementary-material

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Corrigendum: Urine L-selectin reflects clinical and histological renal disease activity and treatment response in lupus nephritis across multi-ethnicity

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A Corrigendum on

Urine L-selectin reflects clinical and histological renal disease activity and treatment response in lupus nephritis across multi-ethnicity

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In the published article, there was an error. In the results section of the abstract, there is an error in the statement.

A correction has been made to the Abstract, Results section, Sentence 2. This sentence previously stated:

"uL-selectin positively correlated with global and renal disease activities as well as histological activity index and chronicity index (CI)."

The corrected sentence appears below:

"uL-selectin positively correlated with global and renal disease activities and was significantly associated with histological activity index and chronicity index (CI)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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A systematic review and meta-analysis of neopterin in rheumatic diseases

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Introduction: Novel biomarkers of inflammation and oxidative stress might enhance the early recognition, management, and clinical outcomes of patients with rheumatic diseases (RDs). We assessed the available evidence regarding the pathophysiological role of neopterin, the oxidation product of 7,8-dihydroneopterin, a pteridine generated in macrophages activated by interferon-γ, by conducting a systematic review and meta-analysis of studies reporting its concentrations in biological fluids in RD patients and healthy controls.

Methods: We searched electronic databases for relevant articles published between inception and 31 August 2023. The risk of bias and the certainty of evidence were assessed using the Joanna Briggs Institute Critical Appraisal Checklist and the Grades of Recommendation, Assessment, Development and Evaluation Working Group system, respectively.

Results: In 37 studies, when compared to healthy controls, RD patients had significantly higher concentrations of neopterin both in plasma or serum (standard mean difference, SMD=1.31, 95% CI 1.01 to 1.61; p<0.001; moderate certainty of evidence) and in the urine (SMD=1.65, 95% CI 0.86 to 2.43, p<0.001; I^2 = 94.2%, p<0.001; low certainty of evidence). The results were stable in sensitivity analysis. There were non-significant associations in meta-regression and subgroup analysis between the effect size and age, male to female ratio, year of publication, sample size, RD duration, C-reactive protein, erythrocyte sedimentation rate, specific type of RD, presence of connective tissue disease, analytical method used, or biological matrix investigated (plasma *vs*. serum). By contrast, the effect size was significantly associated with the geographical area in studies assessing serum or plasma and with the type of RD in studies assessing urine.

Discussion: Pending additional studies that also focus on early forms of disease, our systematic review and meta-analysis supports the proposition that neopterin, a biomarker of inflammation and oxidative stress, can be useful for the identification of RDs. (PROSPERO registration number: CRD42023450209).

Systematic review registration: PROSPERO, identifier CRD42023450209

KEYWORDS

neopterin, rheumatic diseases, inflammation, oxidative stress, biomarkers, metabolism

Introduction

Rheumatic diseases (RDs) is an umbrella term that includes a wide number of chronic, disabling conditions characterized by inflammation and oxidative stress affecting the musculoskeletal system and other organ and tissues. Broadly speaking, RDs can have a predominantly autoimmune (e.g., progressive systemic sclerosis, pSS, rheumatoid arthritis, RA, systemic lupus erythematosus, SLE, Sjogren's syndrome, SSj, systemic sclerosis, SSc, and idiopathic inflammatory myositis, IIM), mixed-autoimmune-autoinflammatory (e.g., ankylosing spondylitis, AS, axial spondylarthritis, axSpA, psoriatic arthritis, PsA, and Behcet's disease, BD), or autoinflammatory component (e.g., familial Mediterranean fever, FMF) (1–3). The availability of a wide range of anti-inflammatory and immunomodulatory medications has revolutionised the management of clinically overt RDs over the last 20-30 years, with significant improvements in symptom control and quality of life of affected patients (4–7) (8–10). However, despite these advances, significant challenges remain with the identification of early forms of RD. This issue, in turn, prevents the implementation of strategies for the rapid control of dysregulated immune and inflammatory pathways and, potentially, the achievement of more favourable long-term clinical outcomes (11–16). Therefore, a significant body of research has been conducted to identify novel biomarkers of RDs which could better assist physicians to make an early diagnosis, in addition to clinical assessment and conventional biomarkers of inflammation, e.g., C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) (17–25).

In the ongoing search for novel cellular and biochemical pathways underlying the pathophysiology of RDs, increasing attention has been given to the pleiotropic effects of the cytokine interferon-γ (26). When produced in excess, interferon-γ exerts detrimental effects on the homeostatic control of inflammatory and immune pathways in a range of experimental and clinical studies of RDs (27–30). Therefore, the identification of biomarkers that adequately reflect the activation of interferon-γ might be particularly useful for diagnosis and management. One such biomarker is neopterin, a pteridine analogue generated from the oxidation of 7,8-dihydroneopterin, a potent radical scavenging and chain-breaking antioxidant derived from the interferon-γ-mediated conversion of guanosine-5'-triphosphate (GTP) by GTP cyclohydrolase-1 in activated macrophages (Figure 1) (31–34). The potential advantages of measuring neopterin in the clinical evaluation of RDs include, in addition to its role as a marker of macrophage activation, the determination in different biological fluids and its rapid elimination by the kidney, which allows assessing fluctuations in disease activity and early effects of treatment (35–40).

Therefore, we investigated the potential clinical utility of neopterin by conducting a systematic review and meta-analysis of studies investigating the concentrations of this pteridine metabolite in different biological fluids in patients with RD and healthy controls. We also investigated associations between the effect size of the differences in neopterin concentrations and several parameters, including RD duration, type of RD (autoimmune, mixed autoimmune-autoinflammatory, or autoinflammatory disease), CRP, and ESR.

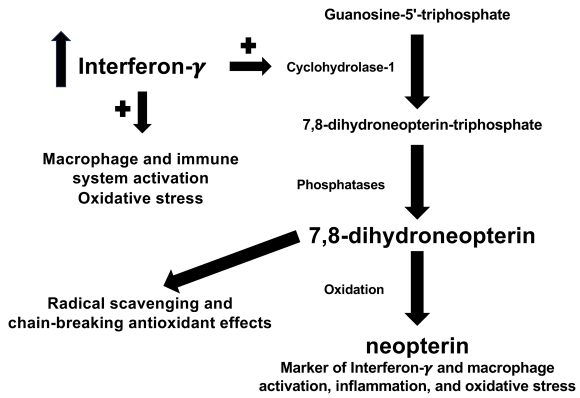

FIGURE 1
Biochemical pathways involved in the formation of neopterin.

Materials and methods

Search strategy, eligibility criteria, and study selection

We systematically searched for relevant publications in the electronic databases PubMed, Web of Science, and Scopus from inception to 31 August 2023 using the following terms and their combination: "rheumatic diseases" OR "rheumatoid arthritis" OR "psoriatic arthritis" OR "ankylosing spondylitis" OR "systemic lupus erythematosus" OR "systemic sclerosis" OR "Sjogren's syndrome" OR "connective tissue diseases" OR "vasculitis" OR "Behçet's disease" OR "idiopathic inflammatory myositis" OR "polymyositis" OR "dermatomyositis"AND "neopterin". Two investigators independently reviewed each abstract and, if relevant, the full-text articles and their references for additional studies. The eligibility criteria included: (i) the assessment of neopterin concentrations in biological fluids (plasma/serum, urine, synovial fluid, saliva, and cerebrospinal fluid, (ii) the comparison between patients with RDs and healthy controls conducted in case-control studies, (iii) the inclusion of patients ≥18 years of age, and (iv) the availability of the full-text of the publication in English language.

The following study and patient variables were independently extracted from selected manuscripts in an *ad hoc* standardized form for further analysis: first author, year of publication, study country, sample size, age, male to female ratio, CRP, ESR, RD duration, sample matrix investigated (serum or plasma), and assay method used to measure neopterin.

We assessed the risk of bias using the Joanna Briggs Institute Critical Appraisal Checklist for analytical studies. Studies addressing ≥75%, ≥50% and <75%, and <50% of checklist items were considered as having a low, moderate, and high risk, respectively (41). We also assessed the certainty of evidence using the Grades of Recommendation, Assessment, Development and Evaluation (GRADE) Working Group system. GRADE assesses the

study design (randomized *vs.* observational), the risk of bias (JBI checklist), the presence of unexplained heterogeneity, the indirectness of the evidence, the imprecision of the results (sample size, 95% confidence interval width and threshold crossing), the effect size (small, SMD <0.5, moderate, SMD 0.5-0.8, and large, SMD >0.8) (42), and the probability of publication bias (43). The results were presented according to the guidelines provided in Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 statement (Supplementary Tables 1 and 2) (44). The review protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO registration number: CRD42023450209) (45).

Statistical analysis

We generated forest plots of standardized mean differences (SMDs) and 95% confidence intervals (CIs) to assess differences in neopterin concentrations between RD patients and healthy controls ($p<0.05$ for statistical significance). If necessary, the mean and standard deviation values were extrapolated from medians and interquartile ranges or medians and ranges (46, 47). The heterogeneity of the SMD across studies was tested by using the Q statistic ($p<0.10$ for statistical significance). Heterogeneity was considered low when the I^2 value was ≤25%, moderate when the I^2 value was >25% and <75%, and high when the I^2 value was ≥75% (48, 49). A random-effect model based on the inverse-variance method was used in the presence of high heterogeneity. Sensitivity analysis was conducted to investigate the stability of the results by assessing the influence of individual studies on the overall risk estimate (50). Publication bias was assessed using the Begg's adjusted rank correlation test and the Egger's regression asymmetry test ($p<0.05$ for statistical significance) (51, 52). The "trim-and-fill" method was used to further test and eventually correct the occurrence of publication bias (53). Univariate meta-regression and subgroup analyses were conducted to investigate the presence of associations between the effect size (SMD) and the following parameters: year of publication, study continent, sample size, age, male to female ratio, CRP, ESR, disease duration, sample matrix investigated, and analytical method used to measure neopterin. Statistical analyses were performed using Stata 14 (Stata Corp., College Station, TX, USA).

Results

Systematic search and characteristics of the included studies

A flow chart describing the screening process is presented in Figure 2. We initially identified 659 articles. A total of 608 were excluded after the first screening because they were either duplicates or irrelevant. After a full-text review of the remaining 51 articles, a further 14 were excluded because of missing data (n=4), duplicate data (n=4), incorrect study design (n=3), non-English language used (n=2), and inclusion of children or adolescents (n=1). Therefore, 37 studies (43 study groups, 34 investigating plasma/serum, seven urine, one saliva, and one synovial fluid) were selected for analysis (Table 1) (54–90).

Serum or plasma neopterin

Study characteristics

Thirty studies (34 study groups) reported serum or plasma neopterin concentrations in a total of 2,618 RD patients (mean age 43 years, 32% males) and 5,318 healthy controls (mean age 42 years, 47% males) (55, 59, 61, 64–67, 69–87, 89, 90).

Twenty studies were conducted in Asia (55, 59, 65, 66, 69, 70, 72–74, 77, 78, 80–82, 84–89), six in Europe (61, 62, 64, 67, 79, 90), three in Africa (71, 75, 83), and the remaining one in America (76). Ten study groups included patients with RA (55, 76, 78–81, 83, 86, 89, 90), nine with SLE (55, 65, 70, 71, 75–77, 82, 84), eight with BD (59, 65, 66, 69, 72–74, 87), three with IIM (62, 88), two with pSS (64, 67), one with SSc (61), and one with AS (85). The analytical methods used for measuring neopterin included an enzyme-linked immunosorbent assay (ELISA) in 18 studies (61, 64–67, 69–71, 74–76, 78, 79, 81, 85, 86, 88, 90), liquid chromatography with fluorimetric detection in 10 (55, 72, 73, 77, 80, 82–84, 87, 89), and radioimmunoassay in two (59, 62). Serum was analysed in 26 studies (55, 59, 61, 62, 64–67, 69–74, 76–78, 81–85, 87–90), and plasma in the remaining four (75, 79, 80, 86). RD duration, reported in 11 study groups, ranged between four and 11 years (61, 67, 71, 73–75, 78, 80, 81, 83, 90).

The risk of bias was low in 14 studies (61, 69, 75–77, 79, 81, 82, 84, 85, 87–90), and moderate in the remaining 16 (55, 59, 62, 64–67, 70–74, 78, 80, 83, 86) (Supplementary Table 3). All studies had an initially low certainty of evidence given the cross-sectional design (rating 2, ⊕⊕⊖⊖) (55, 59, 61, 64–67, 69–87, 89, 90).

Results of individual studies and syntheses

RD patients had significantly higher neopterin concentrations compared to healthy controls (SMD=1.22, 95% CI 0.99 to 1.44, $p<0.001$; I^2 = 91.8%, $p<0.001$; Figure 3). In sensitivity analysis, the corresponding pooled SMD values were not influenced when individual studies were sequentially removed, with the effect size ranging between 1.14 and 1.27 (Figure 4). The effect size was also similar to the primary analysis after removing three studies accounting for 65% of the overall participant population (SMD=1.31, 95% CI 1.01 to 1.61; $p<0.001$; I^2 = 91.2%, $p<0.001$) (76, 80, 90).

Publication bias

A significant publication bias was observed (Begg's test, p=0.004; Egger's test, p=0.006). The "trim-and-fill" method identified ten missing studies to be added to the left side of funnel plot to ensure symmetry (Figure 5). The resulting effect side was attenuated yet still significant (SMD=0.78, 95% CI 0.54 to 1.02, $p<0.001$).

Meta-regression and sub-group and analysis

There were non-significant associations between the effect size and age (t=0.13, p=0.90), male to female ratio (t=-0.34, p=0.73), year of publication (t=-0.51, p=0.61), sample size (t=-0.53, p=0.60),

TABLE 1 Characteristics of the studies included in the meta-analysis.

				Healthy controls				Patients with RDs			
Study	Disease	Matrix	Method	n	Age (years)	M/F	Neopterin Mean ± SD (nmol/L)	n	Age (years)	M/F	Neopterin Mean ± SD (nmol/L)
Hannonen P et al., 1986, Finland (54)	RA	U	LC	67	NR	NR	218 ± 83[§]	67	53	14/53	342 ± 133[§]
Hagihara M et al. (a) 1990, Japan (55)	RA	S	LC	21	56	NR	26.13 ± 9.72	21	56	NR	21.63 ± 3.32
Hagihara M et al. (b) 1990, Japan (55)	SLE	S	LC	21	56	NR	26.13 ± 9.72	23	49	NR	43.08 ± 13.3
Krause A et al., 1990, Germany (56)	RA	SF	RIA	12	NR	NR	10.3 ± 25.0	17	48	6/11	41.0 ± 37.0
Leohirun L et al., 1991, Thailand (57)	SLE	U	LC	43	NR	NR	112 ± 40	43	18-42	7/36	925 ± 282
Lim KL et al., 1993, UK (58)	SLE	U	LC	65	45	2/63	158 ± 53	68	43	3/65	505 ± 326
Yoon J et al., 1993, Korea (59)	BD	S	RIA	30	NR	20/10	3.63 ± 0.88	67	38	34/33	6.36 ± 2.52
Altindag Z et al., 1994, Turkey (60)	BD	U	LC	14	20-34	7/7	125 ± 44	21	31	12/9	184 ± 119
Csipo I et al., 1995, Hungary (61)	SSc	S	ELISA	46	NR	NR	0.9 ± 2.3	29	50	NR	10.8 ± 4.5
Samsonov MY et al. (a) 1997, Austria (62)	DM	S	RIA	31	NR	NR	5.2 ± 1.8	15	35	NR	11.3 ± 4.6
Samsonov MY et al. (b) 1997, Austria (62)	PM	S	RIA	31	NR	NR	5.2 ± 1.8	13	39	NR	20.6 ± 11.3
Altindag ZZ et al., 1998, Turkey (63)	RA	U	LC	20	49	1/19	111 ± 34	36	50	2/34	331 ± 319
Andrys C et al., 1999, Czech Republic (64)	pSS	S	ELISA	26	NR	0/26	7.6 ± 2.3	17	58	2/15	17.9 ± 6.4
Keser G et al. (a) 2000, Turkey (65)	BD	S	ELISA	10	35	3	2.1 ± 0.7*	50	36	35/15	3.2 ± 1.9*
Keser G et al. (b) 2000, Turkey (65)	SLE	S	ELISA	10	35	NR	2.1 ± 0.7*	20	NR	NR	10.5 ± 8.5*
Kökçam I et al., 2002, Turkey (66)	BD	S	ELISA	25	NR	NR	12.16 ± 3.77*	25	31	13/12	17.34 ± 6.2*
Sfriso P et al. (a) 2003, Italy (67)	pSS	S	ELISA	20	48	0/20	5 ± 2.06	30	47	0/30	8.12 ± 3.36
Sfriso P et al. (b) 2003, Italy (67)	pSS	Sa	ELISA	20	48	0/20	2.83 ± 1.47	30	47	0/30	7.5 ± 7.61
de Castro MR et al., 2004, Brazil (68)	SLE	U	LC	49	NR	NR	295 ± 179	49	NR	NR	787 ± 145
Coskun B et al., 2005, Turkey (69)	BD	S	ELISA	30	32	15/15	8.7 ± 2.2*	40	33	21/19	14.3 ± 3.9*
Jin O et al., 2005, China (70)	SLE	S	ELISA	20	NR	NR	0.26 ± 0.19°	22	NR	NR	1.39 ± 1.1°
Mahmoud RAK et al., 2005, Egypt (71)	SLE	S	ELISA	10	26	0/10	5.76 ± 2.52	40	27	0/40	28.36 ± 13.19
Kose O et al., 2006, Turkey (72)	BD	S	LC	17	27	12/5	4.56 ± 0.45	68	26	64/4	7.74 ± 3.63

(Continued)

TABLE 1 Continued

				Healthy controls				Patients with RDs			
Study	Disease	Matrix	Method	n	Age (years)	M/F	Neopterin Mean ± SD (nmol/L)	n	Age (years)	M/F	Neopterin Mean ± SD (nmol/L)
Ozkan S et al., 2007, Turkey (73)	BD	S	LC	21	39	6/15	12 ± 4.4	23	40	8/15	13.4 ± 3.6
Erturan I et al. (a) 2009, Turkey (74)	BD	S	ELISA	45	38	21/24	6.03 ± 3.46	45	39	21/24	12.68 ± 4.67
Erturan I et al. (b) 2009, Turkey (74)	BD	U	ELISA	45	38	21/24	104 ± 48	45	39	21/24	168 ± 149
Salem SAM et al., 2010, Egypt (75)	SLE	P	ELISA	20	26	2/18	9.4 ± 1.1	50	26	6/44	21.2 ± 5
Rho YH et al. (a) 2011, USA (76)	SLE	S	ELISA	177	47	45/232	5.87 ± 1.7	148	40	14/134	8.1 ± 2.44
Rho YH et al. (b) 2011, USA (76)	RA	S	ELISA	177	47	45/232	5.87 ± 1.7	166	54	52/114	6.97 ± 2.67
Bahrehmand F et al., 2012, Iran (77)	SLE	S	LC	101	37	22/82	6.5 ± 2.9	109	36	19/90	28.8 ± 38.1
Ozkan Y et al., 2012, Turkey (78)	RA	S	ELISA	20	62	4/16	7.14 ± 5.15	32	59	5/27	8.47 ± 7.8
D'Agostino LE et al., 2013, Italy (79)	RA	P	ELISA	38	37	9/29	5.62 ± 2.22	27	36	7/20	8.92 ± 4.83
Shahmohamadnejad S et al., 2015, Iran (80)	RA	P	LC	397	49	36/363	4.2 ± 2.22	419	50	42/377	5.93 ± 4.81
Gulkesen A et al., 2016, Turkey (81)	RA	S	ELISA	24	43	11/13	1.88 ± 1.84	33	53	9/24	23.98 ± 18.88
Baniamerian H et al., 2017, Iran (82)	SLE	S	LC	98	36	18/80	6.5 ± 2.9	100	37	20/80	25.7 ± 38.1
El-Lebedy D et al., 2017, Egypt (83)	RA	S	LC	100	NR	NR	4.74 ± 1.98	120	44	NR	11.46 ± 3.56
Tanhapour M et al., 2018, Iran (84)	SLE	S	LC	101	37	20/81	6.06 ± 2.08	107	36	19/88	12.77 ± 13.26
Zorbozan N et al., 2018, Turkey (85)	AS	S	ELISA	80	NR	NR	1.12 ± 0.32	160	NR	91/69	1.13 ± 0.39
Iranshahi N et al., 2019, Iran (86)	RA	P	ELISA	42	46	7/35	15.32 ± 9.02	47	51	7/40	17.63 ± 9.68
Akyurek F et al., 2020, Turkey (87)	BD	S	LC	54	37	NR	76.77 ± 38.27	57	36	NR	111.27 ± 37.49
Peng QL et al., 2020, China (88)	DM	S	ELISA	30	NR	NR	4.3 ± 2.0	182	NR	55/127	24.4 ± 15.8
Ekin S et al., 2021, Turkey (89)	RA	S	LC	30	50	11/19	4.19 ± 1.01*	30	52	10/20	25.99 ± 7.27*
Videm V et al., 2022, Norway (90)	RA	S	ELISA	3,415	58	2,053/1,362	5.15 ± 0.76	283	65	180/103	5.98 ± 0.88

AS, ankylosing spondylitis; BD, Behcet Disease; DM, dermatomyositis; ELISA, enzyme-linked immunosorbent assay; F, female, LC, liquid chromatography; M, male; NR, not reported; P, plasma; PM, polymyositis; pSS, primary Sjogren syndrome; RA, rheumatoid arthritis; RIA, radioimmunoassay; S, serum; Sa, saliva; SF, synovial fluid; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; U, urine; §, µmol/mol creatinine; *, ng/mL; °, µg/dL.

disease duration (t=0.83, p=0.42), CRP (t=-0.50, p=0.62), or ESR (t=0.16, p=0.87).

In subgroup analysis, there were non-significant differences (p=0.39) in SMD values between studies conducted in RA patients (SMD=1.01, 95% CI 0.57 to 1.45, p<0.001; I^2 = 95.8%, p<0.001), SLE patients (SMD=1.23, 95% CI 0.90 to 1.55, p<0.001; I^2 = 81.5%, p<0.001), BD patients (SMD=1.08, 95% CI 0.77 to 1.38, p<0.001; I^2 = 62.6%, p=0.006), IIM patients (SMD=1.88, 95% CI

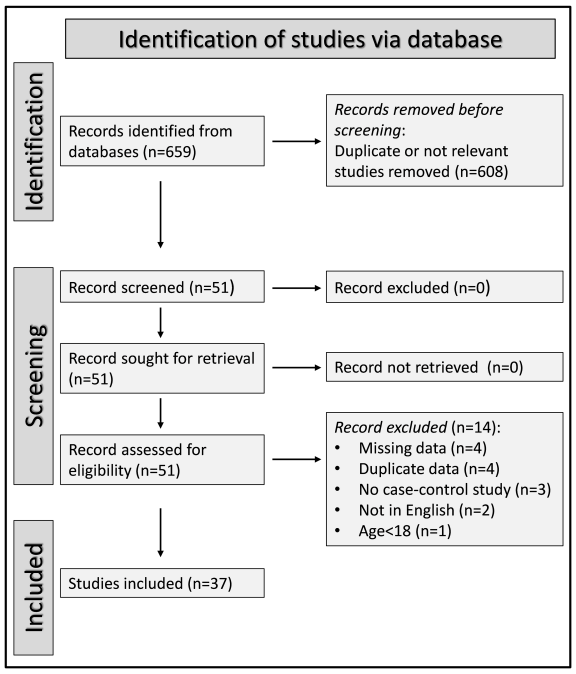

FIGURE 2
PRISMA 2020 flow diagram.

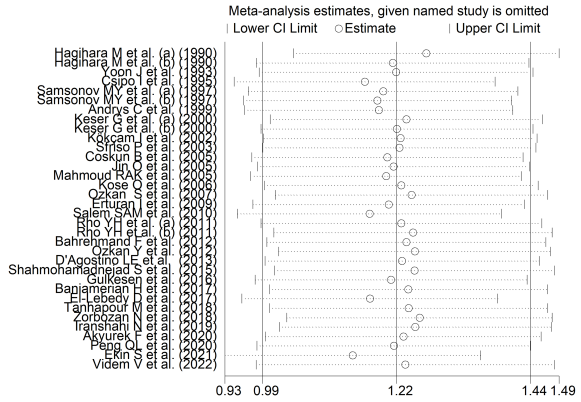

FIGURE 4
Sensitivity analysis of the association between neopterin and RDs in serum/plasma.

1.20 to 2.57, p<0.001; I^2 = 69.6%, p=0.037), and pSS patients (SMD=1.68, 95% CI 0.43 to 2.94, p=0.008; I^2 = 84.1%, p<0.001; Figure 6), with a lower heterogeneity observed in the BD and IIM subgroups. Similarly, the pooled SMD was non-significantly different (p=0.25) between studies conducted in patients with CTD (SMD=1.32, 95% CI 1.06 to 1.59, p<0.001; I^2 = 92.9%, p<0.001) and without CTD (SMD=0.94, 95% CI 0.50 to 1.37, p<0.001; I^2 = 92.0%, p<0.001; Figure 7). By contrast, a significant (p=0.003) increase in the effect size was observed between studies conducted in America (SMD=0.78, 95% CI 0.21 to 1.36, p=0.007; I^2 = 92.3%, p<0.001), Asia (SMD=0.95, 95% CI 0.69 to 1.20, p<0.001; I^2 = 88.5%, p<0.001), Europe (SMD=1.79, 95% CI 1.21 to 2.38, p<0.001; I^2 = 88.8%, p<0.001) and Africa (SMD=2.32, 95% CI 1.94 to 2.69, p<0.001; I^2 = 25.2%, p<0.263; Figure 8), with a lower heterogeneity observed in the African subgroup. There were non-significant differences (p=0.48) in pooled SMD between studies using liquid chromatography (SMD=1.23, 95% CI 0.95 to 1.51, p<0.001; I^2 = 90.0%, p<0.001), ELISA (SMD=1.05, 95% CI 0.61 to 1.49, p<0.001; I^2 = 94.3%, p<0.001), and RIA (SMD=1.86, 95% CI 1.12 to 2.61, p<0.001; I^2 = 72.8%, p=0.025; Figure 9. Finally, non-significant differences (p=0.66) in pooled SMD were also observed between studies investigating serum (SMD=1.25, 95% CI 1.01 to 1.49, p<0.001; I^2 = 90.9%, p<0.001) and plasma (SMD=1.04, 95% CI 0.28 to 1.79, p=0.007; I^2 = 93.2%, p<0.001; Figure 10).

Certainty of evidence

The overall level of certainty was upgraded to moderate (rating 3, ⊕⊕⊕⊖) after taking into account the low-moderate risk of bias in all studies (no rating change), the high but partly explainable heterogeneity (no rating change), the lack of indirectness (no rating change), the relatively low imprecision (confidence intervals not crossing the threshold, no rating change), the large effect size (SMD=1.22, upgrade by one level), and the presence of publication bias which was corrected using the "trim-and-fill" method (no rating change).

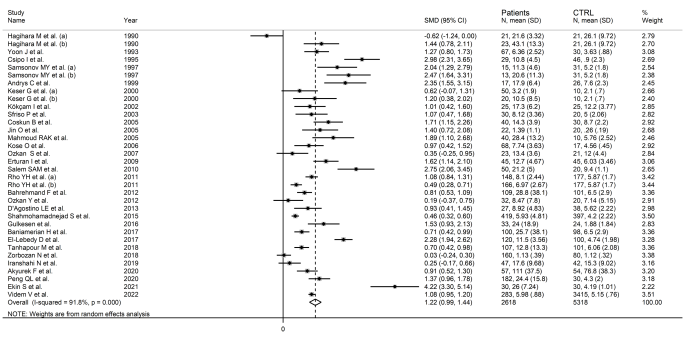

FIGURE 3
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in serum/plasma.

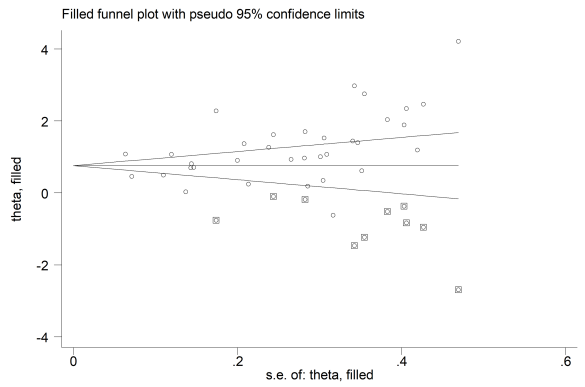

FIGURE 5
Funnel plot of studies investigating the association between neopterin and RDs in serum/plasma after "trimming-and-filling". Enclosed circles and free circles indicate dummy studies and genuine studies, respectively.

in Europe (54, 58), and one in America (68). Three studies investigated patients with SLE (57, 58, 68), two with RA (54, 63), and two with BD (60, 74). Liquid chromatography with fluorimetric detection was used in six studies (54, 57, 58, 60, 63, 68), and ELISA in the remaining one (74).

The risk of bias was considered low in two studies (58, 63), moderate in two (57, 74), and high in the remaining three (54, 60, 68) (Supplementary Table 3). All studies had an initially low certainty of evidence given the cross-sectional design (rating 2, ⊕⊕⊖⊖) (54, 57, 58, 60, 63, 68, 74).

Urine neopterin

Study characteristics

Seven studies investigated urinary concentrations of neopterin in a total of 329 patients (mean age 46.4 years, 21.1% males) and 303 healthy controls (mean age 46.5 years, 20.5% males) (54, 57, 58, 60, 63, 68, 74). Four studies were conducted in Asia (57, 60, 63, 74), two

Results of individual studies and syntheses

The forest plot showed that RD patients had significantly higher urinary neopterin concentrations compared to healthy controls (SMD=1.65, 95% CI 0.86 to 2.43, p<0.001; I^2 = 94.2%, p<0.001; Figure 11). In sensitivity analysis, the corresponding pooled SMD values were not influenced when individual studies were sequentially removed, with the effect size ranging between 1.27 and 1.83 (Figure 12).

Publication bias and meta-regression analysis

Assessment of publication bias and meta-regression could not be performed because of the small number of studies.

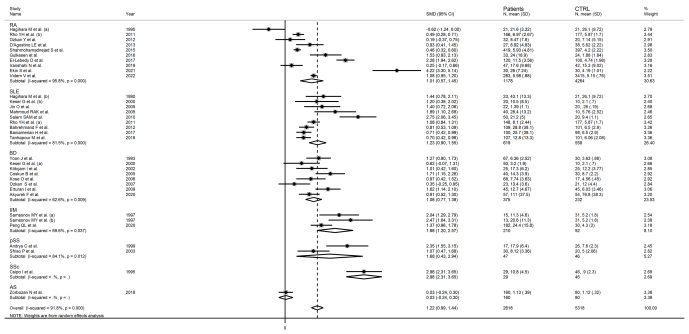

FIGURE 6
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in serum/plasma according to RD type.

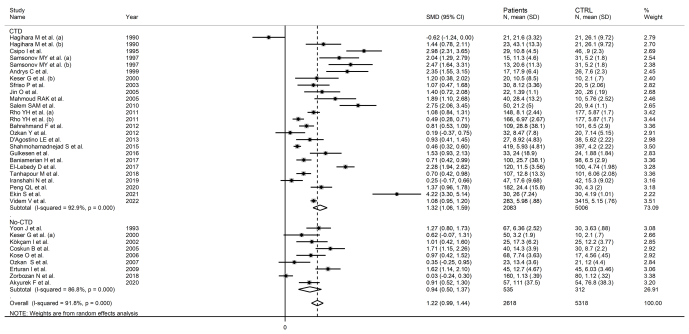

FIGURE 7
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in serum/plasma according to the presence of connective tissue disease.

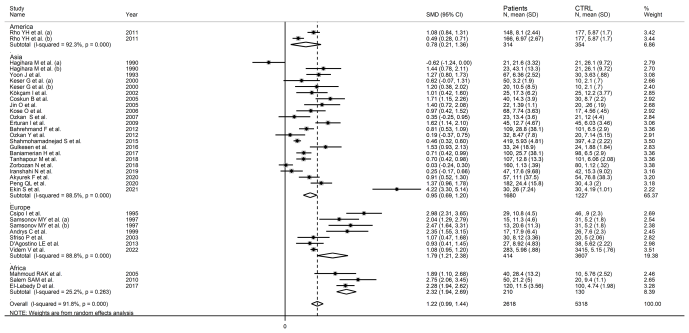

FIGURE 8
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in serum/plasma according to study continent.

Subgroup analysis

There were significant differences (p=0.04) in SMD values between studies conducted in SLE patients (SMD=2.82, 95% CI 1.30 to 4.33, p<0.001; I^2 = 95.5%, p<0.001), RA patients (SMD=1.04, 95% CI 0.73 to 1.35, p<0.001; I^2 = 0.0%, p=0.44), and BD patients (SMD=0.59, 95% CI 0.23 to 0.95, p=0.001; I^2 = 0.0%, p=0.94; Figure 13), with a virtual absence of heterogeneity in the RA and BD subgroups. By contrast, there were non-significant differences (p=0.40) in SMD values between European (SMD=1.29, 95% CI 0.94 to 1.63, p<0.001; I^2 = 40.9%, p<0.001), and Asian studies (SMD=1.50, 95% CI 0.10 to 2.91, p<0.001; I^2 = 94.2%, p<0.001; Figure 14), with a lower heterogeneity in the European subgroup.

Certainty of evidence

The overall level of certainty remained low (rating 2, ⊕⊕⊖⊖) after taking into account the low-moderate risk of bias in the majority of studies (no rating change), the high but partly explainable heterogeneity (no rating change), the lack of indirectness (no rating change), the relatively low imprecision (confidence intervals not crossing the threshold, no rating change), the large effect size (SMD=1.65, upgrade by one level), and lack of assessment of publication bias (downgrade one level).

Neopterin concentration in other biological fluids

One study reported significantly higher salivary concentrations of neopterin in pSS patients when compared with healthy subjects (9.5 ± 7.61 *vs.* 2.83 ± 1.47 nmol/L, p<0.005) (67), whereas another study reported that RA patients have increased concentrations of neopterin in synovial fluid when compared with healthy controls (41 ± 37 *vs.* 10.3 ± 25 nmol/L, p<0.001) (56) (Table 1).

Discussion

The results of our systematic review and meta-analysis have shown that the plasma/serum and urinary concentrations of neopterin, a biomarker of interferon-γ activation, are significantly higher in patients with RDs compared to healthy controls. In meta-regression analysis, the effect size of the between-group differences in plasma/serum neopterin concentrations (SMD) was not associated with a range of study and patient characteristics, including age, male to female ratio, year of publication, study sample size, RD duration, CRP, and ESR. Similarly, in subgroup analysis the SMD was not associated with the type of RD (i.e., RA, SLE, BD, and pSS), the presence of CTD, the analytical method used to determine neopterin, or the matrix used for assessment (plasma

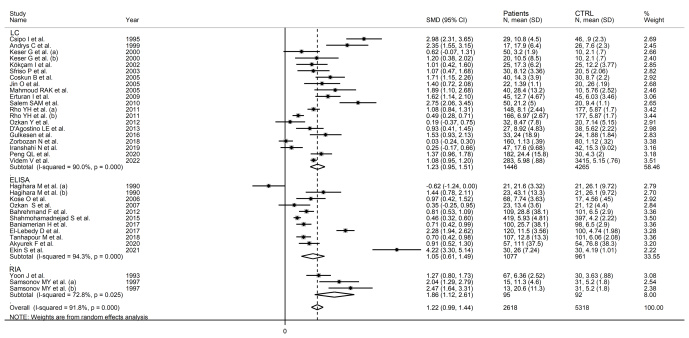

FIGURE 9
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in serum/plasma according to the analytical method used.

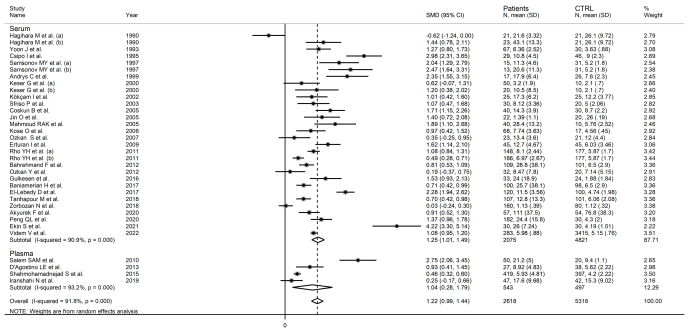

FIGURE 10
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in serum/plasma according to the sample matrix used for assessment (plasma or serum).

vs. serum). By contrast, there was a significant association between the SMD (plasma or urine) and the study geographical location, with progressively higher SMD values in studies conducted in America, Asia, Europe, and Africa, and between the SMD (urine) and the type of RD investigated.

Taken together, these results suggest that neopterin can significantly discriminate between physiological states and different types of RD, including an autoimmune and/or an autoinflammatory component, using a range of analytical methods that can be applied both in plasma/serum and in urine. High-performance liquid chromatography with fluorimetric detection, ELISA, and RIA were the analytical methods most used to measure neopterin in biological fluids. High-performance liquid chromatography with fluorimetric detection offers a particularly high sensitivity, enabling the simultaneous detection of low neopterin concentrations. Its specificity is also high due to compound separation in the sample, which minimize the interference from other molecules. Quantitative accuracy is achievable, particularly when coupled with sensitive fluorimetric detection. However, it demands specialized equipment and expertise for operation and maintenance, and the process is time-consuming and potentially costly (91). ELISA is particularly suitable for the assessment of a large volume of samples due to its capacity to process multiple samples simultaneously. Its execution is relatively straightforward, with many commercially available kits. The broad dynamic range of quantitative values is an advantage, covering both low and high neopterin concentrations. However, specificity relies on the quality of antibodies used, and cross-reactivity with related compounds might limit accuracy. Additionally, sensitivity might be an issue with very low concentrations (38). RIA is known for its high sensitivity, enabling the detection of very low neopterin concentrations. Quantitative accuracy is attainable with proper optimization. Specificity depends on appropriately selected antibodies, which can be highly specific. However, there are also safety concerns due to the use of radioisotopes, requiring proper handling and disposal (92). RIA can involve complex steps due to the separation of bound and free fractions. Overall, the choice among these methods should be based on the required sensitivity, available resources, and safety considerations. High-performance liquid chromatography with fluorimetric detection offers high specificity and sensitivity but requires complex and costly equipment. ELISA is simple, high-capacity, and has a broad dynamic range, but specificity might be limited. RIA provides high sensitivity and precision but carries safety issues and has limitations in reagent availability.

Another interesting observation was the absence of significant correlations between the SMD of neopterin and CRP and ESR, biomarkers that are routinely used to assess inflammation and disease activity in RDs, also suggests that the information provided

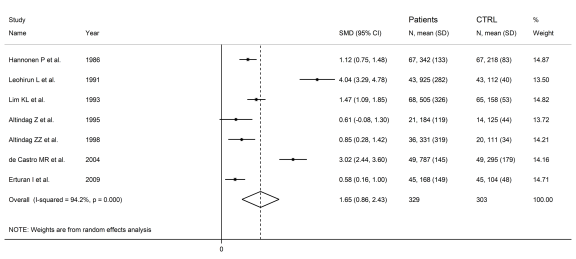

FIGURE 11
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in urine.

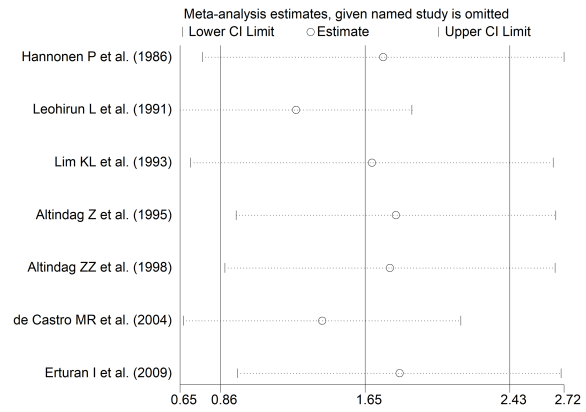

FIGURE 12
Sensitivity analysis of the association between neopterin and RDs in urine.

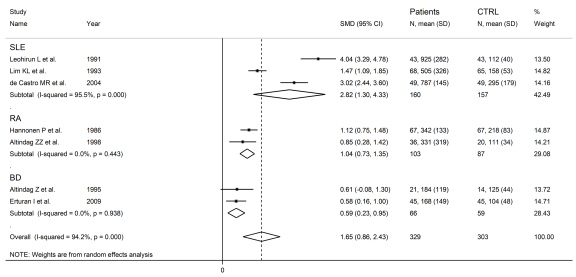

FIGURE 13
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in urine according to RD type.

by neopterin can potentially complement existing knowledge to enhance diagnostic capacity. The presence of significant geographic-related and RD type-related differences in the SMD of neopterin also suggests the potential influence of ethnicity and specific RDs in mediating the associations between interferon-γ, macrophage activation, and inflammatory and immune pathways.

Although interferon-γ is mainly produced by T helper 1 and natural killer cells, macrophages can also contribute to its formation (93, 94). In this context, there is robust evidence that interferon-γ activates macrophages to the creation of a pro-inflammatory phenotype and, at the same time, stimulates the expression of pro-inflammatory cytokines and downregulates anti-inflammatory cytokines (Figure 1) (95, 96). Furthermore, interferon-γ regulates the initial steps of the adaptative immune response by influencing dendritic cells, T-cells, and B-cells (97–99). However, the excessive production of interferon-γ is responsible for the dysregulation of inflammatory and immune pathways, a phenomenon that has been observed in several hyperinflammatory disease states, cytokine release syndromes, and autoimmune conditions (28, 100–103). Notably, in these studies neopterin was measured as a biomarker of interferon-γ activity (28, 100–103). This pteridine analogue is not directly synthesized in macrophages, rather it is the oxidized form of another pteridine analogue synthesized in these cells, 7,8-dihydroneopterin. In activated macrophages, interferon-γ is responsible for the upregulation of GTP cyclohydrolase 1, the enzyme responsible for the bioconversion of GTP into 7,8-dihydroneopterin-triphosphate, which is then transformed to 7,8-dihydroneopterin by the action of phosphatase enzymes (Figure 1) (104–106). 7,8-dihydroneopterin is a known antioxidant and free radical scavenger with protective effects on low-density lipoprotein, other proteins, and lipids (107–109). The scavenging effects of 7,8-dihydroneopterin on free radicals lead to the synthesis of several oxidation products, including neopterin (Figure 1) (110, 111). Although 7,8-dihydroneopterin might theoretically serve as a robust biomarker of immune activation and redox state its physicochemical characteristics, particularly the low fluorescence, present analytical challenges when measured in isolation and in combination with neopterin (total neopterin) (40, 112, 113). Pending further analytical studies to optimize the measurement of 7,8-dihydroneopterin in blood and other biological samples, our systematic review and meta-analysis also warrants further studies to confirm the potential utility of neopterin specifically in the early detection of RDs. In this context, the absence of significant associations between the SMD of neopterin concentrations and RD duration observed in meta-regression analysis suggests that this biomarker can effectively discriminate between physiological states and presence of RDs also in patients with relatively short disease duration.

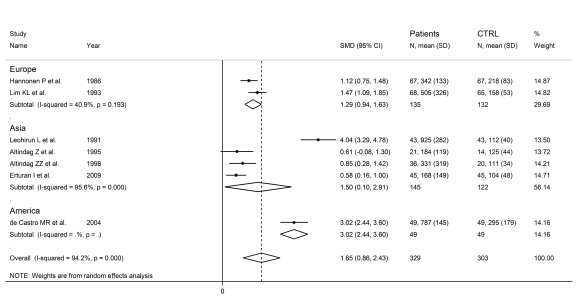

FIGURE 14
Forest plot of studies examining neopterin concentrations in RD patients and healthy controls in urine according to study continent.

Another interesting observation was the presence of significant differences in the SMD of neopterin according to specific geographical locations. Epidemiological studies have shown that in healthy individuals neopterin concentrations can be influenced by age, body mass index, body composition and ethnicity (114, 115). In a study of 426 healthy subjects, black participants, particularly males, had significantly higher concentrations of neopterin than white participants (114). However, opposite results, with higher neopterin concentrations in white compared to black subjects, or no ethnic-related differences were reported in other studies (116, 117). A systematic review and meta-analysis has also investigated the association between a functional polymorphism of the *interferon-γ* gene, +874 T/A, associated with excess production of interferon-γ (118), and the risk of autoimmune disease. In this study, there were significant differences in the frequencies of the T allele across Asian (34.1%), Middle Eastern (47.8%), Latin American (51.5%), and Caucasian subjects (74.2%). Furthermore, the T allele was significantly associated with the risk of autoimmune disease in Latin Americans, but not in Middle Eastern, Asian, or Caucasian populations (119). Clearly, additional research is warranted to investigate the influence of ethnicity on interferon-γ production, macrophage activation, neopterin concentrations, and RDs. The additional observation that the SMD of urine neopterin was significantly associated with specific types of RD also requires further studies to investigate the capacity of urine neopterin to discriminate between different types of RD. At the same time, however, the significantly higher SMD of urine neopterin observed in studies of patients with SLE *vs.* other types of RD opens new opportunities to investigate the utility of this biomarker to diagnose and/or assess the severity of renal involvement, specifically nephritis, often observed in this group (120).

Our study has several strengths, including the assessment of neopterin in different biological fluids in a wide range of RD types, the study of associations between the effect size and several study and patient characteristics, and a rigorous evaluation of the risk of bias and the certainty of evidence. Significant limitations include the paucity of studies investigating specific types of RD (i.e., AS, SSc, FMF, and PsA), and the high heterogeneity observed. However, we identified potential sources of heterogeneity in subgroup analyses (type of RD and study continent for both plasma/serum and urine neopterin). Furthermore, sensitivity analysis ruled out the effect of individual studies on the overall effect size.

In conclusion, this systematic review and meta-analysis has shown the presence of significant alterations in the plasma/serum and urinary concentrations of neopterin, a biomarker of interferon-γ production, macrophage activation, inflammation, and oxidative stress, in patients with RD. Further research is warranted to determine the capacity of neopterin to identify early *vs*. overt RD manifestations and justify its introduction in clinical practice.

Data availability statement

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

Author contributions

AM: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. AZ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1271383/full#supplementary-material

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Molecular profiling of clinical remission in psoriatic arthritis reveals dysregulation of *FOS* and *CCDC50* genes: a gene expression study

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Background: In psoriatic arthritis (PsA), the primary goal of treatment is clinical remission. This study aimed to characterize the molecular profile underlying the induced clinical remission in patients with PsA, comparing the remission state and the healthy condition.

Methods: Whole blood transcriptomic analysis was performed on groups of 14 PsA patients in TNFi-induced clinical remission (DAPSA ≤ 4), 14 PsA patients with active disease (DAPSA > 14), and 14 healthy controls (HCs). Then, all differentially expressed genes (DEGs) derived from remission vs. HC comparison were analyzed for functional and biological characteristics by bioinformatics software. The gene expression of 12 genes was then validated by RT-qPCR in an extended cohort of 39 patients in clinical remission, 40 with active disease, and 40 HCs.

Results: The transcriptomic analysis of PsA remission vs. HCs highlighted the presence of 125 DEGs, and out of these genes, 24 were coding genes and showed a great involvement in immune system processes and a functional network with significant interactions. The RT-qPCR validation confirming the down- and upregulation of *FOS* (FC −2.0; p 0.005) and *CCDC50* (FC +1.5; p 0.005) genes, respectively, in line with their role in orchestrating inflammation and bone metabolism processes, may be related to PsA pathophysiology.

Conclusion: The transcriptomic profile of clinical remission in PsA is similar to a healthy condition, but not identical, differing for the expression of *FOS* and *CCDC50* genes, which appears to play a key role in its achievement.

KEYWORDS

psoriatic arthritis, clinical remission, transcriptomic, *FOS*, *CCDC50*

1 Introduction

Psoriatic arthritis (PsA) is a chronic inflammatory disease characterized by wide clinical heterogeneity due to the variable combination of six major domains, namely, skin and nail psoriatic lesions, peripheral arthritis, axial disease, dactylitis, and enthesitis (1). It is recognized as a potentially disabling disease, as late and inadequate control of disease activity may result in structural damage and disability (2).

According to the European Alliance of Associations for Rheumatology (EULAR) and the Group for Research in Psoriasis and PsA (GRAPPA) recommendations, treatment of PsA should aim primarily at reaching the target of remission by regular disease activity assessment and appropriate adjustment of therapy (3, 4). Although this approach represents one of the strongest and most widely shared recommendations, there are still relevant issues regarding its application in clinical practice. In particular, the definition of remission is still open to discussion among experts and represents a significant challenge in the management of PsA (5). Several definitions of clinical remission, based on composite indices combining objective (e.g., tender and swollen joint count or enthesitis and dactylitis count (6)) and subjective (e.g., scales for pain or general health) measurements of disease activity are currently used in clinical practice and trials (7). However, the clinical heterogeneity of PsA, the potential persistence of subclinical disease activity demonstrated in ultrasonography studies, and the possible progression of structural damage in patients classified as in clinical remission (8), highlight the urgent need for a sensitive and specific biomarker supporting the accurate identification of remission.

Several genetic, circulating, and tissue factors have been studied as biomarkers in the management of different aspects of PsA, including diagnosis and assessment or prediction of disease activity, severity, and response to treatment (9–13). However, none of these has been extensively validated and then translated into routine clinical practice (10, 14). In particular, despite remission being recommended as the primary goal in PsA treatment, to our knowledge, no studies have been specifically designed to identify the underlying molecular mechanisms and potential biomarkers.

Transcriptomic profiling has become a standard technology in searching for biomarkers of susceptibility, disease activity, progression, and response to treatment in several diseases, including PsA (15, 16). However, the transcriptomic approach has yet to be applied so far in the assessment of clinical remission. Since sustained clinical remission without drug treatment is extremely rare in patients with PsA, a substantial molecular difference between clinical remission and the healthy state may be assumed, but it needs to be demonstrated and characterized. In this regard, an intriguing question is whether the achievement of clinical remission reflects a molecular profile closer to healthy individuals rather than PsA active patients, which we refer to as "molecular remission".

This study aimed to identify molecular remission biomarkers by comparing the gene expression profile of PsA patients in clinical remission vs. healthy controls and PsA patients with active disease.

Abbreviations: PsA, psoriatic arthritis; DEGs, differentially expressed genes; EULAR, European Alliance of Associations for Rheumatology; GRAPPA, Group for Research in Psoriasis and PsA; PsA-R, PsA patients in clinical remission; PsA-A, PsA patients with active disease; HCs, healthy controls; CASPAR, classification criteria for psoriatic arthritis; DAPSA, Disease Activity PsA; TNFi, TNF inhibitors; GO, Gene Ontology.

2 Methods

2.1 Patients and controls

The present study was based on the comparative transcriptomic profiling of three groups of subjects: PsA patients in clinical remission for at least 1 year (PsA-R), PsA patients with active disease (PsA-A), and healthy controls (HCs). The PsA patients, recruited from a monocentric cohort, were diagnosed according to the classification criteria for psoriatic arthritis (CASPAR) (17) and classified as in clinical remission or active if they had a Disease Activity PsA (DAPSA) score of ≤4 or >14, respectively (18). To ensure a higher level of homogeneity of the PsA group in clinical remission, all the recruited patients were on treatment with TNF inhibitors (TNFi) after the failure of methotrexate. The treatment regimen of the PsA group with active disease is reported in Table 1. Patients undergoing concomitant treatment with glucocorticoids were excluded from both groups. The healthy control group was matched for mean age and gender ratio with the remission group, as this study was primarily focused on comparing these two conditions.

TABLE 1 Demographic, clinical, and therapeutic data of the recruited PsA patients in clinical remission (PsA-R), PsA patients with active disease (PsA-A), and healthy controls (HCs).

	PsA-R (*n* = 39)	PsA-A (*n* = 40)	HC (*n* = 40)
Demographics			
Male, *n* (%)	30 (76.9)	20 (50.0)	19 (47.5)
Age at enrolment, mean (SD), years	52.0 (12.3)	55.5 (14.9)	52.0 (6.3)
Disease duration, mean (SD), years	10.1 (6.3)	5.6 (5.6)	–
BMI, mean (SD) score	25.5 (3.7)	27.7 (4.9)	
Clinical pattern			
Axial, *n* (%)	11/39 (28.2)	1/40 (2.5)	–
Peripheral, *n* (%)	39/39 (100)	40 (100)	–
Personal history of PsA (%)	37/39 (94.9)	39/40 (97.5)	
Familiar history of PsA (%)	6/38 (15.6)	5/40 (12.5)	

(Continued)

TABLE 1 Continued

	PsA-R (*n* = 39)	PsA-A (*n* = 40)	HC (*n* = 40)
Onychopathy (%)	22/37 (59.7)	22/38 (57.9)	
Dactylitis (%)	26/39 (66.7)	21/40 (52.5)	
Enthesitis (%)	23/38 (60.5)	17/40 (42.5)	
Rheumatoid factor, *n* (%)	8/39 (20.5)	1/34 (2.9)	–
Clinimetrics			
PGA, mean (SD), years	2.9 (7.6)	45.8 (28.0)	–
PtGA, mean (SD), years	15.3 (19.9)	69.0 (20.9)	–
VAS—pain, mean (SD), years	15.1 (21.3)	70.4 (18.2)	–
GH, mean (SD), years	75.2 (21.6)	51.8 (25.4)	–
ESR, mean (SD), years	9.6 (6.4)	29.1 (19.9)	–
CRP, mean (SD), years	1.1 (1.2)	12.5 (22.1)	–
DAS-28, mean (SD), years	1.9 (0.7)	4.6 (1.3)	–
DAPSA, mean (SD), years	3.6 (4.9)	25.8 (10.4)	–
HAQ, mean (SD), years	0.4 (0.4)	1.4 (0.6)	–
Treatment			
NSAID, *n* (%)	11 (28.2)	13 (32.5)	–
cs-DMARDs, *n* (%)	10 (25.6)	23 (57.5)	–
TNF inhibitors, *n* (%)	39 (100)	7 (17.5)	–

BMI, body mass index; PGA, Physician Global Assessment; PtGA, Patient Global Assessment, VAS, visual analog scale; GH: Global Health Assessment; ESR: erythrocyte sedimentation rate; CRP, C-reactive protein; DAS-28, Disease Activity Score-28; DAPSA, Disease Activity Index for Psoriatic Arthritis; HAQ, Health Assessment Questionnaire; NSAIDs, non-steroidal anti-inflammatory drugs; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs.
- means NONE.

The demographic and clinical features of the three study groups are reported in Table 1.

The study was approved by the local ethical committee (PG/2018/16313; 12th November 2018), and written informed consent was obtained from all subjects. All procedures were in accordance with the Good Clinical Practice standards and Helsinki Declaration.

2.2 Study design

The study consisted of three consecutive phases:

I. *Explorative transcriptomic profiling*: To identify a preliminary list of differentially expressed genes (DEGs), transcriptomic analysis was performed on pooled RNAs from peripheral blood in biological duplicates of a group of 14 PsA patients in clinical remission, 14 PsA patients with active disease, and 14 HCs (groups of 7 patients in biological duplicates for each condition, for a total of 6 microarrays).

II. *Functional and biological analysis of dysregulated transcripts*: First, the complete list of DEGs identified by comparing the PsA-R vs. HC groups were analyzed *in silico* for functional and biological characteristics. Then, only mRNAs related to coding genes were selected and re-analyzed *in silico* to select those of greater interest to be assessed in the validation phase.

III. *RT-qPCR validation analysis*: A quantitative reverse transcription PCR (RT-qPCR) for single gene expression analysis of DEGs selected from the previous phases was extended in the whole cohort of 39 PsA patients in clinical remission, 40 PsA with active disease, and 40 HCs.

2.3 Transcriptomic analysis

2.3.1 Target preparation

RNAs were extracted from peripheral blood in RNAlater preservative (Invitrogen) by Ambion RiboPure Kit followed by DNAse treatment. The quality of total RNA was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Extracted RNAs were pooled in groups of seven patients in biological duplicates for each condition (remission, active, healthy controls) for a total of six microarrays.

Biotin-labeled cDNA targets were synthesized starting from 150 ng of total RNA. Double-stranded cDNA synthesis and related cRNA were performed with GeneChip® WT Plus Kit (Affymetrix, Santa Clara, CA, USA). With the same kit, the sense strand cDNA was synthesized before being fragmented and labeled. All steps of the labeling protocol were performed as suggested by Affymetrix. Each eukaryotic GeneChip® probe array contains probe sets for several *Bacillus subtilis* genes that are absent in the samples analyzed (lys, phe, thr, and dap). This Poly-A RNA Control Kit contains *in-vitro* synthesized, polyadenylated transcripts for the *B. subtilis* genes that are premixed at staggered concentrations to allow GeneChip® probe array users to assess the overall success of the assay. The Poly-A RNA Control final concentrations in each target are as follows: lys, 1:100,000; phe, 1:50,000; thr, 1:25,000; and dap, 1:6,667.

2.3.2 DNA microarray hybridization

This was performed using the GeneChip® Hybridization, Wash and Stain Kit. It contains a mix for target dilution, DMSO at a final concentration of 7%, and premixed biotin-labeled control oligo B2 and bioB, bioC, bioD, and cre controls (Affymetrix cat. #900299, Santa Clara, CA, USA) at a final concentration of 50 pM, 1.5 pM, 5 pM, 25 pM, and 100 pM, respectively. Targets were diluted in a hybridization buffer at a 25-ng/µL concentration and denatured at 99°C for 5 min, incubated at 45°C for 5 min, and centrifuged at maximum speed for 1 min before introduction into the GeneChip® cartridge. A single GeneChip® Human Transcriptome Array 2.0 was then hybridized with each biotin-labeled sense target. Hybridizations were performed for 16 h at 45°C in a rotisserie oven. GeneChip® cartridges were washed and stained with the

GeneChip® Hybridization, Wash and Stain Kit in the Affymetrix Fluidics Station 450 following the FS450_0002 standard protocol, including the following steps: 1) (wash) 10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C; 2) (wash) 6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C; 3) stain of the probe array for 5 min in SAPE solution at 35°C; 4) (wash) 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C; 5) stain of the probe array for 5 min in antibody solution at 35°C; 6) stain of the probe array for 5 min in SAPE solution at 35°C; 7) (final wash) 15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C; and 8) fill the probe array with Array Holding buffer.

2.3.3 Image acquisition, data processing, and bioinformatics analysis

GeneChip arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA) using default parameters. Affymetrix GeneChip® Command Console Software (AGCC) was used to acquire GeneChip® images and generate.DAT and.CEL files, which were used for subsequent analysis with proprietary software (Partek Genomics suite V6.6).

To identify differentially expressed transcripts (concordantly on both biological duplicates of each profiled condition), a fold change (FC) ± 1.5 cutoff and a *p*-value of 0.05 were set.

2.4 Functional and biological analysis of dysregulated transcripts

For the bioinformatics Gene Ontology (GO) analysis, only differentially expressed transcripts between the PsA-R group vs. HC, with paired RefSeq, were included. Then, gene set enrichment analysis of other represented GO classes was made by fold enrichment and associated *p*-value (absolute count of identified transcripts vs. expected) for macro- and microcategories. Additionally, coding DEGs were represented in a chromosomic map to visualize their distribution. From the comparative list of DEGs in the PsA-R vs. HC condition, coding mRNAs were selected, interactions were analyzed by the STRING software (free version, V 10.5), and biological functions and annotations were determined by Gene Ontology.

2.5 RT-qPCR validation analysis

Twelve genes were selected from the abovementioned analysis considering literature, GO, and STRING data results and included in the validation phase completed in a larger PsA cohort (39 PsA-R + 40 PsA-A) and 40 HCs.

Extracted RNAs from whole blood were quantified by Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and retrotranscribed by a High-Capacity RNA-cDNA kit (Invitrogen, Vilnius, Lithuania). The qPCR reactions were prepared in a final volume of 10 µL, with 5 µL of 2× TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 µL of each 20× primer, and 1 µL of sample (5 ng of cDNA template per reaction). Thermal profiling consisted of a first cycle at 50°C for 2 min, a second cycle at 95°C for 2 min, followed by 40 cycles of amplification at 95°C for 1 s and 60°C for 20 s. qPCR reactions were run in triplicate on a thermal cycler StepOne Plus (Applied Biosystems, Foster City, CA, USA).

Gene expression was measured using the following TaqMan Gene Expression Assay primers (Applied Biosystems, Foster City, CA, USA): *FCAR* (Hs02572026_s1), *CEACAM8* (Hs00266198_m1), *FOS* (Hs04194186_s1), *BPI* (Hs01552756_m1), *DEFA1B* (Hs07287122_m1), *ANPEP* (Hs00174265_m1), *ALPL* (Hs01029144_m1), *CHI3L1* (Hs01072228_m1), *PADI2* (Hs01042505_m1), *KLRB1* (Hs00174469_m1), *CCD50* (Hs01047000_m1), and *TNSF14* (Hs00542476_g1). Glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene (Hs02758991_g1).

Gene expression quantification was made by the $2^{-\Delta\Delta Ct}$ method for relative quantification (RQ), and the fold change (FC) cutoff was ±1.5 for RQ comparative analysis between groups.

2.6 Statistical analysis

Categorical variables were expressed as absolute values and frequencies (%). Normally and non-normally distributed continuous variables were reported as the mean ± standard deviation (SD) and median and IQR, respectively. Student's *t*-test was applied in the validation phase to compare the mean relative quantification values in the three study groups. The following comparisons were performed: PsA-R vs. HC, PsA-R vs. PsA-A, and PsA-A vs. HC. A *p*-value of <0.05 was considered statistically significant.

3 Results

3.1 Transcriptomic profiling of the remission state

More than 1,000 transcripts differentially expressed in at least one of the three comparisons were identified. The hierarchical clustering with heatmap is reported in Figure 1A. In particular, 125 DEGs (65 up- and 60 downregulated) were identified comparing the PsA-R vs. the HC group, 1,184 (753 up- and 431 downregulated) comparing the PsA-R vs. the PsA-A group, and 378 (378 up- and 314 downregulated) comparing the PsA-A vs. the HC group. The numbers of DEGs for each comparison and the respective overlaps are represented in the Venn diagram in Figure 1B, and the complete list of all DEGs identified in the comparison object of this study is reported in Supplementary Material 1.

3.2 Biological function analysis of DEGs in clinical remission

The bioinformatics gene set enrichment analysis by the GO software of the 125 DEGs identified in the PsA-R vs. the HC comparison showed that they were primarily involved in the "immune system processes" (Figure 1C). A subanalysis on

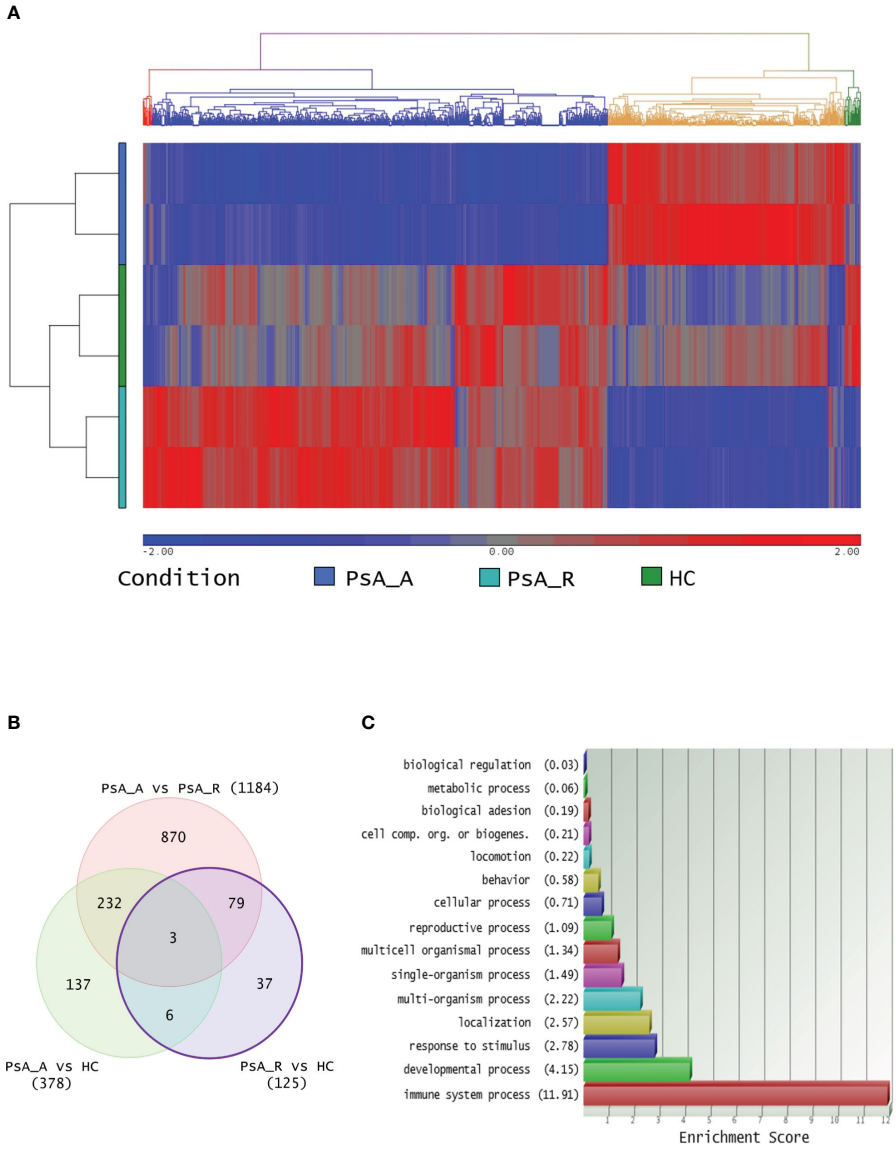

FIGURE 1
(A) Transcriptomic analysis and clustering. Hierarchical clustering of 1,364 differentially expressed transcripts in at least one comparison in analysis (FC 1.5 and *p*-value 0.05). The profiled conditions are in the rows (in duplicates, two rows/condition. PsA_A, active psoriatic arthritis; PsA_R, remission psoriatic arthritis; HCs, healthy controls), and the transcripts are in the columns by a pseudocolor scale with expression values normalized to zero, SD = 1 (blue, lower abundance; red, higher abundance), as indicated in the legend scale. Four clusters are represented by four colors in the upper dendrogram, suggesting that these conditions have distinct signatures (or similarities). **(B)** Venn diagram illustrating the overlap between 1,364 transcripts differentially expressed on three comparative lists (identified with a minimum HR of 1.5 and *p*-value 0.05, no FDR correction applied). **(C)** DEG Gene Ontology analysis. For the bioinformatics Gene Ontology (GO) analysis, only differentially expressed transcripts between PsA-R condition vs. HC, with paired RefSeq, were included. The enrichment analysis about more represented GO classes (histogram bars) was made by fold enrichment and associated *p*-value (the reported enrichment score in brackets is the absolute count of identified transcripts vs. expected), both for macro- and microcategories.

"microcategories" of the immune system process related to the DEGs is reported in Supplementary Material 2.

Out of the 125 DEGs identified by comparing the PsA-R vs. the HC group, only 25 were coding genes. Thus, according to the preset methodology, they were selected for the in-depth functional and biological analysis. The respective hierarchical clustering with the heatmap is reported in Figure 2A, and similar to the previous GO analysis, the biological function study of the 25 coding DEGs demonstrated their primary involvement in the "immune system processes" (Figure 2B; for the complete list of symbols and annotated functions by the Partek software, see Supplementary Material 3). Moreover, when such DEGs were further analyzed for functions and interactions, the bioinformatics STRING software tool built an interaction network between 24 putative proteins, with more significant interactions than expected (Figure 3). For the STRING raw data analysis, legend, settings, and results, see Supplementary Material 4. Lastly, in Supplementary Material 5, the karyomap figure shows these genes' chromosomic mapping.

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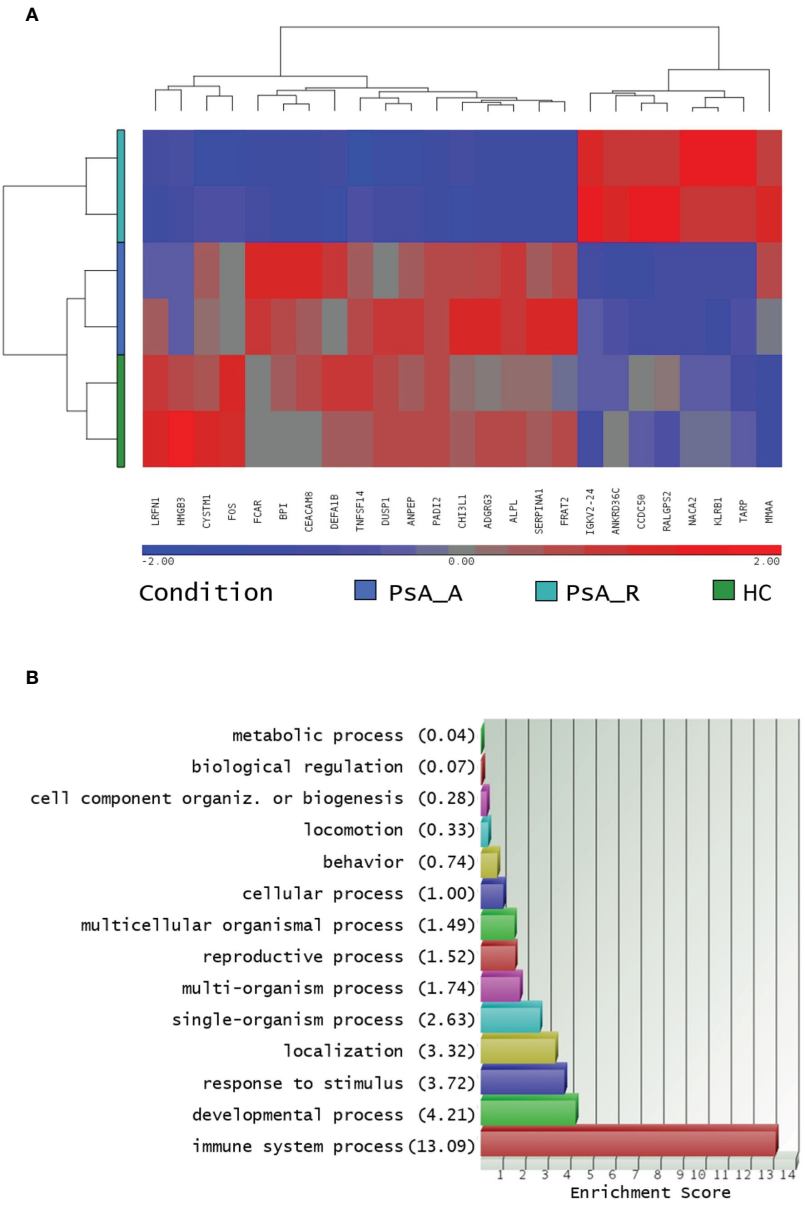

FIGURE 2
(A) Hierarchical clustering of coding DEGs in remission state. Heatmap of 24 filtered coding DEGs in the PsA remission state and their expression in all profiled conditions, analysis in biological duplicates (cutoff FC ± 1.5, *p*-value 0.05). The profiled conditions are in the rows (in duplicates, two rows/condition: PsA_A, active psoriatic arthritis; PsA_R, remission psoriatic arthritis; HCs, healthy controls), and the DEGs are in the columns by a pseudocolor scale with expression values normalized to zero, SD = 1 (blue, lower abundance; red, higher abundance), as indicated in the legend scale. **(B)**: coding DEGs Gene Ontology analysis. Only coding DEGs in the PsA-R condition vs. HC, with paired RefSeq, were included. The enrichment analysis about more represented GO classes (histogram bars) was made by fold enrichment and associated *p*-value (the reported enrichment score in brackets is the absolute count of identified transcripts vs. expected).

3.3 Extended gene expression analysis of coding DEGs in the remission state

Matching our bioinformatics data with the current evidence regarding the 25 coding DEGs identified in the PsA-R vs. the HC comparison, 12 were selected for the validation phase by gene expression quantification. Their symbols, RefSeq, annotated functions, and chromosomic position are described in Table 2.

The RT-qPCR validations in the large PsA cohort (39 PsA-R + 40 PsA-A patients) and 40 HCs measured the expression of all selected genes in all subjects as shown in Table 3.

In the clinical remission vs. healthy condition comparison, data obtained in the single-gene expression dosage significantly confirmed the downregulation (FC −2.0; *p* 0.005) of *FOS* and the upregulation (FC + 1.5; *p* 0.005) of *CCDC50* (alias *YMER*) genes in the PsA-R state (Figure 4). For further analysis, we evaluated the

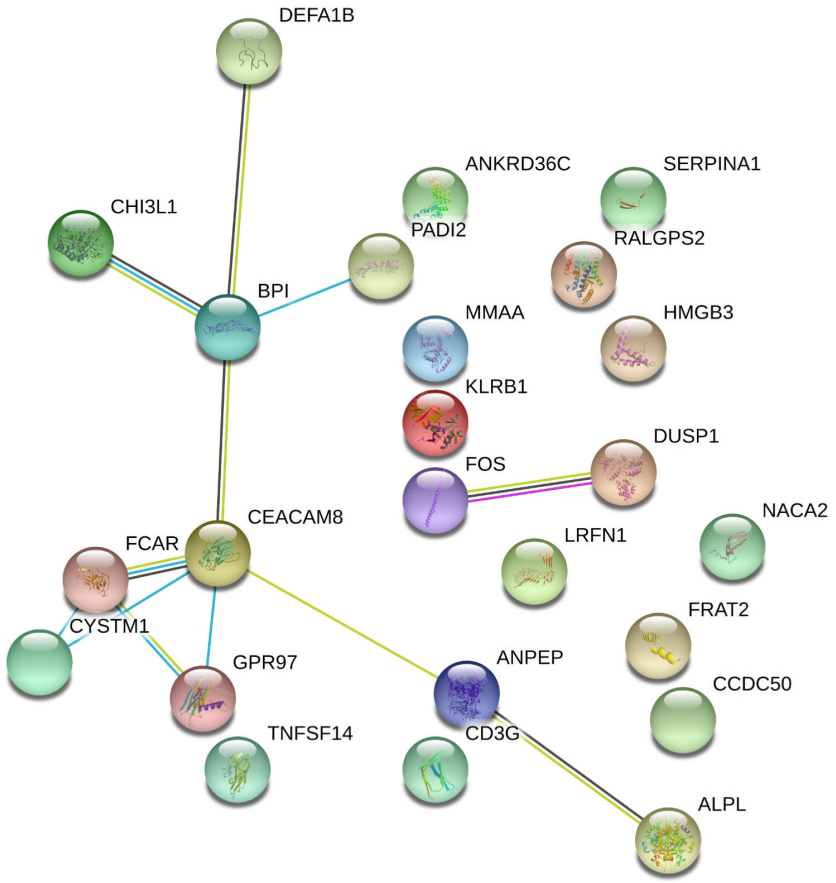

FIGURE 3
Interactome of coding DEGs misregulated on PsA clinical remission. Differentially expressed genes (DEGs) in the remission (PsA-R) group vs. healthy controls (HCs) analyzed for multiple protein interactions by the STRING software V10.5 (raw data and coordinates in Supplementary Material 4). Network nodes representing proteins, splice isoforms, or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Edges represent protein–protein associations and are drawn as follows: red line = presence of fusion evidence; green line = neighborhood evidence; blue line = cooccurrence evidence; purple line = experimental evidence; yellow line = textmining evidence; light blue line = database evidence; black line = coexpression evidence. Edge associations are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other. Represented network stats: number of nodes = 24; number of edges = 12; average node degree = 1; avg. local clustering coefficient = 0.369; expected number of edges = 3; PPI enrichment *p*-value = 3.17e−05.

association between CRP levels and RQ values of both these genes, demonstrating a significant negative and positive correlation, respectively, with *CCDC50* [Pearson's correlation coefficient (*r*): −0.240; $p = 0.035$] and *FOS* (*r*: 0.386; $p = 0.001$).

The differential analysis between groups also showed a significative misregulation of other genes in other comparisons (PsA-R vs. PsA-A; PsA-A vs. HC) (see Table 3). In particular, there was a significative downregulation of *KLRB1* (FC −1.6; p 0.001) in the active disease vs. healthy condition, while the comparison between the remission vs. active PsA exhibited the overexpression of *CCDC50* (FC 1.8; $p < 0.001$) and *KLRB1* (FC 1.6; $p < 0.001$) (RQ and FC values of all validated DEGs are shown in Table 3).

4 Discussion

This is the first gene expression study specifically designed to explore the molecular mechanisms underlying the clinical remission in PsA patients through an investigative approach primarily based on comparing the remission and the healthy condition.

The comparative transcriptomic analysis showed that clinical remission was similar but not identical to the healthy state. Indeed, the presence of 125 DEGs suggests that the TNFi-induced clinical remission is not synonymous with molecular disease inactivation leading to a "back to a healthy state," but it is a condition characterized by several misregulated transcripts that, on the one hand, may represent the persistence of underlying disease activity and, on the other hand, may mean the activation of mechanisms sustaining disease remission.

The subsequent phase of bioinformatics analysis showed that the coding DEGs in clinical remission were strictly correlated to each other in a strong interaction network and were primarily involved in functions related to immune system processes. These *in-silico* predictions were confirmed by the validation phase of this study, where the RT-qPCR single-gene expression analysis showed

TABLE 2 All validated DEGs listed for their gene symbol, full name, synonyms, cytoband, and functions annotated by RefSeq and UniProt sources.

Gene symbol	Full name	Synonyms	Annotated functions	Cytoband
CCDC50	Coiled-coil domain containing 50	YMER; C3orf6; DFNA44	Encodes a soluble, cytoplasmic, tyrosine-phosphorylated protein with multiple ubiquitin-interacting domains that may function as a negative regulator of NF-κB signaling and as an effector of epidermal growth factor (EGF)-mediated cell signaling.	3q28
KLRB1	Killer cell lectin-like receptor B1	CD161, CLEC5B, NKR, NKR-P1, NKR-P1A, NKRP1A, hNKR-P1A	Plays an inhibitory role in natural killer (NK) cell cytotoxicity. Activation results in sphingomyelinase/SMPD1 stimulation, also leads to enhanced T-cell proliferation induced by anti-CD3. Binds also to CLEC2D/LLT1 as a ligand and inhibits NK cell-mediated cytotoxicity as well as interferon-gamma secretion in target cells.	12p13.31
ANPEP	Alanyl aminopeptidase, membrane	APN; CD13; LAP1; P150; PEPN; GP150	Involved in the processing of various peptides including peptide hormones, angiotensins III and IV, neuropeptides, and chemokines. May also be involved in the cleavage of peptides bound to major histocompatibility complex class II molecules of antigen-presenting cells.	15q26.1
DEFA1B	Defensin alpha 1B	HP1; HP-1; HNP-1	Family of antimicrobial and cytotoxic peptides involved in host defense, abundant in the granules of neutrophils and also found in the epithelia of mucosal surfaces such as those of the intestine, respiratory tract, urinary tract, and vagina.	8p23.1
BPI	Bactericidal permeability increasing protein	rBPI; BPIFD1	Belongs to the BPI/LBP/Plunc superfamily. The cytotoxic action of BPI is limited to many species of Gram-negative bacteria.	20q11.23
CHI3L1	Chitinase 3 like 1	ASRT7, CGP-39, CHI3L1, CHIL1, GP-39, GP39, HC-GP39, HCGP-39, HCGP-3P, YKL-40, YKL40, YYL-40	Chitinases catalyze the hydrolysis of chitin. The protein is secreted by activated macrophages, chondrocytes, neutrophils, and synovial cells. The protein is thought to play a role in the process of inflammation and tissue remodeling.	1q32.1
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	AP-1, C-FOS, p55	Nuclear phosphoprotein forms a complex with the JUN/AP-1 transcription factor with an important role in signal transduction, cell proliferation, and differentiation. Forms a multimeric SMAD3/SMAD4/JUN/FOS complex at the AP1/SMAD-binding site to regulate TGF-beta-mediated signaling. Has a critical function in regulating the development of cells destined to form and maintain the skeleton notably involved in the osteoclastogenesis by RANK ligand signaling, in inflammatory bone and skin disease.	14q24.3
ALPL	Alkaline phosphatase, biomineralization associated	HOPS; TNAP; TNALP; APTNAP; TNSALP; AP-TNAP	Encodes a member of the family of phosphatases: intestinal, placental, placental-like, and liver/bone/kidney. The mature enzyme may play a role in bone mineralization. Mutations in this gene have been linked to hypophosphatasia, a disorder that is characterized by hypercalcemia and skeletal defects.	1p36.12
PADI2	peptidyl arginine deiminase 2	MKIAA0994, PAD-H19, PAD2, PADI2, PDI, PDI2	Encodes a member of the family of enzymes, which catalyze the post-translational deimination of proteins by converting arginine residues into citrullines. Known substrates for this enzyme include vimentin in skeletal muscle and macrophages.	1p36.13
TNFSF14	TNF superfamily member 14	LTg; CD258; HVEML; LIGHT	The protein encoded is a member of the tumor necrosis factor (TNF) ligand family: a cytokine ligand for TNFRSF14 may function as a costimulatory factor for the activation of lymphoid cells and as a deterrent to infection by herpesvirus. This protein has been shown to stimulate the proliferation of T cells and trigger apoptosis of various tumor cells.	19p13.3
FCAR	Fc fragment of IgA receptor	CD89; FcalphaRI; CTB-61M7.2	This gene encodes a receptor for the Fc region of IgA, a transmembrane glycoprotein present on the surface of myeloid lineage cells where it mediates immunologic responses to pathogens and stimulation of the release of inflammatory mediators.	19q13.42
CEACAM8	CEA cell adhesion molecule 8	CD67; CGM6; CD66b; NCA-95	Belongs to the immunoglobulin superfamily. Cell surface glycoprotein that plays a role in cell adhesion. Heterophilic interaction with CEACAM8 occurs in activated neutrophils.	19q13.2

in all profiled conditions the dysregulation of genes strictly involved in inflammatory and immune processes.

The primary analysis of this study, based on comparing the clinical remission with the healthy condition, revealed the down- and upregulation of *FOS* and *CCDC50* genes, respectively, which are both reported as having a significant role in the inflammatory process and osteoclastogenesis.

FOS (Fos Proto-Oncogene, AP-1 Transcription Factor Subunit) is a protein-coding gene. The *FOS* gene family consists of four members: *FOS, FOSB, FOSL1*, and *FOSL2*. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1 (19). After being induced by several extra- and intracellular stimuli, the immediate early gene product Fos translates into the regulation of downstream target genes implicated in various cellular processes, including inflammatory response and osteoclastogenesis regulation (20). In this regard, it has been described how Fos/AP-1 has an important role in the induction of NFAT-dependent genes coding

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TABLE 3 Gene expression quantification of DEGs on PsA remission state.

	FC microarray	FC RT-qPCR (*p*-value)		
DEG	**PsA-R vs. HC**	**PsA-R vs. HC**	**PsA-R vs. PsA-A**	**PsA-A vs. HC**
FCAR	−1.56	−1.1 (*0.362*)	−1.3 (*0.114*)	1.1 (*0.295*)
CEACAM8	−2.3	−1.2 (*0.542*)	−1.5 (*0.241*)	1.2 (*0.542*)
FOS	−1.51	**−2.0 (*0.005*)**	−1.4 (*0.150*)	-1.5 (*0.103*)
BPI	−1.57	−1.1 (*0.596*)	−1.5 (*0.151*)	1.27 (*0.400*)
DEFA1B	−2.3	−1.4 (*0.366*)	−2.7 (*0.067*)	1.9 (*0.170*)
ANPEP	−1.68	1.0 (*0.618*)	−1.1 (*0.220*)	1.1 (*0.065*)
ALPL	−1.7	−1.1 (*0.763*)	−1.25 (*0.035*)	1.3 (*0.120*)
CCD50	1.5	**1.5 (*0.005*)**	1.8 (*<0.001*)	−1.25 (*0.006*)
PADI2	−1.54	−1.1 (*0.631*)	−1.1 (*0.388*)	1.0 (*0.773*)
KLRB1	1.52	−1 (*0.184*)	1.6 (*<0.001*)	−1.6 (*0.001*)
CHI3L1	−1.59	−1.1 (*0.425*)	−1.0 (*0.809*)	−1.0 (*0.595*)
TNSF14	−1.51	1.1 (*0.157*)	−1.1 (*0.213*)	1.2 (*0.027*)

In the gray column, the fold change (FC) microarray values of differentially expressed genes (DEGs) in the remission state (PsA-R) vs. healthy condition (HC); in the green column, the same DEGs validated by the RT-qPCR technique [TaqMan chemistry, $2^{-\Delta\Delta Ct}$ method for relative quantification (RQ)]; in the white columns, the FC values of DEGs in the other comparisons. Differential analysis between groups (39 PsA-R vs. 40 HC vs. 40PsA-active) was made by fold change (FC) cutoff ±1.5 to estimate gene dysregulation (overexpressed ≥1.5; −1.5 ≥ downregulated); the p-value cutoff for significance is ≤0.05.

many cytokines such as IL-2 and IL-3, granulocyte–macrophage colony-stimulating factor, IL-4, IL-5, IL-13, IFNγ, TNFα, CD40L, FasL, CD5, Igκ, CD25, and the chemokines IL-8 and MIP1α (21). Furthermore, previous studies reported that AP-1 could affect the severity of inflammation through other mechanisms, including the regulation of naive T-cell differentiation into T helper 1 (Th1) or Th2 cells and modulation of the activity of the innate immune system (22–24).

Aside from its role in the inflammatory process, there is much evidence that *FOS* has an important role in the regulation of osteoclastogenesis by RANK ligand signaling (25, 26), which in turn is demonstrated to have a crucial role in developing joint/bone destructive lesions in inflammatory arthropathies, such as rheumatoid arthritis and psoriatic arthritis (27). After binding with its receptor, RANKL triggers a signaling cascade leading to the activation of key transcription factors such as NF-κB and Fos, leading to the expression of osteoclast-specific target genes (25, 26). In particular, activation of RANKL/Fos is required for the expression of Nuclear Factor for activation of T cells c1 (NFATc1) and interferon-b (IFN-b), two critical actors in

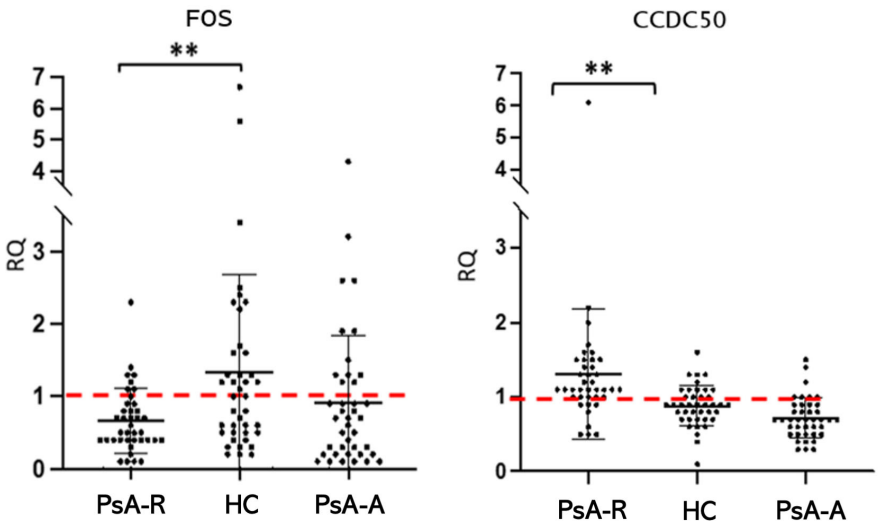

FIGURE 4
The *FOS* and *CCDC50* dysregulation in the PsA clinical remission. Mean (SD) of relative quantification (RQ) of *FOS* and *CCDC50* genes in the remission (PsA-R), active (PsA-A), and healthy condition (HC). Analysis by the $2^{-\Delta\Delta Ct}$ method. **p-value = 0.005.

osteoclast differentiation (28, 29). Notably, AP-1 activity can also affect the severity of primary arthritis with mechanisms different from the regulation of osteoclastogenesis, such as induction of MMP production (30).

A scarce amount of data is available on the potential role of *FOS* in PsA. Interestingly, data are available on rheumatoid arthritis (RA), where previous studies reported that Fos/AP-1 and interleukin 1β (IL-1β) influence each other's gene expression and activity, resulting in an orchestrated cross-talk that, in turn, seems to have an important role in the accrual of joint damage in experimental RA models characterized by the enhancement of Fos/AP-1 activity. For this purpose, Yukiko et al. designed and synthesized a selective inhibitor of Fos/AP-1 to resolve arthritis in a mouse model of the RA disease (31).

Our analysis shows a downregulation of *FOS* in remission and active PsA patients vs. healthy controls (remission < active < healthy), suggesting that such misregulation may occur to counterbalance its pro-inflammatory and pro-osteoclastogenic functions. The fact that *FOS* is slightly downregulated in PsA-A vs. HC could suggest that this mechanism is also established in patients with active disease, but not sufficiently to maintain homeostasis.

CCDC50 (alias *YMER*) encodes a soluble, cytoplasmic, tyrosine-phosphorylated protein with multiple ubiquitin-interacting domains that may be multifunctional in several signaling pathways (32). *CCDC50* overexpression attenuates NF-κB, a critical regulator of innate and adaptive immunity, in collaboration with A20 deubiquitinase (33), that, in turn, plays an important role in the termination of NF-κB signaling and the resolution of inflammation. In particular, *CCDC50* harbors a ubiquitin-binding domain (UBD) that may act as an adaptor molecule for A20, a mechanism important for NF-κB inhibition (33). In fact, the ubiquitin-modifying protein A20 is a broadly expressed cytoplasmic protein induced by TNFα stimulation, and it has been identified as an inhibitor of TNF-induced NF-κB activation or apoptosis. In the literature, it is established that A20 is a critical negative regulator of NF-κB (34), and A20-deficient cells fail to terminate TNF-induced NF-κB signaling (35). Furthermore, *CCDC50* was identified as a gene whose expression is highly decreased in osteoclastogenesis upon myostatin treatment *in vitro*. It could inhibit the function of myostatin in osteoclastogenesis by blocking NF-κB and MAPK pathways. In this model, overexpression of *CCDC50* diminishes NF-κB signaling, whereas knockdown of endogenous *CCDC50* upregulates NF-κB signaling, suggesting that CCD50 functions as a negative regulator for NF-κB signaling (36).

To our knowledge, no specific data are currently available on the role of *CCDC50* in PsA. In affected patients in clinical remission from our cohort, the upregulation of *CCDC50* could have a protective role, contributing to bone homeostasis recovery and avoiding the most aggressive disease outcome, such as articular erosion, by inhibiting the osteoclastogenesis process. This assumption is further supported by the demonstration that *CCDC50* is downregulated in active patients compared with those in clinical remission. Therapeutic strategies targeting *CCDC50* may be conducive to treating diseases related to its aberrant expression.

Taken together, the opposite function of the two inversely misregulated genes, *FOS* and *CCDC50*, in PsA patients in clinical remission would confirm that the former did not represent "a back to the healthy condition," but it is probably the result of a new balance between inhibition of pro-inflammatory and pro-osteoclastic processes and enhancement of protective mechanism against the same inflammatory and osteogenic phenomena. In particular, clinical remission might be molecularly driven by *FOS* gene downregulation, determining the minor activation of RANKL and subsequently slight osteoclastogenesis, and *CCDC50* overexpression, which imply major NF-κB inhibition and type I IFN pathway restriction. No data from the literature showed direct interactions between the products of these two genes; however, a cooperation cannot be excluded because of their involvement in the same downstream pathway. In this regard, bioinformatics tools such as the Gene Transcription Regulation Database (GTRD, website http://gtrd20-06.biouml.org/) allow us to hypothesize that the *CCDC50* gene sequence may be a possible target of the Fos transcription factor; however, further studies need to be conducted for this purpose.

The secondary analysis of our study comparing the remission with active disease, and the latter with healthy controls, showed that there was, as expected, a significant difference in terms of the transcriptomic profile also between TNFi-induced remission and the active disease, as well as between the active disease and the healthy condition. The DEG validation extended to these further comparisons showed the misregulation of *DEFA1B* and *KLRB1* (PsA-A vs. HC) and *BPI, CEACAM8, DEFA1B*, and *KLRB1* (PsA-R vs. PsA-A), respectively. However, these comparisons were not the primary objective of this study and deserve further, separate, in-depth investigation.

This work provides previous unreported information on the molecular characterization of the clinical remission in PsA, describing for the first time in this condition the dysregulation of two key genes notably involved in inflammatory and bone metabolism processes. These findings pave the way into a research field that is of clinical interest and provide data to the debate about considering remission as a condition with molecular disease inactivation leading to a "back to a healthy state." In the precision medicine era, more molecular data about PsA disease activity assessment are needed: in the near future, the biomarker discovery of a molecular remission state achievement, for a better and precise assessment of the actual major goal of PsA management, should be improved.

This study has some limitations. First, the enrolment of PsA patients in clinical remission solely induced by TNFi prevents a generalization of the results of the remission induced by other treatments. However, this methodological choice assumed that different treatments might induce different molecular mechanisms sustaining remission. Thus, for this reason, a homogeneous PsA cohort in remission with the most widely used first-line bioDMARD was selected (37). It is noteworthy that remission induced by TNFi is particularly consistent with the result of our study and corroborates their validity, as both *FOS* and *CCD50* are involved in the regulation of pathways where TNF has a key role. Second, in the validation phase of the study, only the coding

transcripts were evaluated, aiming to assess the interaction between gene products in existing biological pathways and processes. Finally, in the RT-qPCR validation phase, 12 coding genes out of 24 mRNAs were evaluated. Although this selection was based on previous *in-silico* investigations (GO gene set enrichment and STRING software) and an in-depth literature review, also the remaining genes potentially may have a role in sustaining the TNFi remission, deserving further research.

To date, no successful models of TNFi prediction in PsA are available clinically (38), and the research field of biomarker discovery related to molecular remission achievement is only at an early stage (39–41). Whole-blood transcriptomic profiling performed in this study suggests that TNFi-induced clinical remission in PsA is similar to a healthy condition, but not identical, differing for a list of 125 transcripts and particularly for the *FOS* and *CCDC50* gene expression amount. The molecular characterization of PsA disease activity may have a crucial role in identifying biological as well as clinical remission, favoring a more effective application of the prospective treat-to-target strategy.

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 local ethical committee (PG/2018/16313; 12th November 2018). 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

MMA: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Writing – original draft. AF: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. IC: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. MC: Data curation, Formal Analysis, Investigation, Writing – review & editing. EC: Data curation, Formal Analysis, Investigation, Writing – review & editing. MNR: Data curation, Formal Analysis, Investigation, Writing – review & editing. MP: Data curation, Formal Analysis, Investigation, Writing – review & editing. AC: Conceptualization, Data curation, Formal Analysis, Supervision, Validation, Writing – original draft.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1274539/full#supplementary-material

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Circulating exosomal microRNAs as biomarkers of lupus nephritis

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Objective: Disruption in the delicate symphony of genes, microRNA (miRNA), or protein expression can result in the dysregulation of the immune system, leading to the devastating consequences such as lupus nephritis (LN). The capacity of exosomes to transport miRNAs between cells and modify the phenotype of recipient cells implies their involvement in persistent kidney inflammation. This study unveils identifying two previously undiscovered exosomal miRNAs in the serum of LN patients, offering potential solutions to the current challenges in LN diagnosis and management.

Methods: Initially, we used a reagent-based kit to isolate serum exosomes from patients with Systemic lupus erythematosus (SLE) and used Trizol method for total RNA extraction. Subsequently, we employed small RNA sequencing to screen for differential expression profiles of exosomal small RNAs. The RT-qPCR method was used to individually validate samples in both the screening and validation cohorts, enabling the identification of candidate small RNAs; specific to LN. We assessed the diagnostic potency using receiver operating characteristic (ROC) curve, and explored the biological roles of miRNAs using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

Results: Compared to SLE patients without LN, SLE patients accompanied by LN exhibited significantly spiked levels of exosomal hsa-miR-4796-5p and hsa-miR-7974. The duo of miRNAs, hsa-miR-4796-5p and hsa-miR-7974, exhibited promising potential as biomarkers for diagnosing LN, with an AUC exceeding 0.8. Correlation analysis revealed a strong positive association between these miRNAs and proteinuria, as well as the SLE Disease Activity Index (SLEDAI) score. Moreover, the levels of two miRNAs in LN patients were significantly elevated in comparison to other autoimmune nephritis conditions, such as immunoglobulin A nephropathy (IgAN) and diabetic nephropathy (DN). Furthermore, the bioinformatics analysis indicated that this miRNAs duo can play a pivotal role in the regulation of immune processes by modulating signal pathways, such as the mTOR and PI3K-Akt signaling pathway.

Conclusion: This study provides a new ground that serum exosomal miRNAs can effectively identify and predict LN in SLE patients.

KEYWORDS

systemic lupus erythematosus, lupus nephritis, exosomes, miRNA, biomarkers

Introduction

Systemic lupus erythematosus (SLE) continues to pose significant challenges within the realm of medicine, causing characteristic blend of systemic and organ-specific clinical manifestations, coupled with extensive dysfunction of the immune system (1, 2). LN stands out to be one of the most severe organic manifestations of SLE, affecting approximately 30-60% of adults and up to 70% of pediatric lupus patients (3). Furthermore, it is noteworthy that LN contributes significantly to the elevated incidence of SLE, heightened mortality rates, and increased healthcare expenditures (4). According to the guidelines, a reliable criterion for diagnosing LN is the histopathological confirmation obtained through renal biopsy (5, 6). However, kidney biopsy is an invasive procedure associated with the risk of bleeding and is not easily repeatable. Consequently, it poses limitations to rheumatology and immunology physicians in their ability to dynamically monitor and manage the disease progression of SLE. Currently, commonly used laboratory markers for LN include urinary protein, serum creatinine, glomerular filtration rate, anti-dsDNA antibody, and serum complements (7). However, these clinical parameters fall short of meeting the practical demands of clinical settings due to their insufficient sensitivity and specificity (8, 9). Therefore, it is crucial to discover novel non-invasive markers capable of detecting LN activity, predicting relapses, and monitoring treatment responses.

MicroRNAs (miRNAs), tiny non-coding RNAs (18-25 nucleotides) that regulate gene expression by binding to messenger RNA (mRNA), play a crucial role in cell biology and disease (10). This interaction prompts the restraint of mRNA translation and/or hastens its degradation, thus culminating in the curtailment of protein synthesis specific to certain target proteins (11, 12). In 2008, Chen Xi et al. (13) made the initial discovery of miRNA in human serum and provided evidence that it could serve as a new disease marker. In recent years, mounting evidence suggests the involvement of miRNA in the occurrence and development of various diseases, such as Alzheimer's disease (AD), cancers, diabetes and autoimmune diseases (14–17). Extracellular vesicles(EVs) are membrane vesicles released by various cell types and can be categorized into three types: exosomes, microvesicles, and apoptotic bodies (18). Numerous studies have demonstrated that EVs play a significant role in the development of autoimmune diseases through various mechanisms (19). Microvesicles (MVs) are larger membrane vesicles derived from the cell plasma membrane surface. They can carry nuclear autoantigens and form immune complexes (ICs), which activate complements and cause damage to renal tissue (20). Nielsen CT et al. utilized immune electron microscopy technology to provide evidence of co-localization between glomerular deposited immune complexes (ICs) and microvesicles, as well as galectin-3-binding protein (G3BP) in LN (21). Exosomes, which are generated through the exocytosis of endosomal-derived intracellular membrane vesicles into the extracellular space (22), were initially discovered by Johnstone and colleagues in 1983 during the culture of reticulocytes (23). Previous studies have found that exosomes may contribute to a proinflammatory milieu and autoimmune inflammation in LN indirectly, either by directly interacting with their associated proinflammatory components or by triggering other cells to produce proinflammatory cytokines or materials (19, 24). The dysregulation of circulating exosomal miRNAs in autoimmune diseases has been extensively studied, and there is increasing evidence confirming their involvement (25, 26). However, the diagnostic potential of serum exosomal miRNAs in LN has not yet been fully explored.

In this study, our objective was to investigate the differential expression of miRNAs in exosomes derived from the serum of patients with LN. We employed RNA sequencing to screen for differential expression profiles of exosomal small RNAs. Subsequently, we identified the top ten upregulated miRNAs as potential candidate miRNAs. The RT-qPCR method was employed in both the screening and validation cohorts, facilitating the identification of candidate miRNAs specific to LN. To evaluate their potential as biomarkers, we conducted receiver-operator characteristic (ROC) curve analysis. Furthermore, we utilized bioinformatics tools including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to predict the target genes of these miRNAs and explore their potential functions and associated pathways. Our meticulous analysis revealed that serum exosomal miRNAs can be utilized as non-invasive biomarkers for the identification and prediction of LN in individuals with SLE.

Materials and methods

Subjects and study design

This study enrolled 232 subjects from September 2022 to March 2023, including 116 patients with LN and 116 sex-age-matched patients with SLE without LN. All participants were hospitalized

at Drum Tower of Nanjing Hospital and diagnosed by rheumatologists. The inclusion criteria for participants with SLE were based on the modified American College of Rheumatology 1997 revised criteria (27, 28). Individuals with a history of malignant tumors, concurrent infections, metabolic abnormalities, or concurrent other autoimmune diseases were excluded from the study cohort. The subjects were divided into three phases (Figure 1). During the discovery phase, we conducted miRNA sequencing by extracting exosomes from a 10 ml serum pool consisting of 20 LN patients and 20 SLE without LN patients (GEO number: GSE179950). In the training and validation phase, the expression levels of candidate miRNAs were validated in 192 samples using RT-qPCR assay. The activity of the disease was evaluated by the SLE Disease Activity Index (SLEDAI) (29). Approval to conduct this study was provided by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School, with the assigned approval identification number 2020-327-01. Prior to their participation in this experiment, all individuals involved provided written informed consent. The clinical and demographic characteristics of all participants are presented in detail in Table 1.

Library preparation and RNA sequencing

Prior to sequencing, the integrity of RNA samples was validated using agarose gel electrophoresis. The quantification of the RNA samples was achieved using the NanoDrop ND-1000 instrument. To account for the heavy decoration of miRNAs with RNA modifications, several treatments were conducted before library construction. These treatments included 3′-aminoacyl deacylation to 3′-OH for 3′-adaptor ligation, removal of 3′-cP to 3′-OH for 3′-adaptor ligation, 5′-OH phosphorylation to 5′-P for 5′-adaptor ligation, and demethylation of m1A and m3C. The library construction and deep sequencing were carried out by BGI (Shenzhen, China) using the Illumina Next Seq instrument. The sequencing libraries were optimized for RNA biotypes and validated using the Agilent 2100 Bioanalyzer. The sequencing process consisted of 50 cycles.

Exosomes isolation

The serum samples were centrifuged at 2000 g for 30 min to separate cells and debris. Afterwards, they were stored at -80°C and thawed only when needed for use. Exosomes were isolated from serum using a commercial kit named as Total Exosome Isolation reagent (Thermo Fisher scientific, US) according to the manufacturer's instructions. Briefly, a 100 µL serum sample was mixed with 20 µL of kit reagent and incubated at 4°C for 24 h. Then, the sample was centrifuged at 4°C at 10000 g for 10 min. Discarded the supernatant while the exosome pellets were resuspended in 100 µL of 1×phosphate buffer saline (PBS) for further analysis.

Exosomes sizing

For measuring the size, exosomes were first diluted in 1 ml of PBS. The mixture was gently inverted to ensure even distribution and then slowly added to the NTA sample cell using a 1 ml disposable syringe. Three sample videos, each lasting 30 to 60 seconds, were recorded. The ZetaView Nanoparticle Tracking Analysis (NTA) system was utilized to measure the average diameter of the exosomes. This system has the capability to

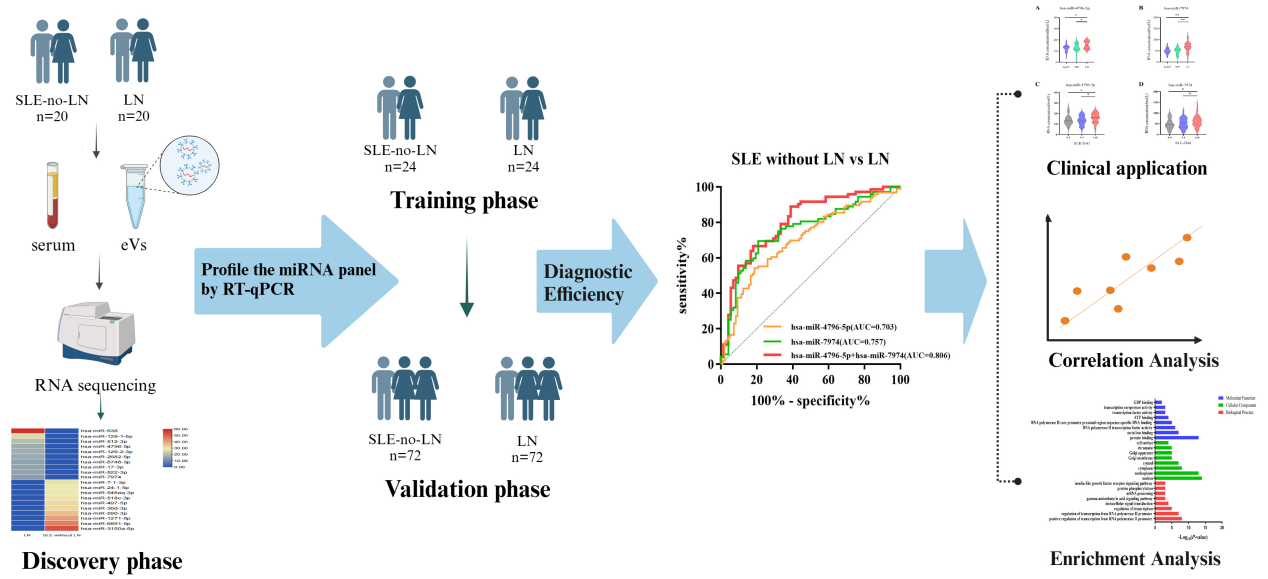

FIGURE 1
Workflow of the study.

TABLE 1 Clinical and demographic characteristics of all participants.

Characteristics	SLE without LN (n=116)	LN (n=116)	*P* value
Age, years	40(29-51)	35(27-47)	0.158
Female, n (%)	97(89.81)	93(86.11)	0.365
Proteinuria, n (%)	34(31.48)	98(90.74)	<0.001***
Hematuria, n (%)	19(17.59)	75(69.44)	<0.001***
Pyuria, n (%)	27(25.00)	50(46.30)	0.003**
Cylinderuria, n (%)	0(0)	42(38.89)	<0.001***
24h proteinuria, median (IQR), mg/24h	137.0(67.0-201.0)	2069.0(699.5-5489.7)	<0.001***
ACR, median (IQR), mg/g	9.2(5.6-24.3)	789.3(189.9-2308.1)	<0.001***
ESR, median (IQR), mm/h	22(10-42)	32(16-57)	0.003**
Total protein, median (IQR), g/L	66.2(63.3-72.5)	55.0(48.8-64.3)	<0.001***
Blood albumin, median (IQR), g/L	37.9(35.4-40.6)	31.9(28.6-36.7)	<0.001***
Globulin, median (IQR), g/L	29.3(25.2-33.8)	22.7(18.4-28.6)	<0.001***
A/G, median (IQR)	1.29(1.12-1.54)	1.39(1.15-1.68)	0.056
GLU, median (IQR), mmol/L	4.46(4.11-4.84)	4.37(3.90-4.86)	0.111
Urea nitrogen, median (IQR), mmol/L	4.9(3.9-6.0)	7.4(5.2-11.5)	<0.001***
CREA, median (IQR), umol/L	49(41-56)	61(47-88)	<0.001***
Uric acid, median (IQR), umol/L	293(208-359)	391(296-452)	<0.001***
Total CO2, median (IQR), mmol/L	24.3(23.0-25.3)	23.6(21.1-25.2)	0.008**
eGFR, median (IQR), ml/min/1.73m^2	132.7(110.6-164.9)	103.5(75.2-145.0)	<0.001***
C1q, median (IQR), mg/dL	15.9(13.7-18.4)	13.2(11.2-17.0)	0.007**
C3, median (IQR), g/L	0.82(0.62-1.01)	0.76(0.47-0.96)	0.041*
C4, median (IQR), g/L	0.12(0.07-0.19)	0.13(0.05-0.19)	0.702
anti-dsDNA, median (IQR), IU/mL	87.43(18.40-253.71)	112.31(19.74-457.58)	0.392
ANA, n (%)	88(81.48)	86(79.63)	0.693
25-(OH) D3, median (IQR), ng/mL	18.50(13.36-24.29)	14.06(8.76-28.19)	0.051
SLE-DAI, median (IQR)	4(2-5)	12(8-15)	<0.001***

Abbreviations: ACR, albumin-to-creatinine ratio; ESR, erythrocyte sedimentation rate; GLU, glucose; CREA, serum creatinine; eGFR, glomerular filtration rate; C1q, complement 1q; C3, complement C3; C4, complement C4; anti-dsDNA, anti-double stranded DNA antibody; ANA, antinuclear antibodies; SLE-DAI, systemic lupus erythematosus disease activity index. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney U test).

characterize the size distribution of small particles in liquid samples and can detect diameters ranging from 20 to 1000 nm (30). The results obtained from processing the Software ZetaView (Zeta View 8.04.02) were expressed as the mean standard deviation (SD) of the three video recordings.

Transmission electron microscopy

A total of 20 µL of exosomes were applied onto the copper grid of the electron microscope and allowed to sit at room temperature for 10 min. Subsequently, 20 µL of 2% phosphotungstic acid was added to the copper grid for negative staining, which was carried out for 10 min. Excess staining was removed using filter paper. Once the copper grid was dried, transmission electron microscopy (TEM) was performed to examine the exosomes. The test was conducted under the at 120KV. A bilayer membrane structure was selected, and particles with diameters ranging from 100 to 200 nm were captured in the photographs.

Western blotting

The exosomes obtained from serum were treated with a lysis solution containing RIPA lysis buffer. After 30 min of lysis on ice, the mixture was centrifuged at 4°C at 12000 g for 10 min. The resulting supernatant was collected, and the protein concentration in exosomes was measured using the Micro BCA protein detection kit (Thermo Fisher Scientific, California, USA). The remaining proteins were added to 5 × SDS loading buffer and heated at 99°C for 5 min. Subsequently, 20 µg of protein was loaded onto a 0.2 µm PVDF

membrane following the manufacturer's protocol. The loading process involved applying a voltage of 80 V for 30 min, followed by 120 V for 1 h. The membranes were then blocked with 5% skim milk at room temperature for 1 h and incubated overnight at 4°C with primary antibodies targeting CD63, TSG101, and Calnexin (Abcam, UK) (21). They were washed four times with 1× TBST for 15 minutes each and incubated with the appropriate secondary antibody for 1 h at room temperature. Finally, the membranes were washed three times with TBST for 10 min each, the ECL exposure solution was applied to the film, and pictures were taken.

RNA extraction and RT-qPCR

Total RNAs were extracted from serum exosomes using the Trizol reagent (Invitrogen, USA) and were dissolved in water treated by diethylpyrocarbonate (DEPC). Briefly, Trizol is a phenol-guanidine isothiocyanate solution that effectively lyses biological material and denatures proteins (31). It is designed to preserve RNA integrity. After adding chloroform and separating the phases, proteins are extracted in the organic phase, DNA is separated at the interface, and RNA is left in the aqueous phase. The aqueous phase can be carefully aspirated and isopropanol can be added to precipitate the RNA. The content and purity of acquired RNA were detected by OneDrop-2000 (Nano Drop Technologies) miRNAs were reverse transcribed into cDNA using miRNA 1st Strand cDNA Synthesis Kit (Vazyme Biotech). The quantitative RT-qPCR reaction was performed in 96-well plates with the miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech). The primer details for the 10 candidate miRNAs can be found in Supplementary Table 1.

Pathway analysis

For a more in-depth insight into the function of the target genes of miRNAs, we conducted gene ontology (GO) and annotations Kyoto Encyclopedia of Genes and Genomes (KEGG). Predictions of miRNAs for LN were performed using the DAVID database (http://david.ncifcrf.gov/), which integrates biological data and analysis tools to offer detailed information on the functional annotations of genes/proteins. To be significant the P-value ($P < 0.05$) was considered.

Statistical analysis

Continuous variables were represented as the median (interquartile range [IQR]) based on the normality test. The Spearman rank test was used to compare miRNA expression and clinical variables between two groups. Receiver operator characteristic (ROC) analysis was performed to calculate the area under the ROC curves (AUC), which evaluated the diagnostic efficiency of the candidate miRNAs. Statistical analyses were conducted using SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA), and GraphPad Prism version 9 (GraphPad Software Inc., La Jolla, CA, USA). The Mann-Whitney U test ($^*P < 0.05$) was used to determine statistical significance. SPSS binary logistic regression was employed to predict the probability of joint diagnosis. The correlation analysis data were analyzed using Spearman rank correlation analysis.

Data and materials availability

miRNA sequencing data, deposited in GEO under GSE179950. The raw data is available upon request from the corresponding author, providing a valuable resource for further research.

Results

Identification and characterization of serum exosomes

The morphology of serum exosomes was verified using TEM and the particle size using NTA. The results showed the presence of elliptical or bowl-shaped particles with an average diameter of 127 nm (Figures 2A, B). Western blot analysis also revealed the presence of typical EV proteins such as CD63 and TSG101 and absence of calnexin (Figure 2C). These findings demonstrate the existence of exosomes in serum, providing a basis for further investigation.

miRNA profiles of serum exosomes from LN patients

To determine the miRNA profile in LN patients, all participants were divided into two groups according to the clinical standard, including SLE with LN and SLE without LN. Exosomes were extracted from the mixed serum of 20 LN patients and 20 SLE patients without LN, and miRNA sequencing was performed. The distribution characteristics and Venn analyses of miRNA revealed a difference between LN and SLE without LN (Figures 3A, B). In comparison to SLE patients without LN, 382 upregulated and 350 downregulated miRNAs were observed in LN, which met the criteria for sequencing detection of log2 fold-change > 2 in scatter plot analysis (Figure 3C). As shown in Figure 3D, the top 10 upregulated miRNAs visualized via hierarchical clustering were investigated as candidate markers of LN.

Validation of RNA sequencing by RT-qPCR in the training phase

To verify the results of sequencing, a total of 24 pairs of subjects (24 LN and 24 SLE without LN patients) were recruited in the training phase, employing the RT-qPCR method. To assess the reliability and repeatability of this method, a standard curve of different concentrations of synthetic miRNA was constructed to determine whether there was a linear relationship between 1 pmol/L and 10 nmol/L (Figure 4A). Out of the top ten upregulated miRNAs, five miRNAs were successfully amplified using the RT-qPCR method (Figures 4B–F). According to Figure 4, the absolute quantitative results indicate a significant increase in three serum

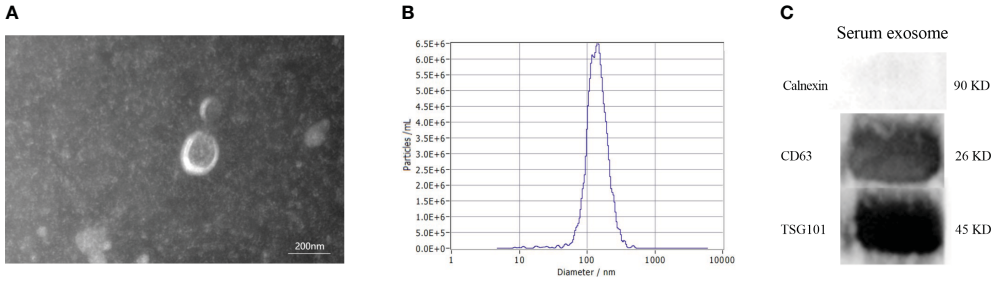

FIGURE 2
Characteristics of serum exosomes. **(A)** Morphology of vesicles under transmission electron microscopy. **(B)** Particle size distribution of vesicles measured by Nanoparticle Tracking Analysis. **(C)** Western blotting revealed the presence of exosomal biomarkers such as CD63, and TSG101.

exosomal miRNAs (hsa-miR-638, hsa-miR-4796-5p, and hsa-miR-7974) in LN patients compared to SLE patients without LN.

Validation of RNA sequencing by RT-qPCR in the validation phase

To further verify the above results, 144 subjects (72 LN and 72 SLE without LN patients) were recruited in the validation phase. According to Figures 5A-C, hsa-miR-4796-5p and hsa-miR-7974 are significantly elevated in LN compared with SLE without LN, while there is no difference in the level of hsa-miR-638 between the two cohorts. The diagnostic performance of two miRNAs, hsa-miR-4796-5p and hsa-miR-7974, was evaluated using ROC analysis. The area under the curve (AUC) values for hsa-miR-4796-5p and hsa-miR-7974 were 0.703 (95% CI: 0.6287-0.7767) and 0.757 (95% CI: 0.6766-0.8373), respectively. These AUC values were obtained for distinguishing between LN and SLE patients without LN, as shown in Figures 5D, E. The AUC for the panel of these two miRNAs combined was 0.806 (95% CI: 0.7348 to 0.8775), as demonstrated in Figure 5F. Serological indicators, such as C1q and CREA, have been found to be closely associated with renal damage caused by SLE.

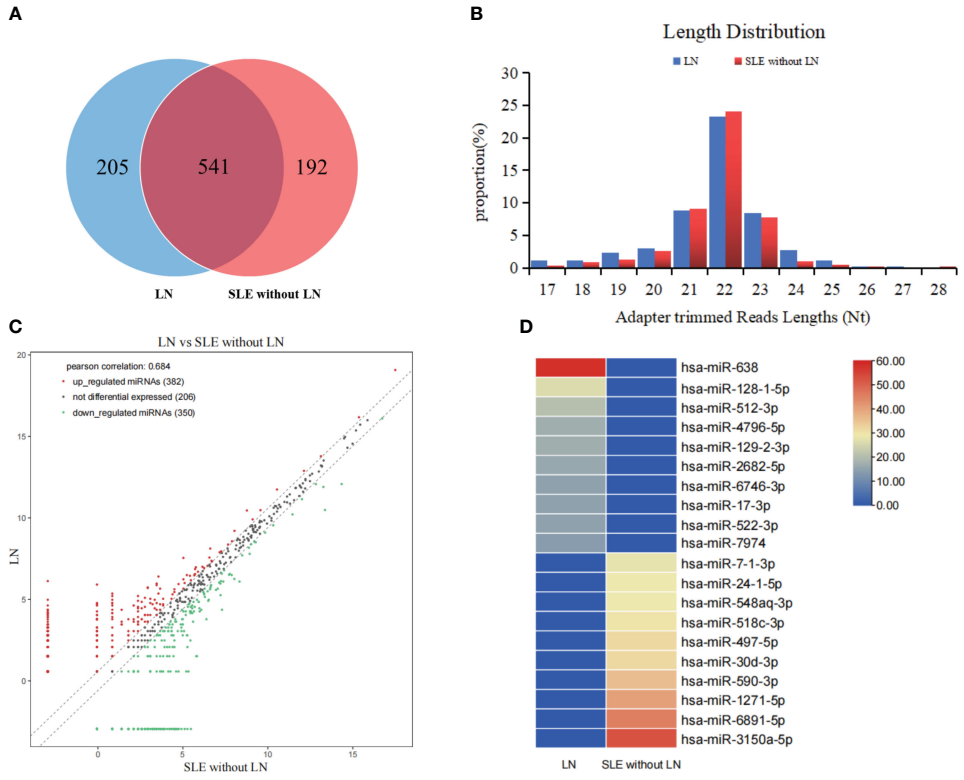

FIGURE 3
Analysis of differentially expressed miRNAs in serum exosomes of LN patients. **(A)** Venn diagram of serum exosomes derived miRNAs in LNs and SLE without LN patients. **(B)** The profiles of various length of miRNAs in serum exosomes between the two cohorts. **(C)** Scatter plots of differentially expressed miRNAs. Red and green dots indicated upregulated and downregulated miRNAs (log2 fold change > 2 between the two compared cohorts), and black dots indicated non-differentially expressed miRNAs. **(D)** Hierarchical clustering indicated the profiles of top 10 upregulated and downregulated miRNAs between two cohorts.

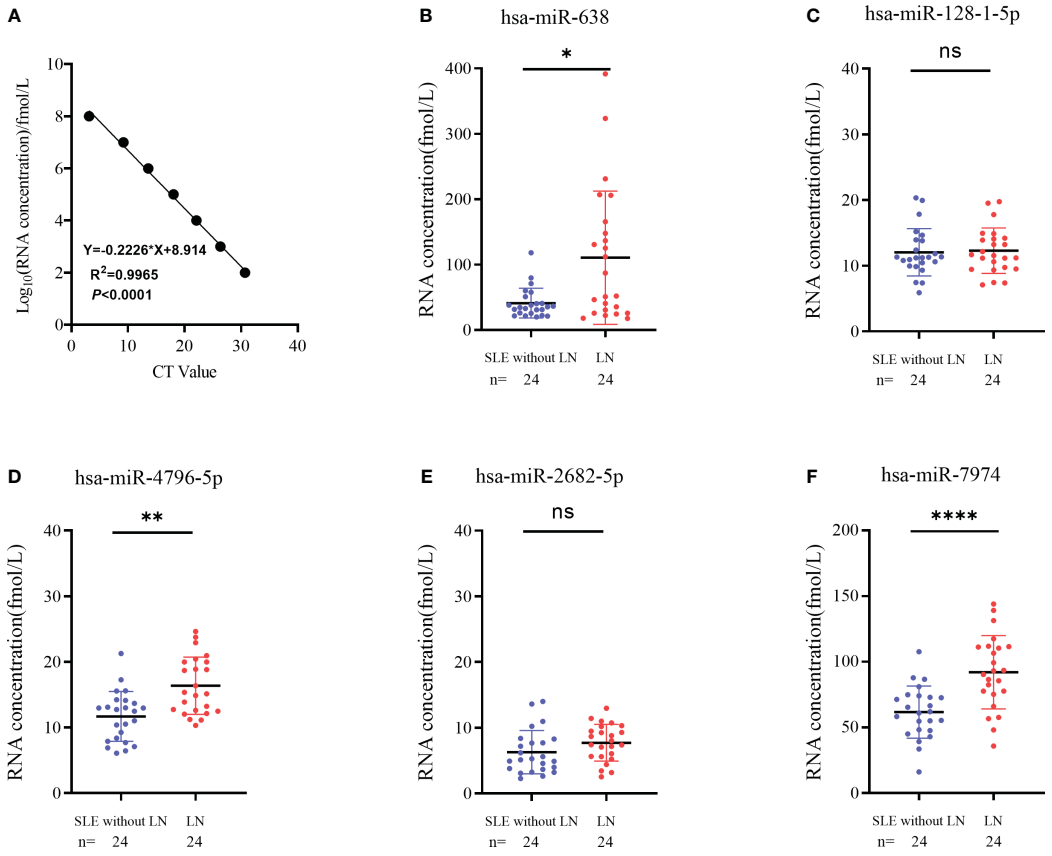

FIGURE 4
Identification of differentially expressed serum exosomes derived miRNAs in LNs and SLE without LN patients. **(A)** Linear standard curve of serum exosomes derived miRNAs concentration. **(B–F)** Differential expression of 5 miRNAs verified by RT-qPCR in LN and SLE without LN patients. hsa-miR-638, hsa-miR-4796-5p and hsa-miR-7974 were significantly upregulated in LNs compared with SLE without LN patients. *P* value of the Mann-Whitney U test: (*P < 0.05, **P < 0.01, ****P < 0.0001; ns, no significant difference).

However, our study suggests that their diagnostic value is limited. In order to improve the accuracy of predictions, we evaluated the combined use of these clinical indicators with a panel of two miRNAs. Encouragingly, the AUCs for the combined approach were 0.837 and 0.844, respectively (Figures 5G–J). Furthermore, our findings revealed a positive correlation between levels of hsa-miR-4796-5p and hsa-miR-7974 with markers such as 24-hour proteinuria, ACR, creatinine, urea nitrogen, and SLEDAI. Conversely, we observed a negative correlation with albumin (Alb), C1q, and vitamin D3 levels (Figure 5K, Supplementary Table 2).

Significance of hsa-miR-4796-5p and hsa-miR-7974 in clinical practice

Autoimmune nephritis encompasses several types of kidney diseases, including immunoglobulin A nephropathy (IgAN), diabetic nephropathy (DN), anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis (GN), Henoch-Schonlein purpura nephritis (HSPN), and LN. In our hospital, the most prevalent types of autoimmune nephritis among patients are IgAN, DN, and LN. To investigate the specificity of miRNAs in diagnosing LN, we conducted experiments to evaluate the effectiveness of candidate exosomal miRNAs. A total of 20 patients with LN, 20 with IgAN, and 20 with DN were randomly enrolled in our study. Our findings revealed significantly higher levels of hsa-miR-4796-5p and hsa-miR-7974 in LN patients compared to those with IgAN and DN. (Figures 6A, B). Our findings reveal a noteworthy correlation between the levels of two specific miRNAs, hsa-miR-4796-5p and hsa-miR-7974, and the SLEDAI score. The correlation coefficients (r) and *p*-values for hsa-miR-4796-5p and hsa-miR-7974 are 0.423 (p=0.003) and 0.398 (p=0.005), respectively (Supplementary Table S2). Notably, the high scoring group exhibited a substantial disparity in miRNA levels when compared to the low scoring group. (Figures 6C, D).

Enrichment analysis of hsa-miR-4796-5p and hsa-miR-7974

To explore the potential functions of hsa-miR-4796-5p and hsa-miR-7974, we performed KEGG pathway enrichment analysis and Gene Ontology analysis. The KEGG results indicated that hsa-miR-4796-5p was associated with HSV-1 infection, the MAPK signaling pathway, and the mTOR signaling pathway (Figure 7A). The GO project of hsa-miR-4796-5p involves intracellular signal

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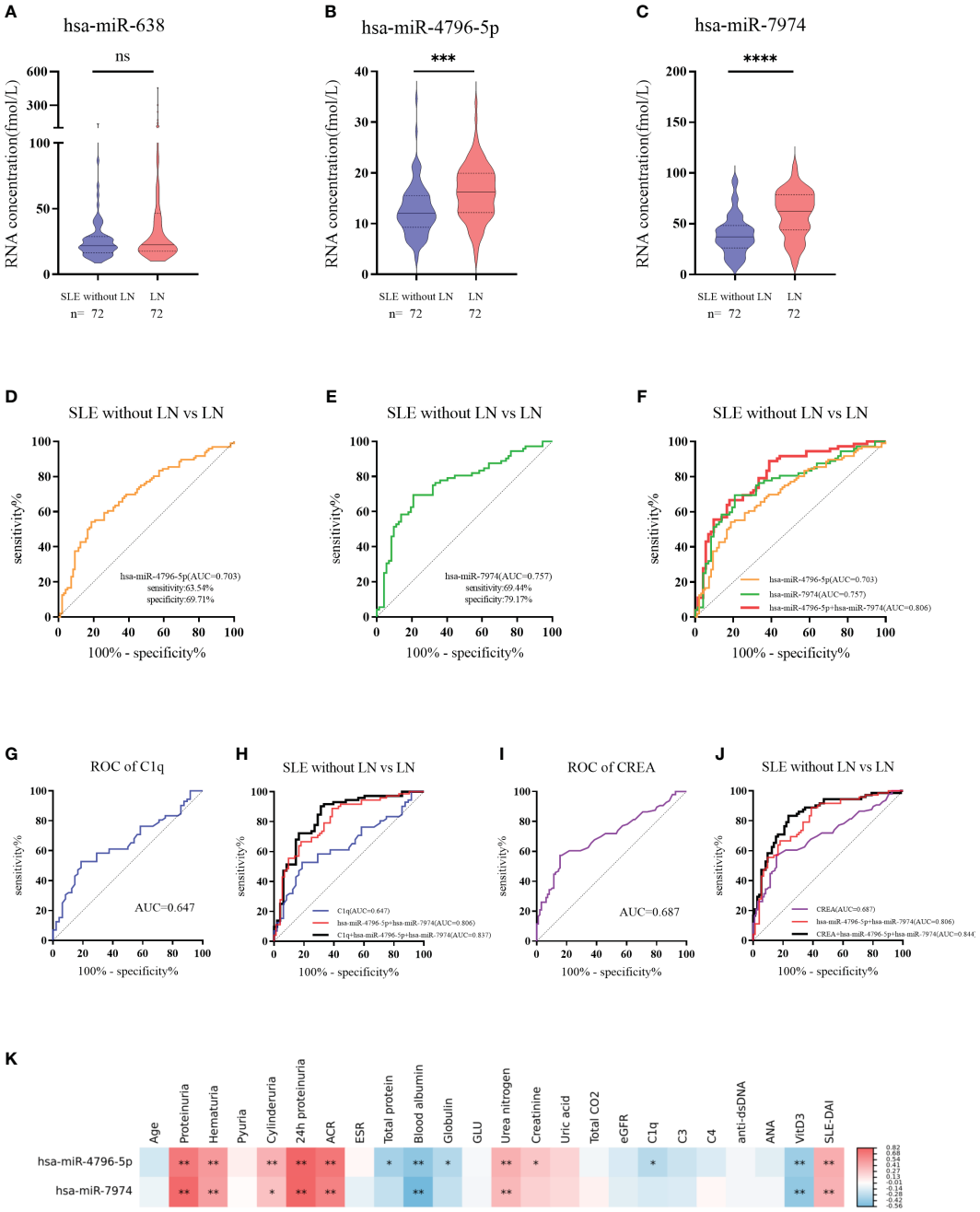

FIGURE 5
Diagnostic value of serum exosomes derived miRNAs in the validation phase. **(A–C)** Expression of 3 miRNAs in LNs compared with SLE without LN patients in large Samples. *P* value of the Mann-Whitney U test: (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; ns, no significant difference) **(D)** Receiver operator characteristic (ROC) curve of hsa-miR-4796-5p in distinguishing LNs from SLE without LN patients. **(E)** Receiver operator characteristic (ROC) curve of hsa-miR-7974 in distinguishing LNs from SLE without LN patients. **(F)** ROC combined diagnostic analyses of hsa-miR-4796-5p and hsa-miR-7974 in discriminating LNs from SLE without LN patients. **(G, H)** ROC curve analysis of a single C1q marker and the combination of C1q with hsa-miR-4796-5p and hsa-miR-7974 markers for distinguishing LNs from SLE without LN patients. **(I, J)** ROC curve analysis of a single CREA marker and the combination of CREA with hsa-miR-4796-5p and hsa-miR-7974 markers for distinguishing LNs from SLE without LN patients. **(K)** Correlation analyses between miRNAs and clinical variables. Red and blue color represent the positive correlation and negative correlation, and the depth of the color represents the degree of correlation. The presence of * indicates an absolute value of the correlation coefficient r >0.3 (*$P < 0.05$, **$P < 0.01$ Spearman rank correlation analysis).

transduction, insulin-like growth factor receptor signaling pathway, protein phosphorylation, and other related processes (Figure 7B). KEGG pathway enrichment analysis of hsa-miR-7974 revealed the involvement of miRNAs in the MAPK pathway, PI3K-Akt signaling pathway, and endocytosis (Figure 7C). The analysis also identified enrichment of GO terms targeted by hsa-miR-7974, such as cellular responses to signal transduction, endocytosis, protein trafficking, and other GO strains (Figure 7D). These results serve as a reminder that miRNA may play an important role in the development of LN by impacting these functions.

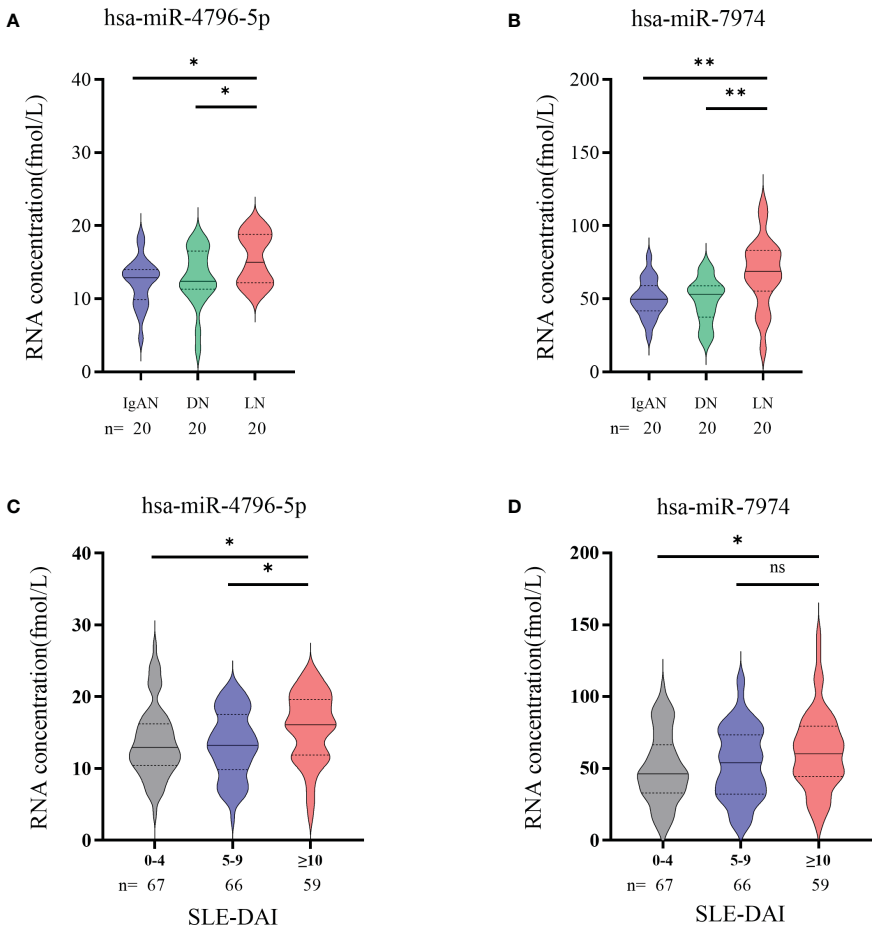

FIGURE 6
Significance of hsa-miR-4796-5p and hsa-miR-7974 in clinical practice. **(A, B)** Differential expression analysis of two miRNAs in different autoimmune nephritis diseases. Statistical significance was determined by the Mann-Whitney U test (*$P < 0.05$, **$P < 0.01$; ns, no significant difference). **(C, D)** Differential expression analysis of 2miRNAs in different LN severity groups. (0-4, no activity; 5-9, mild activity; ≥10, moderate to severe activity).

Discussion

LN is a severe manifestation of SLE, with approximately 40% of patients developing chronic kidney disease and 5-20% progressing to end-stage kidney disease (ESKD) within 10 years of their initial SLE diagnosis (32). Additionally, patients undergoing immunosuppressive treatment for LN may experience various complications such as infection, osteoporosis, cardiovascular issues, and reproductive system problems (4). Clearly, timely diagnosis and accurate evaluation of LN are essential for enhancing outcomes in SLE patients. The commonly used diagnostic methods for LN in clinical practice include 24-hour proteinuria quantification and kidney biopsy. However, these methods have certain limitations. Urine samples may have inaccurate retention time, and there may be poor patient compliance for urine protein testing. Additionally, kidney biopsy, although considered a valuable diagnostic tool, is an invasive procedure that carries the risk of bleeding and is challenging to replicate. Consequently, there is an immediate priority to investigate new non-invasive biomarkers that can effectively differentiate between LN and SLE.

Liquid biopsy is an innovative diagnostic method for analyzing biological material in blood and other bodily fluids to identify disease status (33). In recent years, the detection of miRNAs has been increasingly utilized for studying various autoimmune diseases. MiRNA, a small single-stranded endogenous non-coding RNA, has the remarkable capability to efficiently and precisely suppress the expression of its targeted transcripts, leading to alterations in cellular epigenetics and playing a vital regulatory role in both the innate and adaptive immune systems (34, 35). Current research on miRNAs in SLE primarily focuses on serum miRNAs and PBMC miRNAs, with limited studies on exosomal miRNAs (36, 37). Exosomes, a specific type of extracellular vesicles (EVs), have been identified in majority of body fluids (38–42). Composed of a lipid bilayer, the extracellular surface of exosomes serves as a protective barrier, safeguarding their contents such as proteins, mRNA, miRNAs, and other non-coding RNAs (ncRNAs) from degradation (18). When exosomes circulate, the RNA molecules they contain, especially miRNA, play a vital role in facilitating communication between different tissues through paracrine and endocrine pathways (43). Due to their excellent

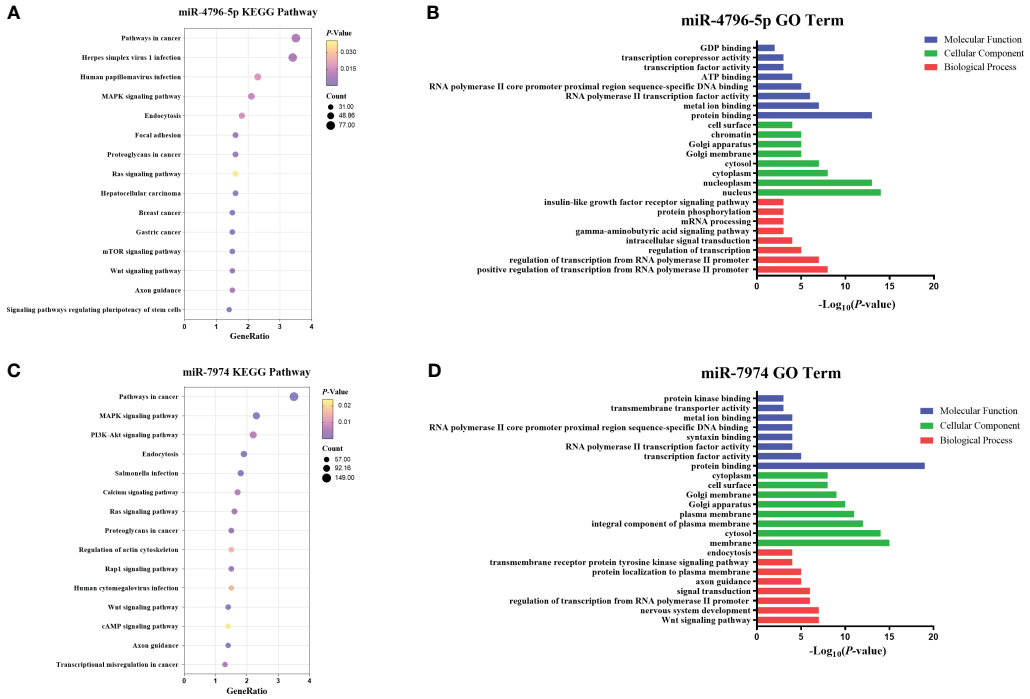

FIGURE 7
Enrichment analysis of hsa-miR-4796-5p and hsa-miR-7974. **(A, B)** KEGG pathway enrichment analysis and GO terms analysis of hsa-miR-4796-5p. **(C, D)** KEGG pathway enrichment analysis and GO terms analysis of hsa-miR-7974. The size of bubbles represents the number of genes enriched in this pathway, and the color of bubbles represents significance. P value represents the significance degree of enrichment.

stability and accessibility, exosomal miRNAs hold potential as non-invasive biomarkers (44).

Our previous research has identified upregulated tsRNAs in the urine exosomes of patients with LN, which have shown promise in distinguishing LN from SLE (45). In this study, we aimed to analyze the levels of circulating exosomal miRNAs in serum to investigate their potential significance in LN. Previous reports have suggested that there may be differences in the miRNA content between blood plasma and serum (46, 47). Liu et al. (48) recommended the use of blood plasma in exosome research, as platelets also contain significant amounts of RNA that could be released into the serum during the coagulation process. However, a recent study (49) discovered that plasma prepared by centrifugation contains platelets and ery-ghosts, which co-isolate with EVs. In this study, we initially conducted RNA sequencing using serum exosomes from patients with LN. We compared the results to SLE patients without LN and identified 382 upregulated miRNAs in LN. Then, we selected the top 10 upregulated miRNAs as potential markers of LN. To validate the expression levels of these candidate exosomal-miRNAs, we used an RT-qPCR assay, which is more sensitive and not limited by sequence-abundance bias compared to the microarray profiling assay (50). Our data demonstrated that the levels of exosomal hsa-miR-4796-5p and hsa-miR-7974 were significantly elevated in patients with LN compared to SLE patients without LN in both the training and validation phases. These two miRNAs demonstrated a significant ability in diagnosing LN in patients with SLE, with an Area Under the Curve (AUC) above 0.8. In order to demonstrate higher diagnostic value, we formed a comprehensive team to integrate these two miRNAs with clinical parameters (such as C1q and CREA). The area under curve (AUC) values for these miRNAs were found to be 0.837 and 0.844, respectively. The SLE Disease Activity Index (SLEDAI) is an important tool for evaluating SLE activity based on clinical symptoms and auxiliary examinations. After scoring the 192 enrolled patients, we observed a significant upregulation of these two miRNAs in moderate to severe cases. Therefore, the exosomal hsa-miR-4796-5p and hsa-miR-7974 in serum have the potential to serve as biomarkers for evaluating disease activity.

In order to assess the specificity of these miRNAs duo in LN, we conducted separate experiments in immunoglobulin A nephropathy (IgAN) and diabetic nephropathy (DN). Our findings revealed that the levels of these two miRNAs were significantly elevated in LN compared to IgAN and DN. Therefore, these miRNAs not only serve as potential biomarkers to distinguish LN in SLE, but also exhibit good specificity in other autoimmune kidney diseases. The KEGG results revealed that hsa-miR-4796-5p and hsa-miR-7974 were found to be associated with various signaling pathways, such as the mTOR signaling pathway and PI3K-Akt signaling pathway which is implicated in the pathogenesis of LN (51–53). GO analysis showed that hsa-miR-4796-5p is enriched in insulin-like growth factor binding, which influences autoimmunity by modulating signaling pathways relevant to Th17/Treg balance (54). These results suggests that miRNAs investigated in this study may play a regulatory role in the progression of LN by engaging in these signaling pathways.

To recap, we have identified two serum exosomal miRNAs signatures in patients with LN. Specifically, we have demonstrated that hsa-miR-4796-5p and hsa-miR-7974, which are derived from serum exosomes, have the potential to be valuable biomarkers for differentiating between LN and SLE patients, as well as the autoimmune nephritis group. Furthermore, our study has shed light on the potential biological functions of these novel serum exosomal miRNAs. These findings provide a foundation for future research to explore the clinical applications and deeper understanding of serum miRNAs. However, it is important to note that our study was limited by the small sample size and single-center experience. Therefore, randomized clinical trials are the next frontier in evaluating these two miRNA signatures for LN diagnosis and prognosis.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA745976.

Ethics statement

The studies involving humans were approved by The Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. 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

FC: Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft. BS: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. WL: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – original draft. JGo: Data curation, Methodology, Writing – original draft. JGa: Data curation, Investigation, Methodology, Writing – original draft. YS: Methodology, Writing – original draft. PY: Funding acquisition, Project administration, Supervision, Writing – review & editing. ZL: Funding acquisition, Resources, Supervision, Conceptualization, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1326836/full#supplementary-material

SUPPLEMENTARY TABLE 1
Specific primer information for 10 candidate miRNAs.

SUPPLEMENTARY TABLE 2
Correlation analysis between levels of hsa-miR-4796-5p and hsa-miR-7974 with laboratory markers and SLE-DAI. ACR, albumin-to-creatinine ratio; ESR, erythrocyte sedimentation rate; GLU, glucose; CREA, serum creatinine; eGFR, glomerular filtration rate; C1q, complement 1q; C3, complement C3; C4, complement C4; anti-dsDNA, anti-double stranded DNA antibody; ANA, antinuclear antibodies; SLE-DAI, systemic lupus erythematosus disease activity index.

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Exploring candidate biomarkers for rheumatoid arthritis through cardiovascular and cardiometabolic serum proteome profiling

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Introduction: RA patients are at higher risk of cardiovascular disease, influenced by therapies. Studying their cardiovascular and cardiometabolic proteome can unveil biomarkers and insights into related biological pathways.

Methods: This study included two cohorts of RA patients: newly diagnosed individuals (n=25) and those with established RA (disease duration >25 years, n=25). Both cohorts were age and sex-matched with a control group (n=25). Additionally, a longitudinal investigation was conducted on a cohort of 25 RA patients treated with methotrexate and another cohort of 25 RA patients treated with tofacitinib for 6 months. Clinical and analytical variables were recorded, and serum profiling of 184 proteins was performed using the Olink technology platform.

Results: RA patients exhibited elevated levels of 75 proteins that might be associated with cardiovascular disease. In addition, 24 proteins were increased in RA patients with established disease. Twenty proteins were commonly altered in both cohorts of RA patients. Among these, elevated levels of CTSL1, SORT1, SAA4, TNFRSF10A, ST6GAL1 and CCL18 discriminated RA patients and HDs with high specificity and sensitivity. Methotrexate treatment significantly reduced the levels of 13 proteins, while tofacitinib therapy modulated the expression of 10

proteins. These reductions were associated with a decrease in DAS28. Baseline levels of SAA4 and high levels of BNP were associated to the non-response to methotrexate. Changes in IL6 levels were specifically linked to the response to methotrexate. Regarding tofacitinib, differences in baseline levels of LOX1 and CNDP1 were noted between non-responder and responder RA patients. In addition, response to tofacitinib correlated with changes in SAA4 and TIMD4 levels.

Conclusion: In summary, this study pinpoints molecular changes linked to cardiovascular disease in RA and proposes candidate protein biomarkers for distinguishing RA patients from healthy individuals. It also highlights how methotrexate and tofacitinib impact these proteins, with distinct alterations corresponding to each drug's response, identifying potential candidates, as SAA4, for the response to these therapies.

KEYWORDS

rheumatoid arthritis, methotrexate, tofacitinib, biomarkers, proximity extension assay (PEA), Olink

1 Introduction

Cardiovascular disease (CVD) has been identified as the primary contributor to premature mortality and sudden death among patients with rheumatoid arthritis (RA). This population has an incidence of CVD at least two times greater than the general population, with cardiovascular mortality being a major cause of death, accounting for 40-50% of RA-related deaths (1). This may be partly attributed to the high prevalence of traditional CVD risk factors (e.g., hypertension, hypercholesterolemia, type II diabetes mellitus, obesity) in conjunction with chronic systemic inflammation (1, 2). In this sense, the prevalence of metabolic syndrome in RA patients is significantly higher (around 30%) compared to the general population. Metabolic Syndrome has been linked to a three-fold increase in the risk of atherosclerotic cardiovascular disease (CVD) (3, 4). Our recent research investigated whether RA-associated inflammatory activity could explain the observed defects in glucose and lipid metabolism in these patients. Our results illustrate that alterations in glucose and lipid homeostasis associated with RA depend on the degree of inflammation, with adipose tissue inflammation identified as the initial target leading to insulin resistance and molecular alterations. Therefore, therapeutic strategies targeting tighter control of inflammation and flare-ups could normalize and/or prevent RA-associated metabolic alterations (5). Circulating proteins have been used as biomarkers of various pathologies for many years. Currently, different technologies are used to measure and analyze proteins in serum or plasma, but accurately measuring and interpreting the complete protein content in a large number of samples is a major challenge. To address this, the Olink Proximity Extension Assay (PEA) has been developed—an advanced, high-throughput method that analyzes up to 92 protein biomarkers with exceptional sensitivity and precision using oligonucleotide-labeled antibodies (6, 7). Despite this, there are still few studies that analyze the serum proteome in RA patients. Early diagnosis of RA is essential for the optimal treatment. According to EULAR recommendations, synthetic DMARDs such as methotrexate should be used as first-line treatment. When the first treatment fails, EULAR recommends that patients with risk factors for severe disease and a high inflammatory burden should receive biologic DMARDs. The introduction of biological therapies has significantly improved the management of RA, allowing for the reduction of symptoms, the prevention of rapid radiological deterioration, and an improvement in the quality of life. On the other hand, Tofacitinib is a targeted synthetic DMARD, reversible, competitive inhibitor that works by blocking the adenosine triphosphate (ATP) binding site in the catalytic cleft of JAK1, JAK2, JAK3 and TYK2 and it has been approved for the treatment of RA in many countries. It has been shown to reduce HAQDI and promote ACR20 responses in patients that had an inadequate response to conventional synthetic DMARDs or TNFα

Abbreviations: CVD, cardiovascular disease; RA, rheumatoid arthritis; PEA, proximity extension assay; EULAR, European Alliance of Associations for Rheumatology; DMARDs, disease-modifying antirheumatic drugs; ATP, adenosine triphosphate; TNF-α, tumor necrosis factor-alpha; CV, cardiovascular; HDs,healthy donors; DAS-28, disease activity score 28; CRP, C-reactive protein; RF, rheumatoid factor; ACPAs,antibodies to citrullinated protein antigens antibodies; PCR, polymerase chain reaction; NPX, normalized protein expression; SD, standard deviation; AUC, area under the curve; HF, heart failure; HLA-B27, human leukocyte antigen B27; SNPs, single-nucleotide polymorphism; MBDA, multi-biomarker disease activity.

inhibitors. However, its efficacy in improving CV risk and reducing cardiovascular risk factors, including mediators of metabolic syndrome, is yet to be determined. This study aims to evaluate the changes in the cardiometabolic and cardiovascular serum proteome in two cohorts of active RA patients: newly diagnosed (naïve- treated) and those with well-established disease and to analyze the association with the clinical characteristics. Furthermore, the study analyses the modulation of the levels of these proteins by methotrexate and tofacitinib.

2 Materials and methods

2.1 Patients

A cross-sectional study was conducted on 50 patients with RA the from the Rheumatology Department of the Hospital Universitario Reina Sofia in Cordoba, the Hospital Universitario La Paz in Madrid and the Hospital Clínico Universitario de Santiago de Compostela in Santiago de Compostela, Spain. The patients fulfilled the American College Rheumatology 2010 criteria for RA and were divided in 2 independent cohorts: a cohort of 25 newly diagnosed RA patients (disease duration = 0 years) and a second cohort of 25 patients with established RA (disease duration > 20 years). None of the patients had a history of previous CV events (ischemic heart disease, stroke, peripheral arterial disease or heart failure). Additionally, 25 age- and -sex matched healthy donors (HDs) were included as a control group, none of which had a history of other autoimmune diseases or cardiovascular diseases/events. All recruited subjects provided written informed consent, which was specifically approved by the hospital ethics committee (ethics committee of the Reina Sofia Hospital, the University Clinical Hospital of Santiago de Compostela, and La Paz University Hospital). Demographic and clinical data were collected, including disease duration and DAS-28 (disease activity score 28), inflammation (CRP, mg/L), and levels of autoantibodies (rheumatoid factor and ACPAs). To assess disease activity, we computed the modified Disease Activity Score including a 28-joint count (DAS28), incorporating information on tender and swollen joints, the patient's global assessment of disease activity on a visual analogue scale (VAS), and acute phase response. As we utilized CRP values (8), it will be subsequently referred to as DAS28-CRP. On the other hand, a longitudinal study was carried out in the 25 newly-diagnosed RA patients which were treated with methotrexate and the 25 RA patients with established disease that were treated with tofacitinib (5mg twice daily) in combination with conventional DMARDs, both according to the daily clinical practice for 6 months. Treatment response was determined after 6 months of treatment by the change in DAS28 (CRP) based on European Alliance of Associations for Rheumatology (EULAR) criteria (9). It considers that a patient has a good response to treatment when, having a DAS28 ≤3.2, it is reduced by at least 1.2. Moderate responders include three scenarios: patients who have a DAS28 ≤3.2 and the decrease in activity is between 0.6 and 1.2; those having a DAS28 >3.2 ≤ 5.1, the diminution is higher than 0.6; and those with a DAS28 >5.1 and a reduction higher than 1.2. The occurrence of cardiovascular events was recorded. At baseline and follow-up visits, peripheral blood samples were collected.

2.1.1 Measurements

2.1.1.1 Blood sample collection and isolation of serum

Peripheral venous blood samples were collected from RA patients before and after treatment and from HDs. Samples were collected early in the morning and after an 8-hour fasting period. The samples were centrifuged for 10 minutes at 3500 rpm and at room temperature to obtain the serum, which was then aliquoted and stored at -80°C until use.

2.1.1.2 Proximity extension assay

Serum samples from the baseline and follow-up visits were subjected to high-throughput analysis of 184 proteins, 92 of which were cardiometabolic-associated and 92 cardiovascular disease-related. This analysis was conducted using proximity extension immunoassay (PEA) provided by Olink Proteomics, Uppsala, Sweden, and was performed in a 96-well plate format by Cobiomic Bioscience S.L, Cordoba, Spain. The PEA is a dual-recognition immunoassay, that uses matched antibodies each of them labeled with unique DNA oligonucleotides, to simultaneously bind to a target protein in solution. This allows the two antibodies to converge and their DNA oligonucleotides to hybridize, serving as a template for a DNA polymerase-dependent extension step. This creates a double-stranded DNA "barcode" which is unique for the specific protein. The hybridization and extension are followed by PCR amplification. The resulting concentration of the PCR product is directly proportional to the initial concentration of the target protein. The relative levels of proteins were reported on an arbitrary Log2-based NPX (normalized protein expression) scale. The samples were completely randomized and distributed across two plates, maintaining the representation of groups/treatments in proportion to the study. Within each 96-plex panel, there are 96 assays, including four internal controls for quality control, systematically monitoring various stages of the process in every sample. Additionally, interplate controls are incorporated to compute normalized expression (NPX) values, along with negative controls and a duplicated external control. In the intensity normalization process, data were adjusted so that the median NPX for a protein on each plate aligned with the overall median. This ensured that each plate was adjusted to have the same median for all assays across the two plates, thereby enhancing the reliability and consistency of the analysis.

2.1.1.3 Statistical analysis

All data analyses were performed using SPSS statistical software package (Iberica, Madrid, Spain), GraphPad Prism9 (version 9.0.1) and MetaboAnalyst 5.0. Graphical representation of the statistical analysis is carried out using Prism9 (version 9.0.1) and MetaboAnalyst 5.0 software. Data in the text, figures and tables were expressed as the mean ± standard deviation (SD). Normality of variables was assessed using the Kolmogorov-Smirnov test. Clinical

characteristics were compared using Student's unpaired t-test for parametric data and the Mann-Whitney sum test for non-parametric data. Paired samples within the same subjects were compared using the paired Student's t test. Correlations were assessed using Pearson correlation between variables. Chi-square tests were performed to analyze qualitative data. A volcano plot, featuring Benjamini-Hochberg adjusted false discovery rates, provided a nuanced visualization of the differential expression of 184 proteins across distinct study groups. Complementing this, Venn diagram was employed to discern the intersection of commonly altered proteins among the groups, shedding light on shared molecular signatures. To delve deeper into the differences among HDs, early RA, and established RA, an analysis of variance (ANOVA) was executed, with subsequent Bonferroni adjustments ensuring robustness in multiple comparisons in the commonly altered proteins. Receiver operating characteristic (ROC) curves, representing the true positive rate (sensitivity) versus false positive rate (1-specificity) at various thresholds, and area under the curve (AUC) analysis were used to determine sensitivity, specificity and corresponding cut-off values. Statistical significance levels were designated as follows: (****) <0.0001 *p*-value; (***) <0.001 *p*-value; (**) <0.01 *p*-value; (*) <0.05 *p*-value. This convention was applied in instances where specific *p*-values were not explicitly provided in the graphs.

3 Results

3.1 RA patients show an altered cardiometabolic and CVD profile: candidate biomarkers in human serum

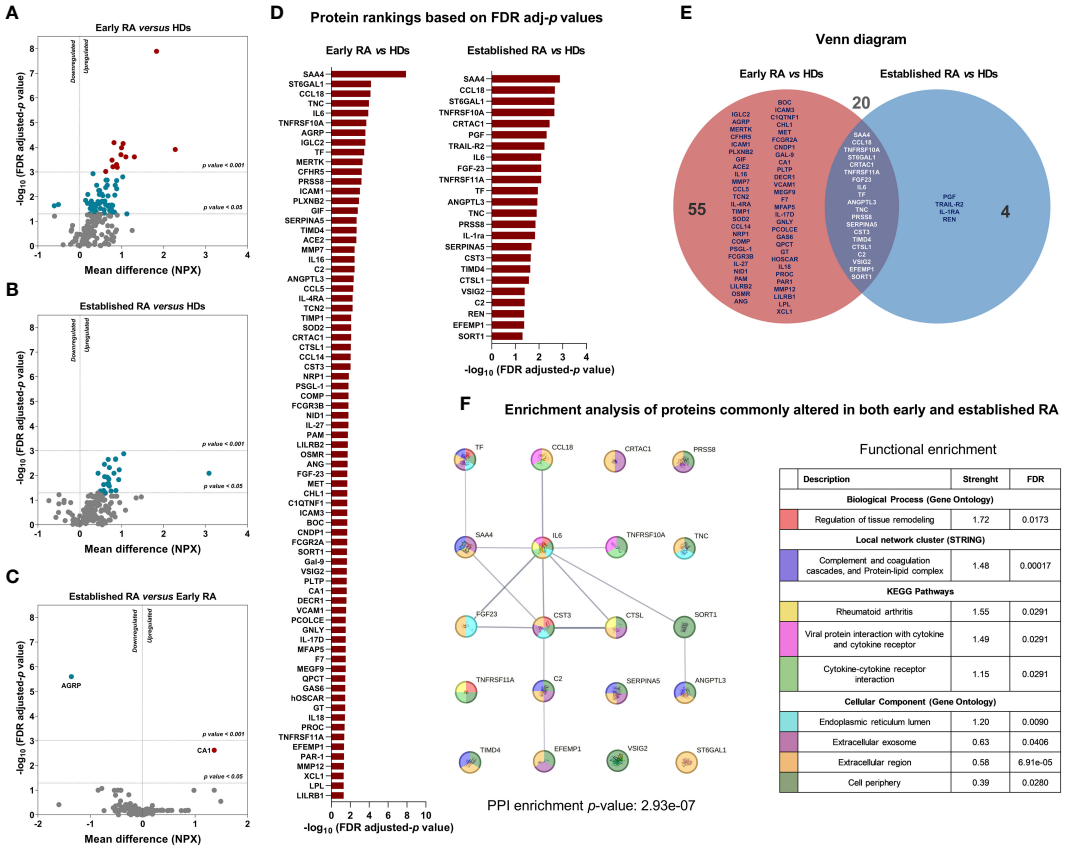
RA patients with less than two years of disease duration (newly diagnosed RA) showed similar age, sex and disease activity compared to RA patients with stablished disease. In addition, RA patients with stablished disease showed a mean disease duration of 37.48 ± 11.79 years and significantly elevated levels of C-reactive protein compared to patients with newly diagnosed RA (Table 1). CVD risk factors were elevated in RA compared HDs.

To analyze the molecular profile that might be associated with CVD in RA patients, we evaluated the levels of 184 proteins related to cardiometabolism and CVD in the serum. Seventy-five proteins were significantly altered in early RA patients compared to HDs (Figure 1A) and 24 proteins were significantly increased in RA patients with established disease *vs* HDs (Figure 1B). In contrast, the comparison of the proteome profile between early and established disease revealed significant alterations in only 2 proteins (Figure 1C). The protein rankings based on FDR-adjusted *p*-values are depicted in Figure 1D. Additionally, a Venn diagram was utilized to identify commonly altered proteins in both early RA and established RA compared to HDs, revealing 20 proteins that were consistently affected in both conditions (Figure 1E). The altered levels of these proteins did not correlate with autoantibodies titers or positivity. We conducted an enrichment analysis to elucidate whether the identified proteins contribute to specific pathways beyond their recognized cardiovascular implications using STRING platform, particularly in the shared alteration pattern observed in both early and established RA disease (Figure 1F). The enrichment analysis revealed a significant protein-protein interaction enrichment (*p*-value: 2.93e-07), underscoring the biological relevance of the identified protein alterations. These proteins were found to play pivotal roles in diverse processes, including the regulation of tissue remodeling, complement and coagulation cascades, and the formation of protein-lipid complexes. Moreover, their implication in pathways directly associated with RA pathology was evident. Notably, these proteins exhibited interactions in viral protein networks and demonstrated associations with various cellular components, such as the endoplasmic reticulum lumen, extracellular exosome, extracellular region, and cell periphery.

TABLE 1 Descriptive clinical data of Rheumatoid Arthritis patients and healthy donors.

	HDs	Early RA	Established RA
Size population	25	25	25
Female/male (%)	78/22	75/25	70/30
Age (years)	58.55 ± 13.12	61.24 ± 12.65	63.75 ± 10.19
Disease duration (years)	–	0	37.48 ± 11.79[b]
Disease activity			
DAS-28 (CRP)	–	5.04 ± 1.20	4.82 ± 0.71
CRP (mg/L)	1.71 ± 1.94	1.48 ± 1.90	8.12 ± 7.39[a,b]
Autoimmunity profile			
RF + (n)	–	16	12
ACPAs + (n)	–	18	21
CVD risk factors			
Arterial Hypertension (%)	12	20	36[a]
Obesity (%)	12	16	36[a]
Smoking habit (%)	12	24[a]	24[a]
Type 2 Diabetes Mellitus (%)	0	8	12[a]
Metabolic profile			
Glucose (mg/dL)	85.06 ± 9.66	89.62 ± 11.89	94.30 ± 22.98
Total-cholesterol (mg/dL)	198.50 ± 24.19	190.25 ± 32.91	202.90 ± 9.66
HDL-cholesterol (mg/dL)	58.65 ± 19.06	55.78 ± 21.83	65.33 ± 12.27
LDL-cholesterol (mg/dL)	119.20 ± 23.44	115.38 ± 32.08	112.86 ± 32.22
Tryglicerides (mg/dL)	99.94 ± 50.54	140.41 ± 94.15	103.73 ± 41.08

Data are represented by mean ± SD. HDs, healthy donors; RA, rheumatoid arthritis; DAS, disease activity score; CRP, c-reactive protein; RF, rheumatoid factor; ACPAs, antibodies to citrullinated protein antigens. [a]Significant differences respect to HDs, p value < 0.05; [b]Significant differences respect to early RA, p value < 0.05.



Description	Strenght	FDR
Biological Process (Gene Ontology)		
Regulation of tissue remodeling	1.72	0.0173
Local network cluster (STRING)		
Complement and coagulation cascades, and Protein-lipid complex	1.48	0.00017
KEGG Pathways		
Rheumatoid arthritis	1.55	0.0291
Viral protein interaction with cytokine and cytokine receptor	1.49	0.0291
Cytokine-cytokine receptor interaction	1.15	0.0291
Cellular Component (Gene Ontology)		
Endoplasmic reticulum lumen	1.20	0.0090
Extracellular exosome	0.63	0.0406
Extracellular region	0.58	6.91e-05
Cell periphery	0.39	0.0280

FIGURE 1
CVD and cardiometabolic serum proteome profile of RA patients in early and established disease. **(A)** Volcano plot of CVD and cardiometabolic proteome profile (184 proteins) in early RA (n=25) respect to HDs (n=25). **(B)** Volcano plot of CVD and cardiometabolic proteome profile (184 proteins) in established RA (n=25) respect to HDs (n=25). **(C)** Volcano plot of CVD and cardiometabolic proteome profile (184 proteins) in established RA (n=25) respect to early RA (n=25). **(D)** Significant protein rankings based on FDR adjusted *p*-values in early RA and established RA compared to HDs. **(E)** Venn diagram illustrating the proteins commonly altered in both early RA and established RA in comparison to HDs. **(F)** Enrichment analysis of proteins commonly altered in both early and established RA using STRING platform (version 12.0, STRING CONSORTIUM 2023). CVD, cardiovascular disease; RA, rheumatoid arthritis, HDs, healthy donors. Annotated names of abbreviated proteins are displayed in Supplementary Table 1.

Notable proteins in the shared alteration (early and established RA conditions) included SAA4, CCL18, TNFRSF10A, ST6GAL1, CRTAC1, TNFRSF11A, FGF23, IL6, TF, ANGPTL3, TNC, PRSS8, SERPINA5, CST3, TIMD4, CTSL1, C2, VSIG2, EFEMP1 and SORT1 (Supplementary Figure 1). Interestingly, elevated levels of CTSL-1 (AUC=0.907), SORT1 (AUC=0.899), SAA-4 (AUC = 0.898), TNFRSF10A (AUC=0.891), ST6GAL1 (AUC=0.855) and CCL18 (AUC=0.854) discriminate RA patients and HDs with high specificity and sensitivity, suggesting the potential role of these proteins to diagnose RA patients. Of note, the combination of all these six proteins could be used as candidate diagnostic biomarkers tool for RA with AUC of 0.970 (Figure 2A). In contrast, we compared the levels of autoantibodies and CRP with the combined proteome to assess these new molecular concepts against the standard criteria for the diagnosis of RA. Hence, ROC analysis demonstrated that elevated levels of RF (AUC=0.915) and ACPAs (AUC=0.845) can effectively discriminate RA patients from HDs. In contrast, levels of CRP (AUC=0.572) did not reach statistical significance (Figure 2B). Notably, the combined serum proteome profile of CTSL-1, SORT1, SAA4, TNFRSF10A, ST6GAL1, and CCL18 exhibited better ROC curve for distinguishing patients from HDs compared to the combination of ACPAS, RF and CRP (Figures 2B, C).

3.2 Effects of methotrexate and tofacitinib on the cardiometabolic and CVD-proteome

We conducted a longitudinal study involving 50 RA patients treated with methotrexate or tofacitinib for six months to analyze the impact of these therapies on the proteome related to cardiometabolism and CVD. After six months of treatment, disease activity and CRP levels decreased with both therapies (Table 2). The group treated with methotrexate (early RA) had an 80% ratio of responders according to EULAR response criteria, while the group treated with tofacitinib (established RA) had a 72% responder ratio (Table 2). No CVD events were observed during the six-month treatment period with either methotrexate or tofacitinib.

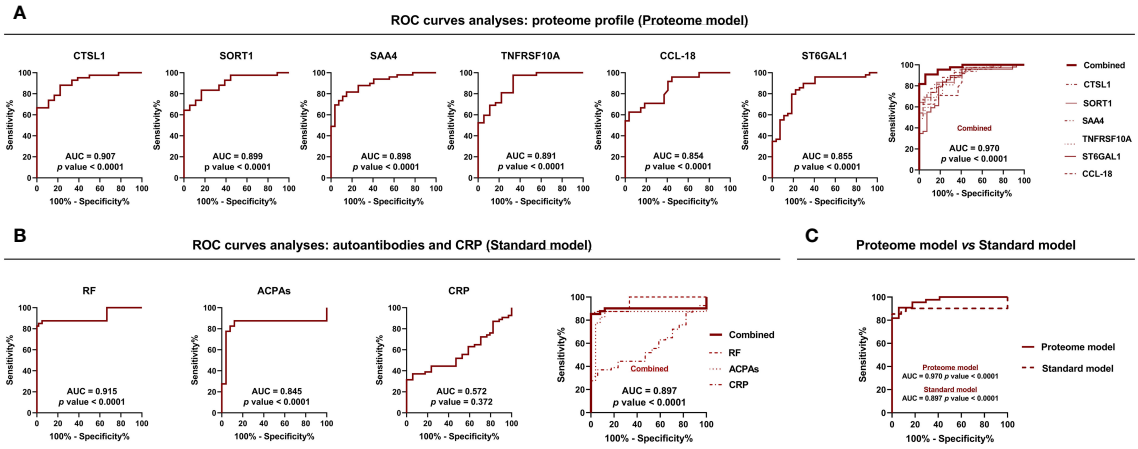


FIGURE 2
Prospective serum biomarkers with diagnostic potential in RA. **(A)** ROC curve analysis of proteins significantly altered in both early and established disease in the whole cohort of RA patients with the best capacity to identify RA (n=50) *vs* HDs (n=25) (Proteome model). **(B)** ROC curve analysis of commonly used diagnostic markers for RA, such as ACPAs, RF, and CRP (n=50) *vs* HDs (n=25) (Standard model). **(C)** Comparison of the ability to differentiate between RA patients and HDs using proteomic and standard models. RA, rheumatoid arthritis; HDs, healthy donors; ACPAs, antibodies to citrullinated protein antigens; RF, rheumatoid factor; CRP, C-reactive protein; ROC, receiver operating characteristic; AUC, area under the curve. Annotated names of abbreviated proteins (Supplementary Table 1).

We then examined the impact of treatments on cardiometabolic and CVD-related proteins that were significantly elevated in the serum of RA patients (described in section 3.1). Methotrexate treatment resulted in a significant reduction of the levels of 13 proteins, including SAA4, ST6GAL1, TNC, NID1, SORT1, TNFRSF10A, IL6, CCL18, IGLC2, TIMD4, CFHR5, IL16 and PSGL-1 (Figure 3A). On the other hand, tofacitinib significantly reduced the levels of 8 proteins including CA1, TNC, SAA4, CCL18, TIMD4, IL16, IL6 and IL18, and increased levels of 2 proteins such as LPL and MMP12 (Figure 3B). Following this, we aimed to gauge the physiological significance of the alterations in protein levels resulting from distinct treatments (methotrexate and tofacitinib). We conducted a comparison of protein levels at the 6-month mark in RA patients to ascertain whether they significantly differed from or mirrored the protein levels in healthy individuals. Among the 13 proteins that were reduced by methotrexate, levels of TIMD4, CFHR5, PSGL-1, IL16 and NID1 were restored at levels seen in HDs (Figure 4A). When comparing the levels of proteins influenced by tofacitinib to those in healthy individuals, we detected significant differences in two proteins. In contrast, eight proteins reached levels resembling those observed in healthy individuals (Figure 4B), underscoring the potential of tofacitinib to revert the observed alterations in established RA patients.

Moreover, we investigated the potential association between baseline protein levels or alterations during methotrexate treatment and the response to therapy. Intriguingly, elevated levels of SAA4 and reduced levels of BNP at the treatment's onset were associated with a positive treatment response (Figure 5A). Conversely, changes in the levels of IL6, TIMD4, CCL18, CFHR5, and TNC following methotrexate treatment were identified as linked to alterations in DAS28, indicating their association with disease activity (Figure 5B). Notably, changes in IL6 levels were specifically linked to the response to methotrexate, with RA responder patients displaying significantly decreased IL6 levels post-treatment compared to non-responder patients. The changes in IL6 levels demonstrated the potential to discriminate between responder and non-responder patients, achieving an AUC of 0.877 (Figure 5C). Upon transitioning to tofacitinib treatment, discernible differences in baseline levels of LOX1 and CNDP1 were noted between non-responder and responder RA patients (Figure 5D). Subsequent analyses revealed that changes in the levels of IL16, CCL18, TIMD4, SAA4, and TNC significantly correlated with alterations in DAS28 (Figure 5E), implying their association with disease activity. Thus, decreased levels of SAA4 or TIMD4 were found in RA responder patients compared to the non-responder group (Figure 5F). Annotated names of abbreviated proteins are stated in Supplementary Table 1.

4 Discussion

This study describes molecular alterations that might be associated to cardiometabolic and cardiovascular disease in the serum of RA patients, using a high-throughput proteomic technology to analyze the serum levels of 184 proteins (Figure 6). Our work identifies novel potential candidate biomarkers of RA diagnosis and therapeutic targets including CTSL-1, SORT1, SAA-4, TNFRSF10A, ST6GAL1 and CCL18, in two different cohort of RA patients. Additionally, it is showed how methotrexate or JAK-inhibitor can modulate these protein alterations and detects biomarkers of response to each therapy. With the use of the PEA technology, we were able to directly analyze a substantial number of proteins that has been described to be involved in cardiovascular and cardiometabolic diseases in the serum of patients with RA. We found 75 proteins significantly altered in the serum of RA patients with early disease, 20 of them were also elevated in RA patients with established disease. These findings may suggest an association of RA with a modification of the molecular profile that might be related to

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TABLE 2 Longitudinal study of early and established Rheumatoid Arthritis patients treated with methotrexate or tofacitinib: clinical details.

Early RA – Methotrexate treatment		
Disease duration (years)	0	
Time	Basal	6 months
Size population (n)	25	
Female/male (%)	75/25	
Age (years)	61.24 ± 12.65	
DAS-28	5.04 ± 1.20	2.94 ± 1.16***
CRP (mg/ml)	1.48 ± 1.90	0.78 ± 0.67*
Responders (%)	80	
Long established RA – Tofacitinib treatment		
Disease duration (years)	37.48 ± 11.79	
Time	Basal	6 months
Size population (n)	25	
Female/male (%)	70/30	
Age (years)	63.75 ± 10.19	
DAS-28	4.84 ± 0.70	3.33 ± 1.12***
CRP (mg/ml)	8.48 ± 7.53	3.51 ± 6.15**
Responders (%)	72	

Data are represented by mean ± SD. HDs, Healthy donors; RA, Rheumatoid arthritis, DAS, disease activity score; CRP, c-reactive protein; *Significant differences vs basal time, p value < 0.05; **Significant differences vs basal time, p value < 0.01; ***Significant differences vs basal time, p value < 0.0001.

CVD, which appears elevated in patients recently diagnosed and persisted over an extended period of evolution. In a previous study conducted by Ferreira and colleagues, a panel of 92 proteins related to cardiovascular disease (Olink proteomics) was assessed in RA patients who had been suffering from the disease for a long period of time and 6.8% of them had heart failure (HF) diagnosis. They identified some biomarkers that were associated with HF (10). Interestingly, some of those proteins increased in patients with HF were also increased in our cohort of RA patients, especially in the cohort of established disease, such as PGF, TNFRSF10A, SPON-2, TF and PRSS8. In our work, we discovered six proteins that exhibit promising potential as biomarkers for the differentiation of individuals with RA from HDs. Specifically, proteins exhibiting alterations in two distinct cohorts of RA patients with both early and established disease, including CTSL-1, SORT1, SAA4, TNFRSF10A, ST6GAL1 and CCL-18. It is noteworthy that the combined use of these six proteins resulted in an improved area under the curve (AUC) of 0.97. In addition, this combined proteome signature exhibited better AUC compared to the performance of stablished biomarkers of RA, such as ACPAs, RF and CRP alone and combined. The data presented herein corroborates recent findings indicating that SAA-4 may serve as a promising serum biomarker for the diagnosis of RA, including cases of seronegative presentation (11–13). ST6GAL1 is a pivotal sialyltransferase enzyme responsible for catalyzing the addition of α2,6-linked sialic acids to glycans' termini. Glycosylation with sialic acid is a notable modification for IgGs. This glycosylation process has been acknowledged for its immunoregulatory impact on various immune cells, including stem cells, B cells, T cells, and macrophages (14). While ST6GAL1 is responsible for adding sialic acid to glycoproteins, the overall sialylation of IgG has been shown reduced in RA patients (15). The elevated levels of ST6GAL1 that we found in the serum of RA patients might be associated with increased inflammatory responses. The reduction in sialylated IgG could be due to several factors independent on the levels of ST6GAL1, such as increased turnover or degradation of sialylated IgG, altered glycosylation patterns driven by other enzymes, or changes in the microenvironment that affect the glycosylation process. In addition, we measured levels of protein, not enzyme activation. Thus, the elevation of ST6GAL1 levels in the serum of RA patients and the reduction in sialylated IgG may be part of the complex molecular and cellular changes associated with RA. Further research is needed to elucidate the precise mechanisms and functional consequences of these alterations.

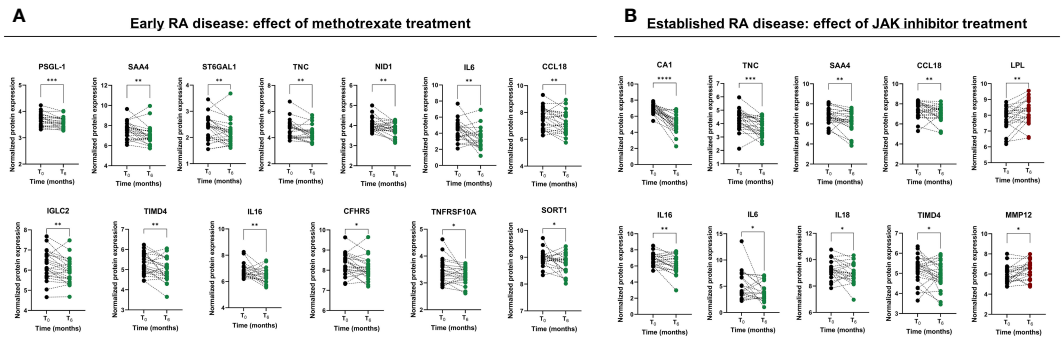

FIGURE 3
Effect of methotrexate and JAK inhibitor treatments on the serum proteome profile in early and established RA patients, respectively. **(A)** CVD and cardiometabolic-related proteins modulated by the treatment with methotrexate after six months in early RA. **(B)** CVD and cardiometabolic-related proteins modulated by the treatment with methotrexate after six months in established RA. RA, rheumatoid arthritis; CVD, cardiovascular disease; ROC, receiver operating characteristic. Annotated names of abbreviated proteins (Supplementary Table 1). Graphs of symbols and lines represent levels of analyzed proteins before and after the treatments. Statistical significance levels were designated as follows: (****) <0.0001 p-value; (***) <0.001 p-value; (**) <0.01 p-value; (*) <0.05 p-value.

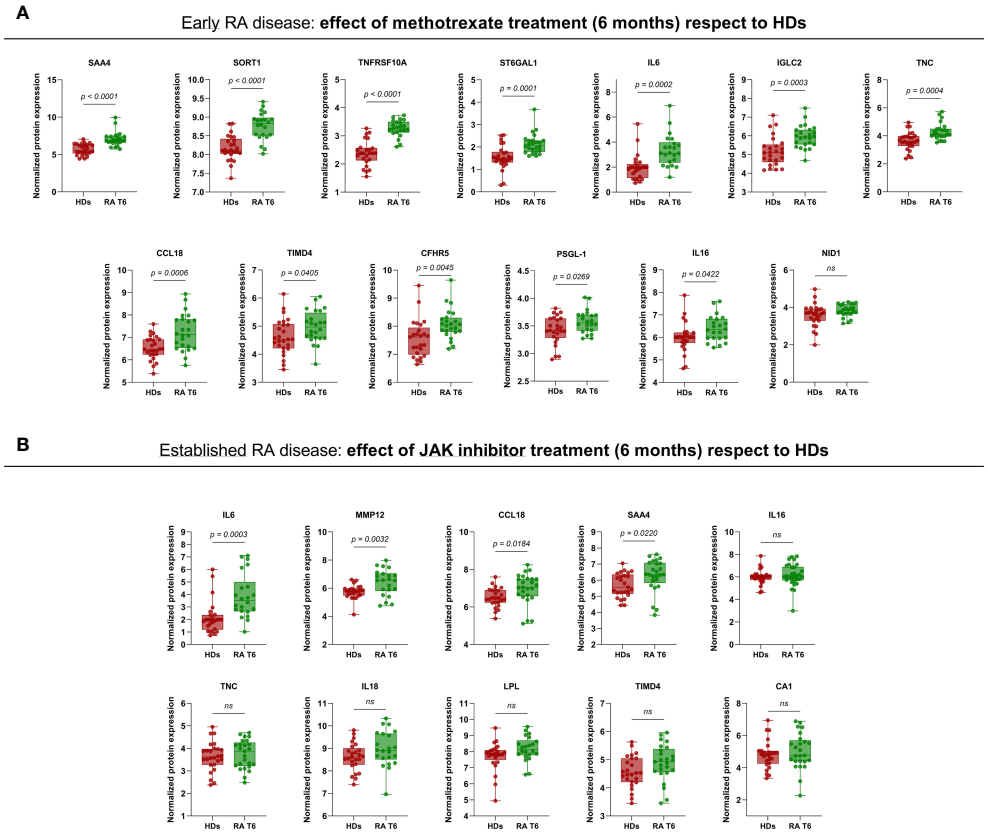

FIGURE 4
Comparison of serum protein levels at 6 months with levels in healthy individuals. **(A)** Levels of proteins influenced by methotrexate in early RA patients compared to serum levels in healthy individuals. **(B)** Levels of proteins influenced by tofacitinib in established RA patients compared to serum levels in healthy individuals. RA, rheumatoid arthritis; RA T6, rheumatoid arthritis time 6 months; HDs, healthy donors; JAK, janus kinase. Annotated names of abbreviated proteins (Supplementary Table 1). Box and whiskers plots represent median and minimum and maximum values of analyzed proteins. The adjusted *p*-values are presented using the Benjamini-Hochberg procedure.

The role of CCL18 in the pathogenesis of RA was investigated some time ago, with studies demonstrating a substantial elevation in the serum levels of this protein among RA patients in comparison with HDs. Moreover, these studies also revealed a positive correlation between the levels of CCL18 and the activity of the disease (16).

TNFRSF10A, also known as TRAIL-R1, presents a complex role in the context of RA synovial fibroblasts, exhibiting dual effects. While it is acknowledged for inducing apoptosis through caspase activation upon TRAIL ligand binding (17), its interaction with other receptors, including TNFRSF10A/TRAIL-R1, can activate NFκB, a transcription factor regulating cell proliferation (18). Morel et al. reported TRAIL's ability to stimulate RA fibroblast proliferation *in vitro* via MAP kinase and PI3 kinase/Akt activation (19). Paradoxically, in RA patients, both serum TRAIL and IL-8 concentrations were elevated, while TRAIL receptor expression, including TNFRSF10A/TRAIL-R1, was reduced in monocytes. This led to decreased TRAIL-induced monocyte apoptosis in RA due to increased TRAIL-induced IL-8 secretion, activating antiapoptotic pathways (20). These findings underscore the potential pathogenic role of TNFRSF10A in RA. Moreover, SORT1, a pivotal regulator of lipid metabolism (21), has been previously noted to be elevated in the serum of RA patients compared to healthy donors. It plays a critical role in immune cell signaling, contributing to the pathogenesis of RA (22). On the other hand, CTSL1, an endosomal proteolytic enzyme involved in extracellular matrix degradation, angiogenesis, and antigen presentation, is elevated in the circulation of RA patients, particularly associated with autoantibodies (23, 24). Our study affirms that TNFRSF10A, SORT1 and CTSL1 are significantly elevated not only in early RA but also in established disease, suggesting their potential as candidate biomarkers for the disease.

The effects of methotrexate therapy on cardiometabolic and cardiovascular alterations in RA patients have yielded inconsistent results in prior studies (25, 26). In our study of patients with early RA disease, we observed that the administration of methotrexate over a six-month period resulted in a significant reduction of 13 molecules that have been related to CVD. Although this reduction did not reach the levels of these proteins in healthy subjects. These results suggest that methotrexate might have beneficial effects on the molecular profile related to CVD. Currently, one of the areas that have garnered considerable attention is the identification of

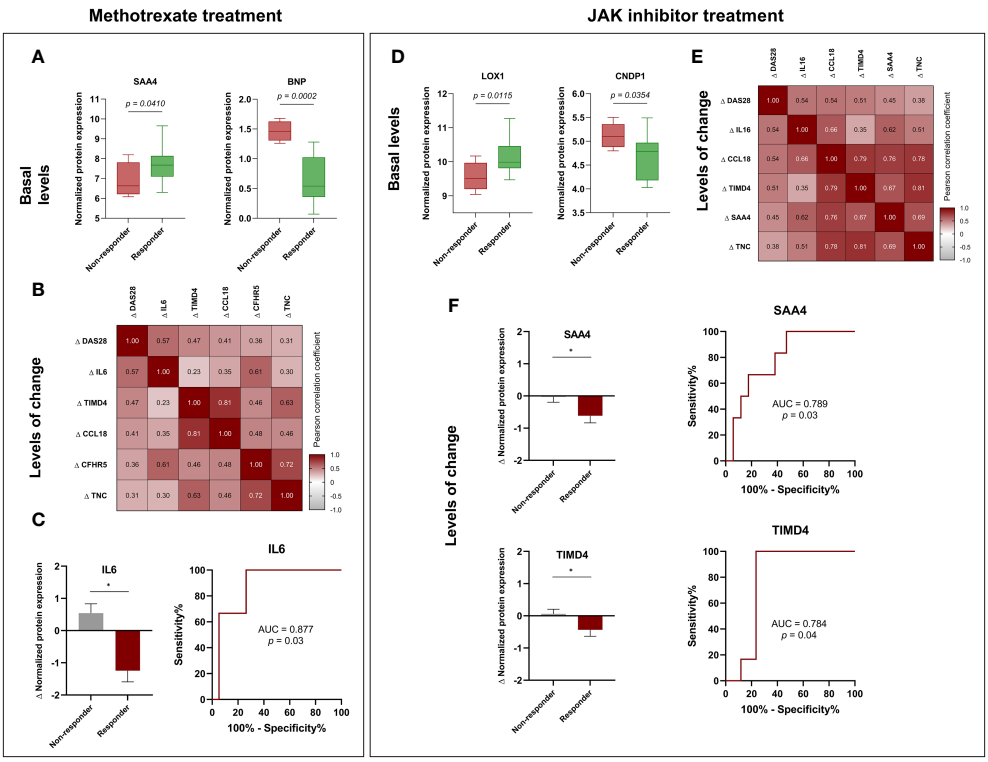

FIGURE 5
Potential biomarkers for predicting response to methotrexate in early RA and JAK inhibitor treatment in established RA. **(A)** Baseline levels of SAA4 and BNP in methotrexate-treated patients: a comparison between responders and non-responders. **(B)** Correlation heatmap of changes in DAS28 and proteins after six months of treatment with methotrexate. **(C)** Comparison of changes (Δ) in IL6 levels in responders and non-responders RA patients and ROC curve of changes in IL6 levels to discriminate responders or non-responders RA patients after six months of treatment with methotrexate. **(D)** Baseline levels of LOX1 and CNDP1 in JAK inhibitor-treated patients: a comparison between responders and non-responders. **(E)** Correlation heatmap of changes (Δ) in DAS28 and proteins after six months of treatment with JAK inhibitor. **(F)** Comparison of changes (Δ) in SAA4 and TIMD4 levels in responders and non-responders RA patients after the treatment with JAK inhibitor and ROC curve analyses. RA, rheumatoid arthritis; ROC, receiver operating characteristic. Annotated names of abbreviated proteins (Supplementary Table 1). Box and whiskers plots represent median and minimum and maximum values of basal proteins. Correlation heatmap represent significant correlations between disease activity and proteins. Numbers in correlation heatmaps include Pearson correlation coefficient (r). Bar graphs represent mean with standard deviation (error bars). *Significant differences: $p<0.05$. The adjusted p-values for the box plots did not demonstrate statistical significance, except in the case of the BNP protein, where the adjusted p-value was 0.037.

biomarkers indicative of the response to therapy among patients diagnosed with RA. This pursuit is primarily aimed at early disease progression, improvement of patient outcome, reducing healthcare costs and promoting a better understanding of the disease. Numerous studies have been conducted to identify clinical or molecular factors or a combination of both, that could predict the effectiveness of methotrexate therapy. The most recent studies have explored the integration of genomics and clinical data through machine learning to predict the response to methotrexate. As such, machine learning methods, which incorporate demographic data, smoking habit, rheumatoid factor, DAS28 and 160 SNPs predicted methotrexate response at 3 months with an AUC of 0.84 (27). With regard to serum biomarkers, prior studies have indicated that four specific proteins may be predictive of methotrexate response in a cohort of RA patients. Notably, high levels of these biomarkers, namely CRP, leptin, TNF-RI and VCAM-1, were found to be associated with low disease activity at 3 months (28). Our research contributes to the biomarker field by identifying serum levels of IL6, SAA4 or BNP as potential predictors of response to methotrexate. It should be emphasized that further research is necessary to confirm these findings and to establish the predictive value of these biomarkers in this regard.

On the other hand, the impact of tofacitinib on CVD risk among RA patients has been a subject of inquiry (29–31). Nonetheless, a considerable cohort study indicated a minimal occurrence of CV events associated with tofacitinib therapy in RA patients (22). A recent study using PEA (Olink proteomics) on a small cohort of RA patients, presented evidence of the modulatory effects of tofacitinib on various inflammatory-related circulating proteins (32). Our study reveals that tofacitinib modulates the levels of 10 proteins potentially associated with CVD in RA patients with established disease. Notably, in six of these proteins, the reduction reached levels comparable to those observed in healthy donors. In this sense, CA1 belongs to the carbonic anhydrase (CA) family, catalyzing reversible hydration and dehydration reactions of CO2/H2CO3 and also can promote the formation of CaCO3. In our

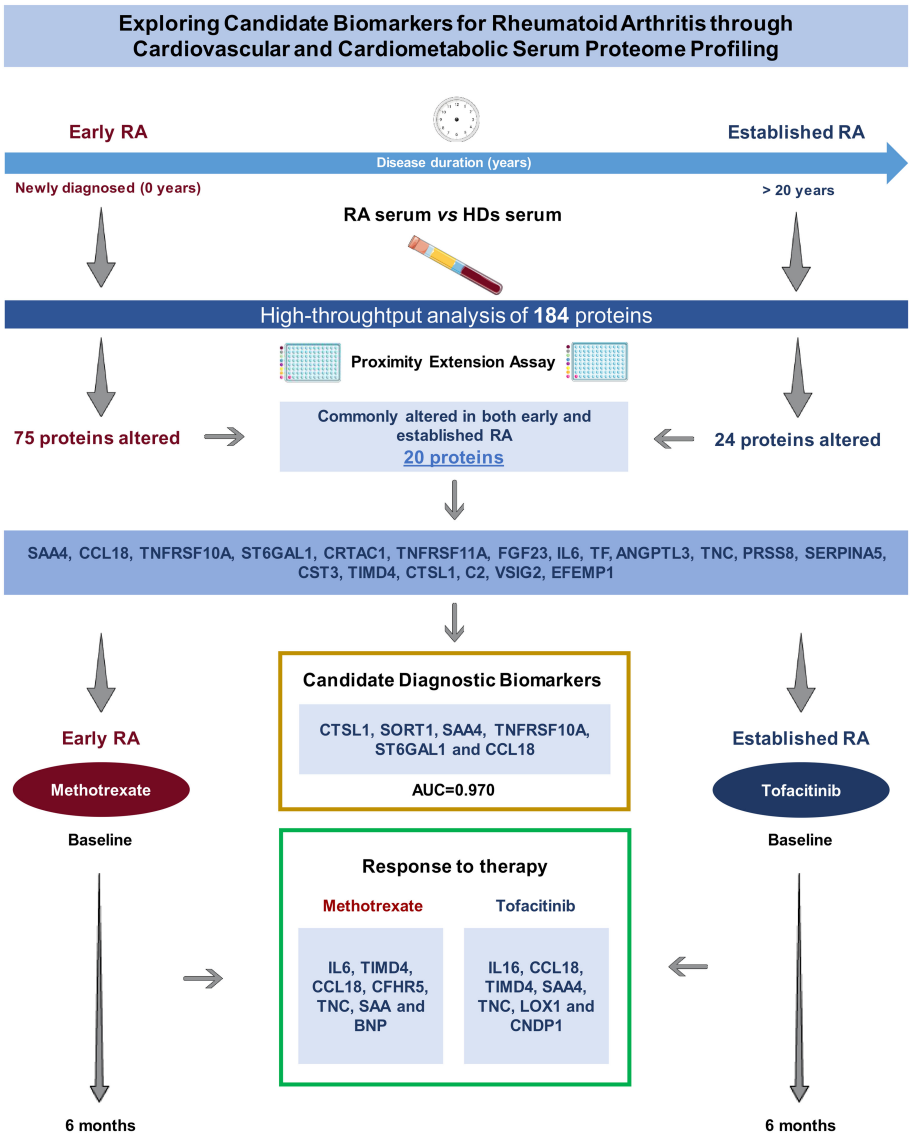

FIGURE 6
Study summary: A high-throughput analysis of 184 proteins across diverse stages of RA (early and established disease) uncovered a consistently altered pattern, indicating potential diagnostic biomarkers, including CTSL1, SORT1, SAA4, TNFRSF10A, ST6GAL1, and CCL18. Methotrexate was administered to early RA patients, while tofacitinib was employed for established RA cases. Post a 6-month treatment period, the levels of proteins exhibiting alterations in both early and established RA were modulated, pinpointing susceptible proteins that may serve as candidate biomarkers indicative of the response to therapy. Annotated names of abbreviated proteins (Supplementary Table 1). RA, rheumatoid arthritis; HDs, healthy donors; AUC, area under the curve.

study, RA patients with established disease exhibited higher levels than those in the early stages of the disease. Notably, treatment with tofacitinib significantly reduced these elevated levels reaching the levels observed in HDs. Our findings align with a study conducted by Zheng and colleagues, demonstrating that overexpression of CA1 accelerated joint inflammation and tissue destruction in a collagen-induced arthritis (CIA) mice model (33). This supports our observation that RA patients with established disease may exhibit higher CA1 levels than those in the early stages of RA. Furthermore, recent research has positioned CA1 as a potential target for managing pain symptoms associated with RA and related inflammatory diseases (34, 35). Our study contributes to the growing body of evidence linking CA1 to RA pathogenesis and treatment response, emphasizing its potential as a therapeutic target in the management of RA. These results could also suggest CA1 as a molecular target for tofacitinib, with the potential to beneficially reduce pain.

Insufficient evidence exists concerning the predictors of clinical response in RA patients undergoing tofacitinib treatment. Recently, a study of 25 RA patients indicated that baseline power Doppler and multi-biomarker disease activity (MBDA) score can forecast the response to tofacitinib (36). Furthermore, early alterations in magnetic resonance may serve as a predictor of the response to therapy, either to methotrexate or tofacitinib (37). Nonetheless, there

is an absence of data on molecular predictors of the response to tofacitinib treatment. Our preliminary study reveals novel findings regarding the serum levels of LOX1, CNDP1, TIMD4 and SAA4 as biomarkers of the response to this therapy.

4.1 Limitations of the study

A key limitation of this study was its small sample size and the lack of randomization in the longitudinal cohort. The non-randomized recruitment from routine clinical practice may have led to uneven distribution among treatment groups, potentially affecting therapy response. Consequently, our results only allow us to make predictive statements about the link between serum proteins and methotrexate or tofacitinib treatment responses. Therefore, larger validation cohorts using complementary techniques are essential to validate these findings.

5 Conclusions

This study aimed to assess the alterations in the serum proteome related to CVD in two cohorts of active rheumatoid arthritis (RA) patients: those who were newly diagnosed (naïve-treated) and those with well-established disease. Our findings revealed that RA is characterized by a distinct and modified CVD proteome, which was observed both at early stages of the disease and throughout its extended progression. This indicates that the alteration in the proteome is a persistent feature of RA. Moreover, we identified a six-biomarker serum panel consisting of CTSL-1, SORT1, SAA4, TNFRSF10A, ST6GAL1, and CCL-18 proteins that effectively distinguished the two cohorts of RA patients from healthy donors. These biomarkers hold potential as valuable candidate diagnostic indicators for RA. Additionally, we observed distinct impacts of methotrexate and tofacitinib on the levels of these proteins, with each treatment modifying a diverse set of proteins. Our results demonstrated that tofacitinib response was associated with baseline levels of LOX1, CNDP1 and changes in SAA4 and TIMD4 proteins. Conversely, methotrexate response showed an association with changes in IL-6 protein levels and the basal levels of SAA4 and BNP. In summary, our study provides insights into the altered proteomic profile associated with CVD in RA patients and identifies a promising six-biomarker panel for RA diagnosis. Furthermore, we elucidated the specific modulation of these proteins by methotrexate and tofacitinib, highlighting potential avenues for personalized treatment strategies in RA management.

Data availability statement

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

Ethics statement

The studies involving humans were approved by ethics committee of the Reina Sofia Hospital, the University Clinical Hospital of Santiago de Compostela, and La Paz University Hospital. 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

LC-L: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. AE-C: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. YH: Data curation, Formal Analysis, Software, Writing – original draft. CP-S: Investigation, Methodology, Writing – review & editing. MR-P: Investigation, Methodology, Writing – original draft. JMM-M: Investigation, Methodology, Writing – original draft. EP-P: Methodology, Writing – original draft. AG: Methodology, Writing – review & editing. CP-R: Methodology, Writing – review & editing. AM-F: Methodology, Writing – review & editing. AB: Methodology, Writing – review & editing. CL-M: Data curation, Formal Analysis, Writing – review & editing. LL-P: Methodology, Writing – review & editing. MR-G: Methodology, Writing – review & editing. RO-C: Methodology, Writing – review & editing. JC-G: Methodology, Writing – review & editing. CL-P: Writing – review & editing. EC-E: Data curation, Formal Analysis, Writing – review & editing. IA-R: Writing – original draft. NB: Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

CP-S, NB, YH, and J-MM-M were co-founders of Cobiomic Biosciences S.L.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1333995/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Comparison of the commonly altered serum proteome in early and established RA compared to HDs. Box and whiskers plots represent median and minimum and maximum values of analyzed proteins. One-way ANOVA and Bonferroni's *post hoc* multiple comparisons tests were conducted for statistical analysis. (****) <0.0001 adj-p-value; (***) <0.001 adj-p-value; (**) <0.01 adj-p-value; (*) <0.05 adj-p-value. RA: rheumatoid arthritis; HDs: healthy donors. Annotated names of abbreviated proteins (Supplementary Table 1).

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Functional significance of DNA methylation: epigenetic insights into Sjögren's syndrome

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Sjögren's syndrome (SjS) is a systemic, highly diverse, and chronic autoimmune disease with a significant global prevalence. It is a complex condition that requires careful management and monitoring. Recent research indicates that epigenetic mechanisms contribute to the pathophysiology of SjS by modulating gene expression and genome stability. DNA methylation, a form of epigenetic modification, is the fundamental mechanism that modifies the expression of various genes by modifying the transcriptional availability of regulatory regions within the genome. In general, adding a methyl group to DNA is linked with the inhibition of genes because it changes the chromatin structure. DNA methylation changes the fate of multiple immune cells, such as it leads to the transition of naïve lymphocytes to effector lymphocytes. A lack of central epigenetic enzymes frequently results in abnormal immune activation. Alterations in epigenetic modifications within immune cells or salivary gland epithelial cells are frequently detected during the pathogenesis of SjS, representing a robust association with autoimmune responses. The analysis of genome methylation is a beneficial tool for establishing connections between epigenetic changes within different cell types and their association with SjS. In various studies related to SjS, most differentially methylated regions are in the human leukocyte antigen (HLA) locus. Notably, the demethylation of various sites in the genome is often observed in SjS patients. The most strongly linked differentially methylated regions in SjS patients are found within genes regulated by type I interferon. This demethylation process is partly related to B-cell infiltration and disease progression. In addition, DNA demethylation of the runt-related transcription factor (RUNX1) gene, lymphotoxin-α (LTA), and myxovirus resistance protein A (MxA) is associated with SjS. It may assist the early diagnosis of SjS by serving as a potential biomarker. Therefore, this review offers a detailed insight into the function of DNA methylation in SjS and helps researchers to identify potential biomarkers in diagnosis, prognosis, and therapeutic targets.

KEYWORDS

Sjögrens syndrome (SjS), DNA methylation, epigenetics, demethylation, autoimmunity, systemic autoimmune disease, T cells

Introduction

Sjögren's syndrome (SjS) is a chronic systemic autoimmune disease distressing a broad range (0.01% to 0.72%) of the general population (1). It predominantly distresses middle-aged individuals, particularly females, in their 40's to 60's (2). The symptoms of SjS evolve from simple dryness in the mouth and eyes to systemic, ultimately leading to lymphoma development (3). There is no radical cure for SjS, as the pathogenesis of SjS is not yet clear. The clinical spectrum is primarily characterized by sicca syndrome, resulting from immune-regulated glandular involvement. Besides that, musculoskeletal pain, fatigue, and systemic symptoms are present in a noteworthy fraction of diseased individuals. Lymphoma, however, presents as a complication in approximately 2%-5% of patients (4, 5). Significant features of the immunopathogenesis of SjS embrace increased B-cell activity, B-cell-T-cell interactions leading to ectopic lymphoid tissue formation, salivary epithelial cell dysfunction with increased apoptosis, autoantigen presentation, sustained elevation of proinflammatory cytokines, and increased level of genes regulated by type I interferon (6). Although the origin of SjS has not yet been completely clarified, it has been understood that environmental, genetic, and epigenetic factors significantly contribute to the progression of SjS, leading to the deregulation of epithelial cell function, inflammation, and autoimmune responses (7).

Genome-wide association studies (GWAS) confirm that genetic variations have been found to be associated with developing SjS in different populations. Genetic factors linked to SjS comprise human leukocyte antigen (HLA) allele subtypes and certain gene polymorphisms (8). However, these mutations only account for a small proportion of susceptibility to SjS. Although environmental factors, such as infections, e.g., the Epstein–Barr virus (EBV) infection (9), are considerably linked to the pathogenesis of SjS, no association was established between the reactivation phase of EBV and the onset of SjS symptoms (9). The specific roles of environmental factors, immunity, lifestyle, and genetics in the pathogenesis of SjS remain unclear. Thus, epigenetic mechanisms, for instance, non-coding RNAs, histone modifications, and DNA methylation, may act as a dynamic bridge between phenotypic expression, genome, and the environment through their regulatory impacts on the expression of genes (10).

A growing number of studies have revealed altered epigenetic landscapes in SjS, suggesting that epigenetic mechanisms contribute to SjS (3, 11–15). Studies in SjS have demonstrated the importance of DNA methylation patterns in the onset of disease and illuminated their role in the pathogenic behavior of various immune cells, cell-specific signaling pathways, and the activation/repression of downstream transcription factors (16, 17). As the cause of SjS is currently unknown and its development is multifaceted, there is still an absence of optimal drugs and treatment approaches. Recently, studies into DNA methylation within the context of SjS have observed a significant surge. This imitates the pivotal function of DNA methylation in the pathogenesis and progression of SjS. Cumulative evidence suggests an increasing focus on unraveling the complex interplay between DNA methylation patterns and the molecular mechanisms underlying SjS, thus identifying its potential as a critical regulator in SjS etiology. This review provides a significant understanding of the basic mechanics of SjS concerning DNA methylation and offers a promising avenue for identifying novel diagnostic, prognostic, and therapeutic targets.

Mechanism of DNA methylation and DNA demethylation

DNA methylation is manifested as a pre-transcriptional epigenetic modification in the DNA, including methylation and demethylation. In the course of DNA methylation, DNMTs, via S-adenosyl-methionine (SAM), transfer a methyl group to the carbon-5 spot of the cytosine pyrimidine ring (5mC) within cytosine-guanine (CpG) dinucleotides (18). Methylation of CpG sites results in structural deviations of chromatin that impede transcription factor binding. CpG islands, with an abundance of CpG base pairs, are primarily located in the first exon regions and promoter regions of the genes. Therefore, DNA methylation of CpG islands in chromatin is typically regarded as an indicator of gene repression (14). The gene regulation through DNA methylation has been illustrated in Figure 1.

The regulation of the DNA methylation group is strictly and dynamically controlled by both 'writers' and 'erasers' who modulate epigenetic marks in opposite directions through enzymatic activity. Enzymes known as DNA methyltransferases (DNMTs) have been identified as a class of enzymes that not only maintain methylated sites following DNA replication (DNMT1) but also place new methylation sites (de novo methyltransferases: DNMT3A and DNMT3B) (19). The ten-eleven translocation (TET) family and the DNA repair enzyme thymine DNA glycosylase (TDG) are the primary enzymes responsible for active DNA demethylation in mammals. Three mechanisms for demethylation have been identified: active demethylation (replication-independent), passive demethylation mediated by TET (replication-dependent), and 5mC deamination. In replication-independent active demethylation, TET dioxygenases oxidize 5mC to 5-hydroxymethylcytosine (5hmC), then to 5-formylcytosine (5fC), and finally to 5-carboxylcytosine (5caC) (20).

Previous research utilizing organisms lacking the DNMT1 enzyme demonstrated significant reductions in the methylation level of genomic DNA at CpG-rich repetitive regions and imprinted genes (21–23). In the deficiency of these enzymes, CpG-rich retroviral and intracisternal A particle (IAP) elements became slightly demethylated, while Igf-2 and Xist turned widely demethylated, according to recent studies using cells deficient in both the DNMT3A and -3B enzymes. This suggests that DNMT1 alone had sequence specificity for sustaining the methylation of these sequences (19).

Most 5mC in mammalian cells happens at DNA sequences involving CpG dinucleotides. Within human somatic cells, 70–80% of CpG sites are methylated, and the majority of unmethylated CpG sites are grouped on the CpG island, which is situated in the gene promoter region (24, 25). DNA methylation has been demonstrated to be crucial for several biological processes, such as X chromosome inactivation, genomic imprinting, chromosomal stability, and gene

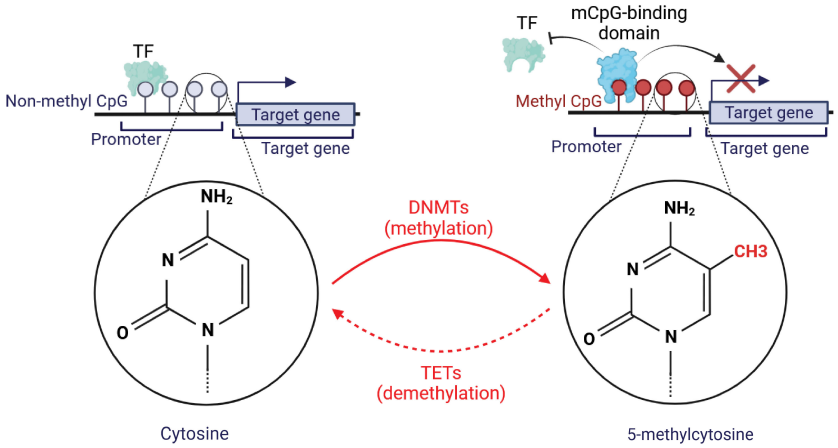

FIGURE 1
Graphical illustration of DNA methylation and its basic mechanism. The formation of novel DNA methylation patterns is modulated by methyltransferases (DNMTs). DNMTs aid in the formation of 5-methylcytosine from cytosine. This was followed by binding a methyl CpG-binding protein to methylated CpG sequences, which limits the ability of transcription factors (TFs) to reach this region, preventing the target genes' transcription. Conversely, Ten Eleven Translocation (TET) proteins may initiate the demethylation and convert 5-methylcytosine to cytosine. This demethylation promotes the binding of TFs with non-methylated CpG to accelerate the expression of target genes.

control (26). Numerous investigations have demonstrated that DNA methylation regulation is essential to mammalian developmental processes (27). A noteworthy example arises from the differentiation of stem cells. Hematopoietic stem cells (HSCs) give rise to all myeloid and lymphoid blood lineages (28). During this process, the methylation status of some genes (e.g., KCNH2, SUSD3) that determine cell destiny is highly controlled (29). On the other hand, several studies have demonstrated the connection between aberrant DNA methylation and the development of several human diseases (26, 30). This includes the activation of tumor promoter genes (such as MAGE and S100P) and the silencing of tumor suppressor genes (such as VHL and MLH1) in a variety of cancers (31, 32). Furthermore, studies have shown that aberrant methylation is a significant factor in the pathophysiology of neurological disorders like autism spectrum disorder, metabolic syndromes like hyperglycemia, and autoimmune diseases like idiopathic human lupus (33–35).

In broad terms, the inhibition of gene expression is tightly linked to DNA methylation in regions adjacent to the transcription start site (TSS) (36, 37). Based on accumulating data, transcriptional suppression of DNA methylation entails either the recruitment of transcription inhibiting factor (MeCP2) or the prevention of transcription activation factor (AP-2) from binding to TSS regions (38–40). Conversely, studies have suggested that increased transcriptional activity may result from gene body methylation (41, 42). Recent research has demonstrated that by ensuring the integrity of mRNA transcription beginning, DNA methylation on gene functions can shield the gene from spurious transcripts (43).

DNA methylation in autoimmune disease

As a crucial regulator in the development and differentiation of immune cells, impaired DNA methylation profiles may substantially induce autoreactivity in the immune cells. This predisposes a person to immune irregularities and increases the possibility of progression in autoimmune anomalies (44–46). Epigenetic modification via DNA methylation recently gained consideration as a potential biomarker (47, 48). Several genes sensitive to DNA methylation and are linked with systemic lupus erythematosus (SLE) and other autoimmune illnesses were discovered to be hypomethylated in lymphocytes, especially $CD4^+$ T cells. These genes include perforin (PRF1), integrin alpha L (ITGAL/CD11a), and lymphocyte function-associated antigen-1 (LFA1), which exhibit indistinguishable characteristics from $CD8^+$ T cells (48–50). It is evident that DNA methylation alteration exhibits a constructive correlation with the activity of autoimmune diseases (51). For instance, MX dynamin-like GTPase 1 (MX1) and type I interferon (IFN) are suggested as latent biomarkers for the activity of SjS and SLE diseases (51, 52), and *IFI44L* is also referred to as a signatory gene of the type I IFN signaling (53). Moreover, the promoter methylation of interferon-induced protein 44-like (*IFI44L*) is a biomarker that can be found in the blood and can be used to monitor changes in the activity of SLE (54). Several investigations have exhibited that RA patients have an altered DNA methylation pattern in T cells, monocytes, B cells, synovial fibroblasts, and PBMCs (55–57). Similar to this, hypomethylated areas in the promoters of the enzymes dual specificity phosphatase 22 *(DUSP22)* and cytochrome P450 2E1 *(CYP2E1)* were linked to erosive and active disease as observed in peripheral blood specimens from patients with RA (58). Less research has been done on DNA methylation's role in spondyloarthropathies (SpA) compared to RA (59, 60). Ankylosing spondylitis (AS) patients' peripheral blood has been found to have altered DNA methylation levels (61–63). In AS patients but not in healthy controls, the inflammatory gene SOCS-1 (suppressor of cytokine signaling 1) is methylated (64). Accumulating evidence suggests that DNA methylation contributes to autoimmune disease through various immune cells, cellular signaling, and the modifications of the

downstream transcriptional factors (54, 56, 58, 65–70). The function of DNA methylation in various autoimmune diseases is listed in Table 1 (54, 56, 58, 65, 67, 69–71). In conclusion, a thorough knowledge of the role of DNA methylation by characterizing its modifications and identifying approaches to modify and achieve the desired level and course of anti-autoimmune responses may provide possible strategies for improved monitoring, diagnosis, and mitigation of disease progression driven by epigenetic modifications.

Role of DNA methylation in SjS

DNA methylation is a reflection of the epigenetic status at a particular point that can impact the activity of disease through the modification of gene-level and downstream pathways (72). By engaging proteins entailed in gene repression or by preventing transcription factors from binding to DNA, DNA methylation controls gene expression (48, 73, 74). An EWAS examined the association of European League Against Rheumatism (EULAR) Sjögren Syndrome Disease Activity Index (ESSDAI) score with DNA methylation and discovered that patients with high ESSDAI had significantly more differentially methylated regions than those with low ESSDAI (75). The etiology of chronic fatigue, a leading reason for disability in patients with SjS, is not well stated. Norheim and colleagues performed an epigenome-wide DNA methylation patterns analysis to examine the possible involvement of DNA methylation in fatigue in SjS. The outcomes revealed 251 CpG sites with differential methylation, with the main finding being hypomethylation of a non-coding RNA in high fatigue (76). When compared to other epigenetic alterations, DNA methylation has a greater degree of stability, making it an effective marker for use as a diagnostic indicator (77). Over the recent years, several research teams have demonstrated that a range of changes in DNA methylation patterns are linked to autoimmune rheumatic diseases. These alterations have been found to be associated with different subtypes of these diseases, as well as with their activity levels and overall severity.

Lymphotoxin-α (*LTA*), belonging to the TNF superfamily and secreted by $CD8^+$, Th17, B cells, and NK, is recognized for its pro-inflammatory properties (78). Mechanistically, *LTA* operates through two distinct signaling routes. Firstly, as an *LTA* trimer, it binds to TNF1 and TNF2 receptors, driving lymphangion genesis and elevating the secretion of chemokines and cytokines (79–82). Secondly, it competes with *lymphotoxin-β (LTB)* to shape a trimeric ligand that binds to the *LTB* receptor on lymphoid cells and stimulates various pathways, such as NF-KB (83–85). The hypomethylation at numerous regions of *lymphotoxin-α (LTA)* has been found (81, 86). It is interesting to note that genetic variations in LTA are linked with vulnerability to SjS, as *LTA* has been found in the salivary glands of SjS patients (87, 88), thereby acting as a critical factor in the progression of SjS (89). It was estimated that deletion of *LTA* in IL-14α transgenic mice resulted in normal salivary gland secretion rate and no lymphocytic infiltration (89). A recent work by Altorok et al. (52) employing genome-wide DNA methylation has shown that naïve T cells of pSjS had hypomethylated *LTA* gene. Additionally, pSjS and several SNP in

TABLE 1 Compilation of studies regarding autoimmune rheumatic diseases and DNA methylation in cells.

Reference	Study design for DNA methylation	Cell types	Main outcomes for DNA methylation
Lu Q, et al. (65)	Bisulfite sequencing was used to determine the methylation status of the *ITGAL* promoter and flanking regions in T cells from lupus patients and healthy subjects.	T cells in SLE	Bisulfite sequencing was used to determine the methylation status of the *ITGAL* promoter and flanking regions in T cells from lupus patients and healthy subjects and in T cells treated with DNA methylation inhibitors.
Lu Q, et al. (71)	Decreased global DNA methylation, T cell DNA was isolated, and bisulfite treated using published protocols, then the promoter and enhancer were amplified using nested primers to determine whether *CD40LG* on the inactive X demethylates and is overexpressed uniquely in women with lupus	T cells in SLE	promoter and enhancer demethylation may cause *CD40LG* overexpression on $CD4^+$ T cells in women but not men with lupus.
Miller S, et al. (67)	Genome-wide DNA methylation of lupus and age, sex, and ethnicity-matched control $CD8^+$ T cells was measured using the Infinium Methylation EPIC arrays	$CD8^+$ T cells in SLE	55% of genes had hypermethylated CpG sites, 38% had hypomethylated CpG sites, and the remaining 7% of genes exhibited a mixed methylation pattern at CpG sites in $CD19^+$ B cells. Genes with significantly different DNA methylation patterns are involved in functional pathways required for B cell signaling, inflammation and autoreactivity.
Zhao M, et al. (54)	*IFI44L* promoter methylation was examined using DNA samples from a discovery set including 377 patients with SLE, 358 healthy controls	Whole blood cells in SLE	The methylation levels of Site1 and Site2 within the *IFI44L* promoter were significantly lower in patients with SLE with renal damage than

(Continued)

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TABLE 1 Continued

Reference	Study design for DNA methylation	Cell types	Main outcomes for DNA methylation
	and 353 patients with RA		those without renal damage. Patients with SLE showed significantly increased methylation levels of Site1 and Site2 during remission compared with the active stage.
Sun X, et al. (69)	Bisulfite sequencing was performed to determine the methylation status of the *FOXP3* proximal promoter sequences	$CD4^+$ T cells in systemic sclerosis	in $CD4^+$ T cells from patients with systemic sclerosis, treatment with all-*trans* retinoic acid, a natural derivative of vitamin A, increases the expression of *FOXP3* and, subsequently the proportion of T_{reg} cells by promoting demethylation of the *FOXP3* promoter.
Julià A, et al. (70)	CpG methylation in isolated B lymphocytes was assayed on the Illumina HumanMethylation450 BeadChip in a discovery cohort of RA patients (N =50) and controls (N=75). Differential methylation was observed in 64 CpG sites	B cells in RA	in $CD19^+$ B cells, many relevant genes are differentially methylated in patients with RA compared to healthy individuals. These genes include *CD1C*, *TNFSF10*, *PARVG*, *NID1*, *DHRS12*, *ITPK1*, *ACSF3* and *TNFRSF13C*, all of which were identified in a discovery cohort of patients with RA and validated in an independent cohort.
Mok A, et al. (58)	Fluorescence-activated cell sorting was used to separate the cells into 4 immune cell subpopulations ($CD14^+$ monocytes, $CD19^+$ B cells, $CD4^+$ naive T cells, and $CD4^+$ memory T cells), and 229 epigenome-wide	$CD14^+$ monocytes, $CD19^+$ B cells, $CD4^+$ naive T cells, and $CD4^+$ memory T cells in RA	Differential methylation of CpG sites associated with clinical outcomes was observed in all 4 cell types. Hypomethylated regions in the *CYP2E1* and

(Continued)

TABLE 1 Continued

Reference	Study design for DNA methylation	Cell types	Main outcomes for DNA methylation
	DNA methylation profiles were generated using Illumina HumanMethylation450 BeadChips		*DUSP22* gene promoters were associated with active and erosive disease, respectively.
Rodríguez-UbrevaJ, et al. (56)	High-throughput DNA methylation analyses of patients with RA and controls and in vitro cytokine stimulation were used to investigate the underlying mechanisms behind DNA methylation alterations in RA as well as their relationship with clinical parameters, including RA disease activity	peripheral blood monocytes in RA	The DNA methylomes of peripheral blood monocytes displayed significant changes and increased variability in patients with RA with respect to healthy controls. Changes in the monocyte methylome correlate with DAS28.

SLE, systemic lupus erythematosus; ITGAL, lymphocyte function-associated antigen-1 (LFA1), integrin alpha L; CpG sites, cytosine guanine dinucleotide (CpG) sites; IFI44L, interferon-induced protein 44-like; FOXP3, forkhead box protein 3; RA, Rheumatoid arthritis; CYP2E1, cytochrome P450 2E1; DUSP22, dual specificity phosphatase 22; DAS28, Disease Activity Score in 28 joints.

the *LTA/LTB/TNFα* locus have been shown to strongly correlate as found by single-nucleotide polymorphism (SNP) analysis (87).

Meanwhile, *IFI44L* (interferon-induced protein 44-like) promoter undergoes DNA methylation in various autoimmune diseases (51, 90). Also, *IFI44L* is a recently discovered gene that has been implicated in predisposing people to certain infectious diseases. The analysis of genome-wide DNA methylation patterns identified hypomethylation of I*FI44L* in whole blood in SjS as the most significant finding in these two large-scale studies. *IFI44L* has been identified as a signature gene for the IFN-I pathway in SjS (52, 53, 75). It was estimated that methylation of the *IFI44L* promoter could accurately differentiate between patients with SLE and healthy individuals with a high degree of sensitivity and specificity (54). However, a recent study used the microarray and sc-RNA analysis and concluded that *IFI44L* is a highly expressed shared gene in SLE and SjS (91). Additionally, several genes, including *BLK* (B lymphoid tyrosine kinase), *STAT4* (signal transducer and activator of transcription 4), *CXCR5* (C-X-C chemokine receptor type 5), *IL12A* (interleukin-12 subunit alpha), *TNIP1* (TNFAIP3-interacting protein 1), and *IRF5* (interferon regulatory factor 5), have been suggested as potential gene candidates for susceptibility to SjS in various studies (92). The pathophysiology underlying SjS is complex. Thus, only a few studies have explored the attribution of DNA methylation in SjS. It has been reported that the most frequent modification observed in the genome of SjS patients is the demethylation of several sites (6, 93, 94).

Type I interferon (IFN-I) is known to perform a crucial function in the pathophysiology of autoimmune diseases,

particularly in the progression of SjS (95, 96). Interestingly, studies indicate that the most strongly linked differentially methylated positions and regions in SjS patients are situated within genes regulated by type I interferon (13). Interferon-activated Myxovirus-resistance proteins (Mx) are an excellent tool for assessing IFN-I in autoimmune disorders (97), especially distinguishing the SjS using serum and saliva (98). Among various Mx proteins, MxA is the most applicable and suitable biomarker for SjS (99). MxA levels are linked to signs of active disease and decrease in patients treated with hydroxychloroquine, indicating the potential clinical usefulness of MxA in categorizing patients based on IFN positivity (99, 100).Activation of the IFN-I pathway is observed as one of the vital pathways in the pathophysiology of SjS, and it is particularly pronounced in individuals who have antibodies to SSA/SSB (101, 102). However, the connection between IFN-regulated genes and DNA methylation is complex and multifaceted, especially in SjS. Both SLE and SjS patients have demonstrated significant cell-specific epigenome-wide and genomic-wide hypomethylation of IFN-regulated genes in the epithelial cells from minor salivary gland as well as multiple tissues, with numerous sites being linked to augmented levels of IFN-regulated genes (13, 100, 103). The interferon regulatory factor 5 (IRF5) gene, which encodes a transcription factor that contributes to the modulation of IFN-induced genes and synthesis of IFN-α, is the most important genetically susceptible locus for SjS irrespective of the HLA region (104, 105). An earlier study examined the differential methylation positions and regions in whole blood. It was found that IFN-I-regulated genes were enriched in the differentially methylated positions and regions with the strongest associations. Additionally, these areas were found to be improved in pathways associated with extracellular matrix assembly and collagen metabolism. Moreover, the identified epigenetic signatures were exclusively detected in patients who tested positive for anti-SSA/Ro antibodies (106). Likewise, another study identified significant genome-wide hypomethylation of IFN-modulated genes in B cells and whole blood (100).

Besides the importance of IFN-I in SjS, the calcium and Wnt pathways were identified as important regulatory molecules in the salivary gland epithelial cells (SGECs) in the course of SjS progression. Cell-specific epigenome-wide analysis showed that genes involved in these pathways are enriched for hypomethylation and hypermethylation at differentially methylated CpG sites (DMCs) (103). The alteration in the DNA methylation of critical pathways provides a theoretical basis for a therapeutic target in SjS (Table 2) (66, 68, 75, 76, 105, 107–110, 112).

Our understanding of the relationship between these pathways involved in DNA methylation and gene regulation is evolving, as epigenetic regulation is a multifaceted process prejudiced by various factors. Meanwhile, the interplay between these signaling pathways and DNA methylation is more likely depending on the context and is highly intricate. Further research is required to uncover more details about these interactions and their implications for SjS. Interestingly, besides providing novel therapeutic targets, using DNA methylation arrays in a clinical setting can also advance researchers understanding of classifying SjS phenotype (112).

TABLE 2 Compilation of studies regarding Sjögren's syndrome and DNA methylation in cells.

Country Reference	Study design for DNA methylation	Cell types	Main outcomes for DNA methylation
Norway BrækkeNorheim K et al. (76)	Methylation analysis of patients in pSS patients with high or low fatigue	Whole blood	251 differentially methylated CpG sites were associated with fatigue. The CpG site with the most pronounced hypomethylation in pSS high fatigue annotated to the SBF2-antisense RNA1 gene. The most distinct hypermethylation was observed at a CpG site annotated to the lymphotoxin alpha gene.
France Thabet Y et al. (66)	Methylation analysis and transcript levels of DNMTs in patients with and without SS	Peripheral B and T cells; SGECs	Global demethylation and reduction in DNMT1 transcript levels in SGECs of SS patients. No differences in methylation profile for B and T cells.
France Gestermann N et al. (105)	Methylation analysis of IRF5 promoter region to determine if this could be the cause for increased IRF5 mRNA expression in patients with SS	Peripheral B and T cells	DNA methylation profile of T CD4+ and B lymphocytes did not differ between SS patients and controls.
France Miceli-Richard C et al. (75)	Methylation comparison in profiles in the $CD4^+$ T cells and $CD19^+$ B cells of pSS patients and controls	$CD19^+$ B cells	55% of genes had hypermethylated CpG sites, 38% had hypomethylated CpG sites, and the remaining 7% of genes exhibited a mixed methylation pattern at CpG sites in $CD19^+$ B cells. Genes with significantly different DNA methylation patterns are involved in functional pathways required for inflammation.
China Yu X et al. (68)	Methylation analysis of *FOXP3* promoter region to determine whether the *FOXP3* promoter is hypermethylated and whether aberrant *FOXP3*	$CD4^+$ T cells	Hypermethylation at the promoter of *FOXP3* in $CD4^+$T cells of pSS patients. A decrease in expression in protein *FOXP3* mRNA and protein in $CD4^+$T cells of pSS patients.

(Continued)

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TABLE 2 Continued

Country Reference	Study design for DNA methylation	Cell types	Main outcomes for DNA methylation
	expression occurs in $CD4^+$ T cells from patients with pSS		
China Yin H et al. (107)	Methylation analysis of TNFSF7 promoter region	$CD4^+$ T cells	Hypermethylation at the promoter of TNFSF7 in pSS $CD4^+$T cells. Demethylation of the CD70 promoter regulatory elements contributes to CD70 overexpression in pSS $CD4^+$ T cells and may contribute to autoreactivity.
France Belkhir R et al. (108)	methylation analysis of CD40L promoter region	$CD4^+$ T cell	No significant differences in methylation profiles between patients and controls.
Greece Mavragani CP et al. (109)	Methylation analysis of LINE-1; expression analysis of DNMT1, DNMT3A, DNMT3B, MeCP2 in SS patients and controls	SGECs	↑ levels of DNMT1, DNMT3B and MeCP2 transcripts in SS patients.
France Konsta OD et al. (110)	Methylation analysis at SSB promoter region in patients with pSS and cell cultures	SGECs	↓ global DNA methylation levels in patients with SS. ↓ global methylation associated with lymphocyte infiltration in MiSG; ↓ global methylation associated with anti-La/SSB positive SS cases demethylation at SSB promoter caused higher levels of transcripts and SSB super expression in 5-Aza-C-treated HSG cells.
France Konsta OD et al. (111)	Methylation analysis of the KRT19 locus to investigate epigenetic regulation of expression in patients with SS	SGECs	↓ global DNA methylation in pSS patients is associated with demethylation of the KRT19 locus as well as with overexpression of the KRT19 protein.
USA Chi C et al. (112)	Methylation analysis in patients with and without SS	SGECs	215 DMRs in SS patients: 169 hypermethylated regions related to nervous system development, cell signaling and transport; and 46 hypomethylated regions related to immune function.
USA Cole MB et al. (113)	Methylation analysis in patients with varying phenotypes of SS	SGECs	7,820 DMPs associated with disease status (5,699 hypomethylated and 2,121 hypermethylated DMPs); 57 of the genes with DMPs are involved with the immune response; extensive hypomethylated region near genes PSMB8 and TAP1.
Chile Sepúlveda D et al. (114)	Methylation and expression analysis in genes of the IRE1α/ XBP-1 pathway in SS and control patients	SGECs	Hypermethylation in IRE1α, XBP-1 and GRP78 promoter region and diminished transcript levels; ↓ protein levels for IRE1α, XBP-1s and GRP78 in SS patients.

SS, Sjögren's syndrome; pSS, Primary Sjögren's syndrome; SGECs, salivary gland epithelial cells; CpG sites, cytosine guanine dinucleotide (CpG) sites; DNMTs, DNA methyltransferase 1; MiSG, minor salivary gland; FOXP3, fork head Box Protein 3; LINE-1, long interspersed repeat element 1; anti, SSB, anti-Sjögren's syndrome antigen B; HSG, human salivary gland; KRT19, keratin 19; DMRs, Differentially methylated regions; DMPs, differentially methylated positions; IRE1α, inositol-requiring enzyme 1alpha; XBP-1s, X-box binding protein 1s; GRP78, glucose-regulating protein 78.

DNA methylation in various cells during SjS progression

The immune system involves various types of immune cells, each with specific functions for the progression of autoimmune diseases (115). In the context of SjS, immune cells, particularly lymphocytes, infiltrate the affected glands and contribute to inflammation and tissue damage. These immune cells release cytokines and other signaling molecules that further promote inflammation and disrupt normal gland function (116, 117). The interaction between SjS and immune cells is complex. While attempting to regulate the autoimmune response, immune cells can inadvertently contribute to the damage to glandular tissues. Additionally, the chronic inflammation associated with SjS can have systemic effects beyond the glands, impacting various organs and tissues throughout the body (7, 118). Recent studies show that DNA methylation is applied to many cell types implicated in the pathophysiology of SjS. Among these, SGECs, lymphocytes, and monocytes (17, 88) are significant factors contributing to the SjS progression after being subjected to DNA methylation. As evidence increases, these differential DNA methylation genes in immune and non-immune cells may be used as candidate biomarkers to predict SjS. Here, we have discussed the importance of DNA methylation in these cell types and how it plays an imperative function in SjS (Figure 2).

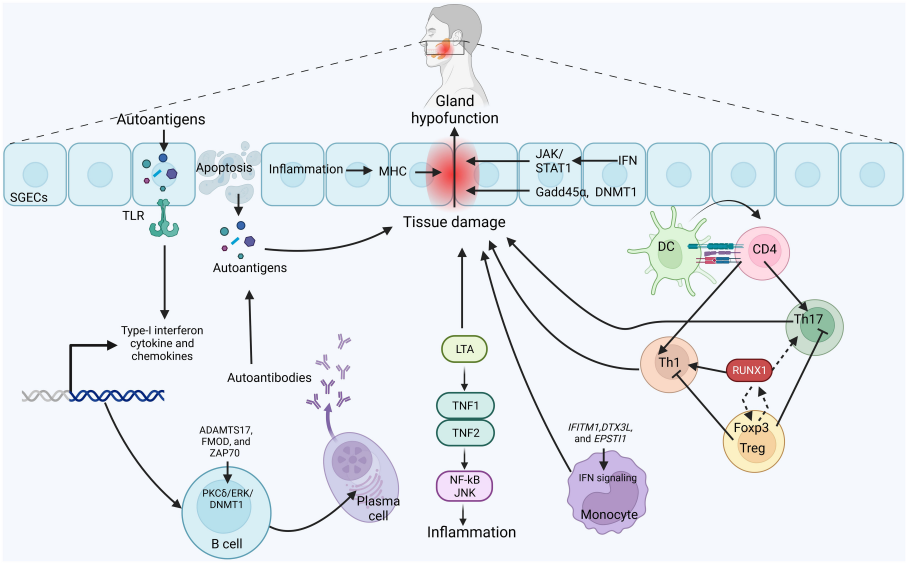

FIGURE 2
Possible mechanism of DNA-methylation in the pathogenesis of SjS: Activation of toll-like receptors (TLRs) signaling, predominantly activated in the salivary gland epithelial cells (SGECs), produces autoantigens which enhances the production of immunocompetent cytokines and chemokines and cytokines. These lead to the apoptosis of SGECs, epithelial hypofunction and tissue damage. Autoantigens can be released from SGECs and presented to immune cells. CD4+ T cells differentiate into inflammatory Th1 and Th17 to participate in the tissue damage. Immunosuppressive Tregs inhibit the activity of inflammatory Th1 and Th17 cells. Methylation of RUNX1 activates the Th1 cells, whereas elevated RUNX1 binds with Foxp3 in Tregs, leading to transcriptional modification and enhancing its expression, which in turn inhibits the function of RUNX1. Meanwhile, LTA binds with TNF1 and TH=NF2 to activate JNK/NF-kB signaling, leading to inflammation. In addition, DNA methylation pattern of ADAMTS17, FMOD and ZAP70 is altered in a PKCδ/ERK/DNMT1-dependent manner. Increased B cells also activate plasma cells to produce antibodies against autoantigens. Meanwhile, DNA methylation of IFITM1, DTX3L and EPSTI1 activates the IFN signaling in monocytes to alleviate inflammation and tissue damage. Strikingly, inflammation-induced DNA methylation regions in major histocompatibility complex, IFN signaling induced JAK/STAT1 signaling, and altered levels of Gadd45α level and DNMT1 in SGECs also participates in the pathogenesis of SjS.

DNA methylation in T cells

T cells, including $CD4^+$ and $CD8^+$ T cells, are overwhelming infiltrators in most phases of the SjS (116). Specifically, the engagement of various T-cell subsets in Sjögren's syndrome (SjS) underscores the remarkable complexity of the disease's pathogenesis (116). It was determined that in SjS, T cell activation results in tissue damage, B cell activation, inflammatory cell infiltration, and metabolic alterations (119). DNA methylation is crucial for the differentiation of T cells (120, 121). In the process of becoming activated and differentiating from naive $CD4^+$ and $CD8^+$ T cells to effector cells, there are alterations in the DNA methylation patterns of the promoters associated with effector genes, such as Il2, Tnfa, and Ifng. These changes in DNA methylation may exhibit a progressive decline throughout differentiation (122). The initial Epigenome-Wide Association Study (EWAS) in SjS focused on examining DNA methylation in immature $CD4^+CD45RA^+$ T cells isolated from 11 female SjS individuals and 11 matching controls. The authors found that there was hypomethylation of multiple sites of *LTA* and IFN-regulated genes, including interferon-induced transmembrane protein 1 *(IFITM1)*, activators of transcription 1 *(STAT1)*, and *IFI44L* (52). Similarly, in another study, four hypomethylated *STAT1*, homo sapiens SH3 and PX domains 2A (*SH3PXD2A*), friend leukemia virus integration (*FLI)37453*, ubiquitin-specific peptidase 18 *(USP18)* and one hypermethylated gene F-box and leucine-rich repeat protein 16 *(FBXL16)* were found in T cells (75). Likewise, Yin et al. found that CD70 overexpression results from demethylation of the CD70 promoter regulatory regions in SjS CD4+ T cells, which may be a factor in their autoreactivity (107).

Among different subsets of $CD4^+$ T cells, regulatory T cells (Tregs) are fundamental in maintaining normal physiology during the progression of SjS (123). The stability and role of $Foxp3^+$ Tregs is highly reliant on DNA methylation (124). It was suggested that reduced expression of *FOXP3* in $CD4^+$ T cells in SjS is associated with DNA hypermethylation (68). RUNX1 has been known to perform essential roles in developing the granular convoluted tubules (125). It was studied that *RUNX1* is tangled in the membrane trafficking of particular proteins of the acinar cells in the submandibular gland, which further permits the proper secretion of saliva (126). Taking into account the function of *RUNX1* in saliva secretion, Altorok and colleagues have reported hypermethylation of the transcriptional factor RUNX1 in SjS individuals (52), which acts on regulating the development of HSCs (hematopoietic stem cells). The CNS2 region of Foxp3 has CpG islands, which undergo differential methylation. Conversely, Runx1, along with Cbfβ, demethylates Foxp3 by directly binding with the CNS2 element of Foxp3 (127, 128), thereby regulating the Foxp3 transcription by altering its chromatin structure (129). Meanwhile, RUNX1 expression has been linked to a susceptibility to cancer, implying a potential link to a predisposition to lymphoma in SjS patients (130). Collectively, RUNX1 enhances Foxp3, which in turn inhibits the RUNX1 activity and transforms RUNX1 from an activator into a

repressor, or Foxp3 also contributes to Runx in activating or repressing its downstream target genes (129). Additional research is required to clarify the epigenetic processes underlying the pathophysiology of SjS since the function of DNA methylation in T cells is still unclear. These investigations have the possibility of significantly improving our understanding of SjS and establishing the groundwork for innovative, tailored therapeutic interventions.

DNA methylation in B cells

A multistep mechanism leads to the overactivation of B lymphocytes, which is crucial in the pathophysiology of SjS (117). Salivary epithelial cells, the targets for SjS, continue to interact with subpopulations of B cells, which in return help to activate an autoimmune reaction in tissues by producing autoantibodies and consequently form immune complexes (131). It has been reviewed that aberrant expression of translational factors and modifications in the epigenetics in B cells is highly correlated with anomalous B cell functions in multiple diseases, including autoimmune diseases (132). For instance, analysis of differentially expressed and methylated genes shows the alterations of expression patterns and DNA methylation patterns of *ADAMTS17*, *FMOD*, and Z*AP70* in chronic lymphocytic leukemia (CLL) (133). They found that *ADAMTS17* was hypermethylated in the gene promoter region and hypomethylated in the gene body region. In contrast, *FMOD* and *ZAP70* were hypomethylated in the promoter region (133). Regarding the involvement of B cells in SjS, abundant genes with differential DNA methylation in genetic at-risk loci (*HLA-DRA, HLA-DQB1*, *IRF5*) were observed (66, 75). Methylation changes in B cells were common in patients who were positive for autoantibodies in a number of particular pathways, including IFN-modulated genes (75). Miceli-Richard and colleagues conducted an analysis of genome-wide methylation patterns in two distinct immune cell populations, namely peripheral CD4+ T cells and B cells, within a cohort of 26 women diagnosed with SjS and 22 control subjects with same age. Their study revealed more significant differences in DNA methylation patterns among B cells, as opposed to T cells, when comparing patients with SjS to the control group (75). Meanwhile, in an investigation by Altorok et al., only 119 differentially methylated CpG sites (DMCs) were found in $CD4^+$ T cells, which is interesting because they used a lower cutoff level of 0.07 to detect differential methylation between patients and controls. On the other hand, B cells showed a strikingly larger number of 6,707 DMCs, impacting 3,619 genes. In these DMCs, SjS patients had hypomethylation at 44% of the differentially methylated locations, as revealed by genome-wide DNA methylation patterns. Notably, several of the genes activated by IFN were connected with certain hypomethylated CpG sites in B cells, indicating a potential connection between DNA methylation alterations and immunological activation in these cells (52). Imgenberg-KreuzJ et al. conducted a deeper look at the methylation of the whole genome in peripheral B lymphocytes. They determined that 5623 distinct genes had different levels of methylation, with the majority of these hypomethylated regions being assigned to genes that participate in immune response pathways, particularly IFN-regulated *MX1*, *IFI44L*, *poly* (*ADP-ribose*) *polymerase 9 (PARP9)* and *IFITM1* (100). Moreover, Thabet Y et al. conducted a study to analyze the global DNA methylation in the salivary gland epithelial cells (SGEC) and peripheral B and T cells from SS patients. They found that the overall methylation was decreased in SGECs. Surprisingly, by co-culturing human salivary gland cells and B cells, authors have found that SGEC demethylation may be caused in part by invading B cells, as suspected in patients treated with anti-CD20 antibodies to reduce B cells (66). Mechanistically, it was suggested that DNA demethylation mediated by B lymphocytes could be due to changes in the PKCδ/ERK/DNMT1 pathway as using rottlerin, PD98059 and 5-azacytidine to inhibit PKCδ/ERK/DNMT1 signaling reduces global DNA methylation in SGECs; however, when patients get the anti-B cell mAb rituximab, this process can be reversed (66, 111). All these findings suggest that anomalous B cell activation and cytokine secretion contribute significantly to the immunopathogenesis of SjS, and investigating the abnormal DNA methylation changes in B cells may highlight the potential for DNA methylation-targeted therapies to modulate the core anomalies driving the disease.

DNA methylation in monocytes

There is evidence to suggest that the monocyte is a crucial actor linking multiple immune responses (134), as it performs a major role in the SjS (135). Over previous years, interest has grown in the impact of monocytic cells in the development of SjS. There is evidence that IFN-signaling and viral infection-related pathways are highly upregulated in monocytes involved in the pathogenesis of SjS (136). Like B and T cells, the function and biology of monocytes are also influenced by DNA methylation, predominantly in autoimmune diseases (134, 137). In circulating monocytes from individuals with SjS, DNA methylation alterations appear primarily as hypomethylation. Hypomethylation in *IFITM1, myxoma resistance1, PARP9, deltex E3 ubiquitin ligase 3L* (*DTX3L*), and epithelial-stromal interaction 1 (*EPSTI1)*, which eventually effect the IFN signaling in SjS monocytes, was testified. In patients with SSA/SSB autoantibodies, differently methylated genes were present in the ribosome and involved in AMP-activated protein kinase (AMPK) signaling pathway (138). Thus, changes in methylation may have an effect on IgG production through the influence of monocyte differentiation (139). These discoveries underscore the significance of gene regulation by DNA methylation in the dysfunctional classical monocytes across SjS patients.

Association of salivary gland epithelial cells with DNA methylation

Aberrant DNA methylation has also been implicated in non-immune cells. Considering that SGECs play an essential function in the emergence of SjS (140), it has been found that SGECs also play a significant function in the immune regulation in the pathophysiology of SjS and influencing the initiation and

continuation of autoimmune response and inflammation (7, 141, 142). As SjS advances, the fundamental variations in the proteome of SGECs between SjS and healthy controls provide tangible evidence of SGEC transformation into an innate immune cell (143). This shift is coupled with a notable redirection of cellular metabolism. These metabolic shifts primarily center around mitochondrial processes, also reflected in the structural changes observed within the cells (143). Conversely, IFN-γ-meditated ferroptosis of SGEC exacerbates SjS pathogenesis through JAK/STAT1, signifying the function of ferroptosis in SGECs in SjS-associated immunogenicity and inflammatory responses (144). Disparity in DNA methylation in salivary gland tissues can exacerbate the progression of autoimmune diseases (50, 112). Minor salivary gland-based epigenome-wide DNA methylation found a decreased global DNA methylation in the SGECs from SjS patients. SGEC demethylation in SjS patients was linked with a 2-fold upsurge in *Gadd45α* level and a 7-fold reduction in DNMT1 (66). A genome-wide methylation investigation was conducted by Cole et al. using minor salivary glands from 13 SjS patients and 13 control participants. In SjS, a study utilized genome-wide DNA methylation analysis on the human labial salivary gland biopsy specimens and discovered 7820 sites had variable methylation, of which 5699 had hypomethylation, and 2121 had hypermethylation (113). Likewise, genome-wide DNA methylation analysis conducted by Imgenberg-Kreuz et al. found 45 differentially methylation locations in minor salivary gland samples from 15 SjS individuals and 13 controls, with the IFN-induced gene *OAS2* having the most substantially hypomethylated site (100). Similarly, Konsta et al. (2016) showed a potential correlation between abridged DNA methylation in minor salivary glands and up-regulation of the KRT19 (keratin-19) in glandular acini. In a following investigation, the incubation of a human salivary gland cell line with the DNMT antagonist 5-azacytidine led to the amplification of the mRNA level of KRT19 and protein level of cytokeratin-19 (110, 111, 145). Moreover, another study thoroughly performed epigenomic-wide association study and analyzed 131 samples of labial salivary glands (LSGs) and illustrated that the major histocompatibility complex (MHC) region has a large number of DMRs, which are hypomethylated in genomic regions implicated in the immunological response in LSGs. To address the challenges posed by cellular heterogeneity, Charras and colleagues conducted their study using long-term cultured SGECs that were obtained from minor salivary glands from 8 individuals diagnosed with SjS and 4 control subjects. It is noteworthy that 2650 genes had 4662 differential methylation sites, among which 21% exhibited hypomethylated in SGECs from SjS. Interestingly, the data attained from these SGECs was in accordance with the data from whole minor salivary glands, as IFN-regulated genes were postulated as differentially methylated genes (103). HLA region constitutes almost 50% of the altered methylated regions, with the matching methylation quantitative trait loci (meQTLs) in the regions encircling the HLA-DQA2, HLA-DQB1, and HLA-DQA1 loci (146). This research identified unusual DNA methylation changes in SGECs. It highlighted the prospect role of HLA class DNA methylation modifications and other major pathways and genes in the pathophysiology of SjS. Non-immune cell epigenetic changes have been found to be more similar to autoimmune disease-induced inflammatory responses rather than being directly related to SjS (14). It has been suggested that these epigenetic changes may be part of the pathogenesis of SjS, but are not the direct cause (14). It is necessary to consider multiple factors, including autoimmune reactions, inflammatory responses, and non-immune cell epigenetic changes, for the diagnosis and treatment of SjS. Currently, the role of non-immune cell epigenetic changes in SjS is still unknown, which requires further research and exploration.

Targeting DNA methylation as a potent therapeutic approach in SjS

SjS has no cure at present. The clinical treatment approaches currently in use and the available biomarkers can barely halt the advancement of SjS and cannot entirely anticipate how the disease will progress. To meet clinical needs, new biomarkers and molecular targets are instantly desirable. Many clinical trials for SjS-related drugs have failed to meet the primary endpoint due to unclear SjS assessment criteria, making it difficult to determine the extent to which the symptoms reflect underlying pathological biology (147). The emergence of epigenetic regulation, particularly DNA methylation, offers new insights into the treatment of SjS. Drugs related to DNA methylation mainly include traditional DNA hypomethylating agents (HMAs), such as decitabine (DAC) and azacitidine (AZA) (148). Even though epigenetics is a relatively new discipline that gradually emerged in the 1980s, HMAs were only approved in the 21st century for treating hematopoietic system tumors (149). Currently, there is a lack of evidence for the use of passive demethylation agents in SjS treatment.

In the last five years, much consideration has been given to Treg cells in DNA methylation studies of autoimmune diseases. Studies on other autoimmune rheumatic diseases have also demonstrated a correlation between autoimmunity and reduced *FOXP3* promoter DNA methylation. *FOXP3* promoter hypermethylation leads to reduced levels of *FOXP3* in $CD4^+$ lymphocytes in SjS (68). In $CD4^+$ lymphocytes derived from individuals afflicted with Systemic Sclerosis (SSc), the application of a naturally occurring vitamin A derivative (all-trans retinoic acid) upregulates *FOXP3* expression and, consequently, elevates the population of Tregs by inducing *FOXP3* promoter demethylation (69), which may act as a target for treatment in SjS.

Aberrant DNA methylation in B lymphocytes implies their role in the pathogenesis of SjS (100). B cells significantly contribute to most autoimmune conditions, as seen by the prevalence of autoantibodies in autoimmune rheumatic disorders and the efficiency of B cell-depleting therapy in certain conditions (150). The severity of SjS and B cell infiltration has been found to be negatively associated with the DNA methylation levels in SGECs. Furthermore, administering the anti-CD20 monoclonal antibody rituximab has been testified to increase the DNA methylation levels in the SGECs of SjS patients (151). It has been proven that a number of genes transcribed by SGECs, including B-cell activating factor (BAFF), aquaporin-1/5, and IFN pathway, are susceptible to the

effects of rituximab (152, 153). Anti-B cell treatments that indirectly restore DNA methylation in SGECs bring up new therapeutic possibilities for SjS (154).

The activation of the IFN-I system is widely considered a crucial mechanism in the pathophysiology of SjS. Once elevated levels of IFN-α and downstream activation of interferon-stimulated genes (ISGs) were discovered in patients with SjS, strategies targeting IFN-I were promptly developed. Although not specific to hindering type I IFN signaling, various small molecule kinase inhibitors that are targeted at Janus Kinases (JAKs) are being trialed clinically for SjS, such as filgotinib (JAK1 inhibition) and lanraplenib (formerly GS-9876, Tyk2 inhibition) (94, 114, 145).

Conclusion and perspectives

Recent genetic and epigenetic research into SjS has unveiled several underlying genes responsible for the disease, with immune cells playing a crucial role in its development. These important genetic and epigenetic discoveries have the potential to address several clinical needs, including improved diagnosis, patient classification, predictive indicators of associated diseases, for instance, heart disease and lymphoma, and eventually, more effective treatments to alleviate symptoms, halt progression, and restore organ function. These procedures are linked to alterations that modify DNA, known as epigenetic modifications. These changes can affect gene expression and cell behavior. Researchers have tested several treatments for these processes, but many more are still in the discovery phase. In particular, the calculation of epigenetic risk scores offers the probability for improved classification of the subtypes of the disease and must be considered in subsequent clinical studies (155).

DNA methylation changes are being recognized as a crucial component of SjS genesis and progression (48). A notable observation is the limited connection between epigenetic signals and identified genetic risk loci in the context of SjS. This lack of concurrence implies that numerous genes contributing to the heightened risk of SjS likely operate upstream of cellular processes. These genes may trigger epigenetic alterations and disparities in gene expression without undergoing direct epigenetic modifications, presenting intriguing molecular targets for investigation.

Strong evidence implicates interferon pathways in SjS, as evidenced by disease associations with genetic variation in genes within these pathways. SjS patients show significant hypomethylation of ISGs. The majority of hypomethylation sites are related to augmented levels of ISGs. These observations confirm previous findings linking IFN activity to disease activity and emphasize the DNA methylation pattern stability (100, 103). Additionally, there is noteworthy hypomethylation observed in genes regulated by interferons. Genetic and epigenetic insights reinforce the robust correlation between the disease and the human leukocyte antigen (HLA) locus, a recognition spanning decades.

Epigenetic modifications exhibit variations across different tissues and cell types, yet a discernible pattern is beginning to surface within the context of SjS. This pattern entails extensive DNA methylation alterations, affecting T and B cells and the target tissue. A forthcoming challenge involves identifying drugs capable of selectively reverting these epigenetic changes. This sets them apart from currently available medications, which primarily operate in a nonspecific manner. Within immune cells and target tissues, other potential avenues for epigenetic treatment exist. These include modifying non-coding RNAs, altering histone acetylation patterns, and adjusting nucleosome positioning. In addition, the developing field of epitranscriptomics, involving post-transcriptional RNA modifications, shows potential. Although deserving attention, epitranscriptomics has not yet been explored within the realm of SjS. This aspect presents an exciting avenue for future research.

While numerous drugs under development are geared towards interferon pathways, treatments with a specific focus on antigen presentation or the induction of tolerance have not yet been effectively devised for SjS. Hydralazine and procainamide have been recognized to cause SS in rodents and humans by preventing DNA methylation since the 1990s. In light of this, Cole et al.'s observation of a tendency towards DNA hypomethylation in labial salivary gland (LSG) tissue from SjS patients is not surprising (113). The growing body of research confirming the association between DNA methylation and the clinical presentation of SjS individuals highlights the potential of DNA methylation as a clinical marker. The integration of DNA methylation markers may facilitate patient stratification according to disease subtypes. DNA methylation may be a valuable biomarker for monitoring the disease's activity and response to treatment in certain diseases. In addition to the correlation of DNA methylation profiles with various health conditions, DNA methylation-based markers are useful for clinical applications due to the reliability and stability of DNA methylation and the ease of assessing DNA methylation patterns (156). There are currently several high-throughput methods available for studying DNA methylation on a large scale. Single-cell methods are now available to better understand DNA methylation and transcriptional changes in autoimmune rheumatic conditions. This poses new difficulties and opportunities and provides an opportunity to identify novel clinical indicators and therapeutic targets. The system based on CRISPR-Cas9 is also being investigated as a state-of-the-art tool for altering certain epigenetic variants, which has potential as a method for treating and preventing SjS.

Conclusively, the pathogenesis of SjS has been linked to targeting epigenetic dysregulation, for instance, changes in DNA methylation. These epigenetic modifications can profoundly impact gene expression profiles in immune cells, thereby contributing to the disease's chronic inflammation and immune dysfunction. By intervening to rectify these aberrant DNA methylation patterns, it becomes possible to restore a more normalized gene expression landscape in immune cells, potentially mitigating the underlying causes of SjS. As immune cells like T and B cells are key players in the inflammatory and autoimmune response associated with SjS, modulation of DNA methylation in these cells may affect how they are activated, differentiated, and interact with other immune components. By restoring a more balanced immune response, targeting DNA methylation could offer the potential for lasting and meaningful improvements in patient well-being.

Author contributions

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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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Machine learning models predicts risk of proliferative lupus nephritis

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Objective: This study aims to develop and validate machine learning models to predict proliferative lupus nephritis (PLN) occurrence, offering a reliable diagnostic alternative when renal biopsy is not feasible or safe.

Methods: This study retrospectively analyzed clinical and laboratory data from patients diagnosed with SLE and renal involvement who underwent renal biopsy at West China Hospital of Sichuan University between 2011 and 2021. We randomly assigned 70% of the patients to a training cohort and the remaining 30% to a test cohort. Various machine learning models were constructed on the training cohort, including generalized linear models (e.g., logistic regression, least absolute shrinkage and selection operator, ridge regression, and elastic net), support vector machines (linear and radial basis kernel functions), and decision tree models (e.g., classical decision tree, conditional inference tree, and random forest). Diagnostic performance was evaluated using ROC curves, calibration curves, and DCA for both cohorts. Furthermore, different machine learning models were compared to identify key and shared features, aiming to screen for potential PLN diagnostic markers.

Results: Involving 1312 LN patients, with 780 PLN/NPLN cases analyzed. They were randomly divided into a training group (547 cases) and a testing group (233 cases). we developed nine machine learning models in the training group. Seven models demonstrated excellent discriminatory abilities in the testing cohort, random forest model showed the highest discriminatory ability (AUC: 0.880, 95% confidence interval(CI): 0.835–0.926). Logistic regression had the best calibration, while random forest exhibited the greatest clinical net benefit. By comparing features across various models, we confirmed the efficacy of traditional indicators like anti-dsDNA antibodies, complement levels, serum creatinine, and urinary red and white blood cells in predicting and distinguishing PLN. Additionally, we uncovered the potential value of previously controversial or underutilized indicators such as serum chloride, neutrophil percentage, serum cystatin C, hematocrit, urinary pH, blood routine red blood cells, and immunoglobulin M in predicting PLN.

Conclusion: This study provides a comprehensive perspective on incorporating a broader range of biomarkers for diagnosing and predicting PLN. Additionally, it offers an ideal non-invasive diagnostic tool for SLE patients unable to undergo renal biopsy.

KEYWORDS

proliferative lupus nephritis, machine learning, kidney biopsy, predictive model, diagnostic marker

1 Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with an unclear etiology, characterized by the loss of normal immune tolerance to endogenous nuclear components (1, 2). The development of lupus nephritis (LN) in SLE patients is multifactorial, involving dysregulation of the complement system, abnormal production of autoantibodies, environmental influences, and genetic factors (3). LN is defined by the deposition of immune complexes within the renal glomeruli, confirmed through histopathological examination. It represents one of the most common and severe organ challenges in SLE patients (4), posing a significant risk factor for morbidity and mortality (5, 6). In 2003, the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classified LN (7), excluding advanced sclerosing LN (Type VI), into proliferative and non-proliferative types based on renal histopathology. Non-proliferative lupus nephritis (NPLN) includes types I, II, and isolated type V, with milder inflammation and renal damage, leading to a favorable prognosis (8). Conventional treatment tends to be conservative (9). Proliferative lupus nephritis (PLN) refers to type III or IV lesions alone or combined with type V lesions (10–12), indicating a more severe condition compared to NPLN, with a significantly increased risk of progression to end-stage renal disease (ESRD) and poor prognosis (13, 14). Due to its detrimental impact on renal function and prognosis (14), the treatment strategy for PLN involves overall immunosuppression and maintenance therapy to control inflammation and autoimmune reactions (9).

Given the differences in treatment strategies and prognosis between PLN and NPLN, rapid diagnosis and early targeted treatment are crucial for improving renal function prognosis, particularly for PLN (9, 15). However, renal biopsy, as the gold standard for diagnosing PLN, is not always feasible or safe due to potential complications (16), technological limitations in primary healthcare facilities (9, 15), and contraindications for certain patients with specific conditions (17). Therefore, the development of a safe, non-invasive diagnostic method is urgently needed.

Currently, research on using big data analysis to predict clinical factors related to PLN is still quite scarce. There is limited evidence demonstrating the potential of biomarker analysis in predicting PLN risk or identifying individuals who may develop PLN at the onset of their disease. Based on this, we have developed and validated various machine learning models to predict the occurrence of PLN. The development of these models is crucial for achieving early diagnosis of PLN in clinical practice and effectively stratifying PLN from NPLN, thereby improving patient prognosis.

2 Materials and methods

2.1 Study participants

This study was a single-center retrospective study conducted at West China Hospital, Sichuan University, a tertiary teaching hospital. Between 2011 and 2021, a total of 1312 patients diagnosed with SLE with renal involvement underwent renal biopsy.

2.1.1 Inclusion criteria

(1)Patients clinically diagnosed with SLE and renal involvement, with renal involvement manifested by persistent proteinuria (>0.5g protein per day), presence of cellular casts (red blood cells, hemoglobin, granular, tubular, or mixed), urinary protein-to-creatinine ratio >500mg/g (50mg/mmol), or renal dysfunction. (2) Patients who underwent renal biopsy and were pathologically diagnosed with PLN or NPLN according to the 2003 ISN/RPS classification criteria. NPLN includes class I, II, or V LN, while PLN includes class III, IV, or III/IV with V LN (10–12).

2.1.2 Exclusion criteria

(1)Patients with repeat biopsies who underwent clinical intervention between the two biopsy procedures, to ensure model accuracy, patients undergoing their second biopsy were excluded based on the time of renal puncture. (2) Patients with non-LN or unclear pathological diagnosis of LN (such as limited glomerular number in renal biopsy, making classification difficult). (3) Patients with class VI LN or other renal diseases besides LN (such as primary glomerulonephritis, diabetic nephropathy, hepatitis B virus-related nephropathy, drug-induced renal injury, etc.). (4) Patients with

concurrent autoimmune diseases such as rheumatoid arthritis, autoimmune hepatitis, ANCA-associated vasculitis, etc. The flow chart for inclusion and exclusion is provided in Figure 1.

After confirming subjects based on inclusion and exclusion criteria, we collected clinical and laboratory characteristics. Clinical features included renal biopsy pathology, demographics (age, gender), admission physical exam indicators (systolic and diastolic blood pressure, body mass index, pulse). Laboratory features encompassed all indicators detected during hospitalization (hematology, immunology, biochemistry, coagulation, routine exams, etc.). Data were collected from the most recent data before renal biopsy. Features with <30% missing values for laboratory features and <60% for observation samples were selected. Missing values were then addressed using multiple imputation methods.

2.2 Machine learning models overview

This study developed nine models, including generalized linear models such as logistic regression, Least Absolute Shrinkage and Selection Operator(LASSO), ridge regression, and elastic net regression, as well as support vector machines including linear and radial basis kernel functions, and decision tree models such as classical decision trees, conditional inference trees, and random forests. Logistic regression estimates model parameters using Maximum Likelihood Estimation (MLE) (18). LASSO regression, ridge regression, and elastic net regression improve the model by adding an additional shrinkage penalty term to ordinary least squares (OLS). LASSO controls the sum of absolute values of coefficients through L1 regularization, achieving coefficient shrinkage and variable selection, making the final model more concise and interpretable. Ridge regression introduces a penalty term for the sum of squared coefficients through L2 regularization, improving prediction stability and accuracy. However, ridge regression lacks the ability to perform feature selection when dealing with datasets with a large number of features. Elastic net combines L1 and L2 regularization to penalize coefficients in the regression model, enabling feature selection that ridge regression cannot achieve and handling correlations between features that LASSO may overlook (19, 20). These three models using shrinkage

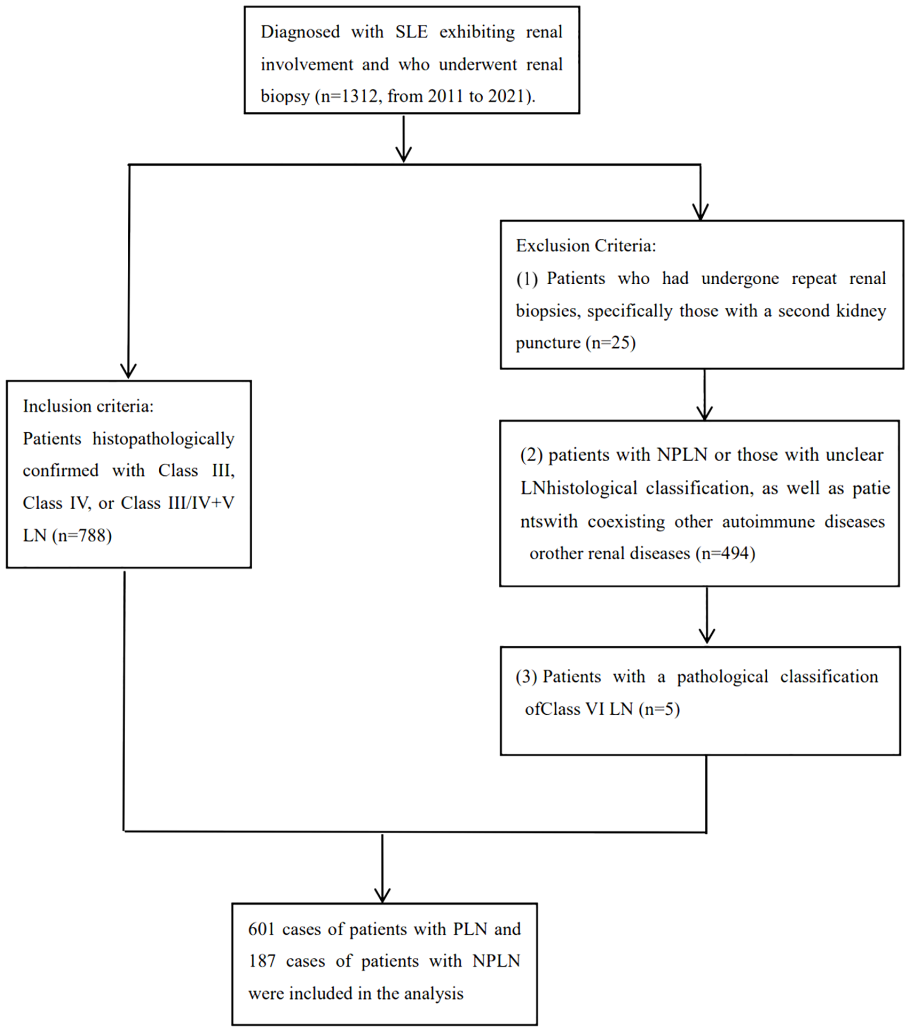

FIGURE 1
The flow chart for inclusion and exclusion.

penalties can avoid multicollinearity and overfitting problems. Support Vector Machine (SVM) maximizes the margin between two classes by hyperplane (decision boundary) in a high-dimensional feature space to distinguish different classes. Linearly separable SVMs are called linear kernel SVMs, while nonlinearly separable SVMs use kernel tricks to map data to higher-dimensional space for linear separability, known as radial basis kernel SVMs (21). Classical decision trees build tree models based on maximizing purity, conditional inference trees select features and build models based on statistical significance tests, and random forests are an ensemble supervised learning algorithm that constructs multiple decision trees by random sampling of samples and features (22). The final prediction classification of a sample is determined by the most frequently occurring class among the predictions of all trees to improve overall prediction accuracy.

2.3 Machine learning models establishment

We randomly split the dataset into training and testing sets in a 7:3 ratio. Machine learning models were built on the training set, with elastic net regression optimizing model parameters using grid search, and the remaining models selecting optimal parameters through ten-fold cross-validation. We chose the point of maximum Youden index as the optimal cutoff value to distinguish between PLN and NPLN.

2.4 Models validation

In this study, the ability of the models to differentiate between PLN and NPLN was evaluated using Receiver Operating Characteristic Curve (ROC) on both the training and testing datasets. The Youden index was used to determine the threshold for assessing accuracy, sensitivity, and specificity. Calibration curves were plotted to evaluate the calibration accuracy of the models, ensuring the reliability of their predictive results. To analyze the clinical utility of the models, the study quantified the net benefit of PLN risk probability at different thresholds using Decision Curve Analysis (DCA) curves, thereby determining the clinical application value of the models.

2.5 Statistical methods

In the study, continuous data for PLN and NPLN groups in the training and testing sets were represented using median and interquartile range (IQR), and compared using the Wilcoxon rank-sum test (Mann-Whitney U test). Categorical data were presented as frequencies (proportions) and compared using the chi-square test. The logistic regression model included LASSO-selected predictor variables or clinically relevant variables as independent variables, while other models used all predictor variables as independent variables. All models were built with PLN or NPLN as the response variable. Model parameters were selected using ten-fold cross-validation or grid search. The optimal cutoff value for distinguishing PLN and NPLN was determined using the point of maximum Youden index. All statistical tests were two-tailed, with significance set at $P < 0.05$. Data analysis was conducted using R (version 4.2.2) and RStudio.

2.6 Ethics statement

This study was approved by the biomedical research ethics committee of West China Hospital (2022–239). The informed content was waived. The study conformed to the Declaration of Helsinki.

3 Results

3.1 Study participants

This study enrolled 1312 SLE patients with kidney involvement, of whom 788 met the inclusion and exclusion criteria for analysis. Data on 7 clinical features (pathological classification, age, gender, systolic and diastolic blood pressure, BMI, and pulse) and 1265 laboratory features were collected. After addressing missing values, analysis included 780 patients and 129 features, with PLN or NPLN as the outcome. 6 clinical features and 122 laboratory features (detailed in Supplementary Material 1) were considered. Baseline characteristics of the training and testing sets (Table 1) showed no significant differences ($P > 0.05$) in age, gender, blood pressure, BMI, and pulse rate. However, significant differences ($P < 0.05$) in blood pressure and 13 other major laboratory features were observed between PLN and NPLN patients in both sets.

3.2 Machine learning models establishment

The logistic regression model utilized ten-fold cross-validation with LASSO variable selection, identifying 11 non-zero potential predictor variables at a lambda value of 0.04171. The classical decision tree model, through ten-fold cross-validation, determined 4 terminal nodes with a complexity parameter of 0.04615385, involving features such as Serum Cystatin C (CysC), anti-double stranded DNA antibodies (Anti-dsDNA) and urinary red blood cells (URBC). The conditional inference tree model considered only four variables: hematocrit (HCT), Anti-dsDNA, systolic blood pressure (BPS), and CysC. In the random forest model, the optimal number of trees corresponding to the minimum error rate was 169. Variable importance was assessed using MeanDecreaseAccuracy and MeanDecreaseGini. The linear kernel support vector machine (LSVM) model explored 21 different cost parameters, with optimal selection achieved at 0.01 through ten-fold cross-validation. The radial basis kernel support vector machine (RSVM) model, utilizing 441 parameter combinations of cost and gamma, identified the optimal combination: gamma of 0.0001 and cost of 100. The LASSO model employed ten-fold cross-validation, selecting a lambda of 0.04171 and identifying 14 non-zero variables. For the ridge regression model, ten-fold cross-

TABLE 1 Comparison of patient characteristics in this study.

	Training Cohort					Test Cohort						Missing rates
	NPLN(n=130)		PLN(n=417)		P1	NPLN(n=55)		PLN(n=178)		P2	P3	
Sex	Female	115(88.5)	Female	352(84.4)	0.254	Female	50(90.9)	Female	147 (82.6)	0.135	0.767	0.26%
	Male	15(11.5)	Male	65(15.6)		Male	5(9.1)	Male	31(17.4)			
Age	33.00[25.00, 43.00]		33.00[24.00, 42.00]		0.408	31.00[26.00, 40.00]		31.00[24.00, 41.75]		0.771	0.665	0%
BPS	120.00[109.00,134.00]		135.00[123.00,150.00]		<0.001*	123.00[115.50, 131.00]		133.00[120.00, 148.00]		<0.001*	0.316	8.59%
BPD	80.00[72.00, 87.75]		88.00[79.00, 98.00]		<0.001*	81.00[75.00, 90.00]		86.00[75.25, 97.75]		0.013*	0.475	8.72%
BMI	Lean	27(20.8)	Lean	51(12.2)	0.047*	Lean	3(5.5)	Lean	23(12.9)	0.203	0.659	43.21%
	Normal	63(48.5)	Normal	252(60.4)		Normal	38(69.1)	Normal	99(55.6)			
	Overweight	26(20.00)	Overweight	73(17.5)		Overweight	8(14.5)	Overweight	39(21.9)			
	Obese	14(10.8)	Obese	41(9.8)		Obese	6(10.9)	Obese	17(9.6)			
pulse	80.00[75.00, 98.00]		84.00[78.00, 94.00]		0.702	86.00[79.00, 97.50]		82.00[78.00, 98.00]		0.375	0.220	14.36%
C3	0.6[0.45, 0.81]		0.39[0.26, 0.54]		<0.001*	0.61[0.46, 0.81]		0.39[0.29, 0.56]		<0.001*	0.313	2.56%
C4	0.13[0.08, 0.20]		0.09[0.05, 0.13]		<0.001*	0.14[0.11, 0.21]		0.09[0.05, 0.13]		<0.001*	0.801	6.28%
IGM	1315.00[790.75, 1785.00]		936.00[649.00, 1400.00]		<0.001*	1230[870.00, 1805.00]		987.50[678.00, 1447.50]		0.017*	0.441	11.79%
RBC	4.30[3.95, 4.72]		3.54[3.06, 4.10]		<0.001*	4.26[4.03, 4.73]		3.64[3.19, 4.07]		<0.001*	0.430	0.13%
Cl	105.80[102.73, 107.47]		108.50[104.90, 111.90]		<0.001*	105.40[102.90, 108.20]		108.00[104.93, 110.68]		0.001*	0.378	3.46%
NEUTP	64.55[57.02, 74.72]		72.70[63.50, 80.40]		<0.001*	64.10[57.05, 72.75]		71.20[62.80, 80.18]		0.003*	0.378	1.03%
CysC	1.04[0.86, 1.26]		1.78[1.30, 2.45]		<0.001*	1.09[0.92, 1.40]		1.79[1.23, 2.57]		<0.001*	0.548	0.13%
Cr	55.00[47.58, 63.50]		86.10[62.00, 138.00]		<0.001*	52.00[45.50, 63.65]		91.20[62.00, 153.62]		<0.001*	0.607	0.26%
HCT	0.39[0.35, 0.42]		0.32[0.27,0.36]		<0.001*	0.38[0.36, 0.44]		0.31[0.27, 0.36]		<0.001*	0.903	0.13%
UPH	6.50[6.00, 7.00]		6.00[6.00, 6.50]		<0.001*	6.50[6.00, 7.00]		6.00[6.00, 6.50]		0.002*	0.241	1.79%
URBC	Normal	37(28.5)	Normal	30(7.2)	<0.001*	Normal	15(27.3)	Normal	12(6.7)	<0.001*	0.795	1.92%
	High	93(71.5)	High	387(92.8)		High	40(72.7)	High	166(93.3)			
UWBC	Normal	87(66.9)	Normal	120(28.8)	<0.001*	Normal	32(58.2)	Normal	60(33.70)	0.001*	0.666	1.79%
	High	43(33.1)	High	297(71.2)		High	23(41.8)	High	118(66.3)			

(Continued)

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TABLE 1 Continued

		Training Cohort				Test Cohort						Missing rates
		NPLN(n=130)		PLN(n=417)		P1	NPLN(n=55)		PLN(n=178)		P2	P3	
Anti-dsDNA	Normal	99(76.2)		Normal	130(31.2)	<0.001*	Normal	44(80.0)	Normal	76(42.7)	<0.001*	0.013*	4.23%
	High	31(23.8)		High	287(68.8)		High	11(20.0)	High	102(57.3)			

For the comparison of characteristics between the two cohorts, PLN and NPLN, continuous variables are presented as median [IQR], and categorical variables are presented as Frequency (proportion). P1 represents the comparison between PLN and NPLN in the training cohort, P2 represents the comparison between PLN and NPLN in the test cohort, and P3 represents the comparison between the training and test cohorts. P-value < 0.05 (*) indicates a statistically significant difference. In the table, BPS and BPD represent systolic blood pressure and diastolic blood pressure, respectively; RBC, HCT, and NEUTP represent red blood cells, hematocrit, and the percentage of neutrophils in whole blood, respectively; C3, C4, IgM, Cl, CysC, Cr, and Anti-dsDNA represent serum levels of complement 3, complement 4, immunoglobulin M, chloride, cystatin C, creatinine, and anti-double-stranded DNA antibodies, respectively; UPH, URBC, and UWBC represent PH, red blood cells, and white blood cells in urine, respectively.

validation determined the optimal lambda as 0.0899. The elastic net model used cross-validation to select optimal alpha and lambda, with alpha at 0.2894737 and lambda at 0.03757956. Except for the classical decision tree model and the conditional inference tree model, the features or the top 15 important features for the remaining models are listed in Figure 2.

3.3 Models validation

In our model training set, all models achieved an AUC exceeding 0.8, indicating strong classification performance. Notably, the ridge regression model stood out with an impressive AUC of 0.953 [95% confidence interval(CI): 0.933, 0.973]. In the testing set, except for the classical decision tree and conditional inference tree, all models maintained AUC above 0.8, with the random forest model performing the best (AUC: 0.880 [95% CI: 0.835, 0.926]). RSVM exhibited the highest sensitivity in the training set (0.923 [95% CI: 0.893, 0.945]), while logistic regression showed the best specificity (0.908 [95% CI: 0.844, 0.948]). Additionally, RSVM achieved the highest accuracy (0.914 [95% CI: 0.887, 0.935]). In the testing set, ridge regression ranked first in sensitivity (0.837 [95% CI: 0.775, 0.885]), while logistic regression had the highest specificity (0.818 [95% CI: 0.695, 0.900]). The ridge regression model also led in accuracy (0.803 [95% CI: 0.747, 0.849]). The differentiation performance of each model in the training and testing cohorts is illustrated in Figure 3 and Table 2.

Calibration curve analysis indicated good consistency between predicted values and actual observations for all models. Particularly, in the training set, the ridge regression model demonstrated the highest prediction accuracy, with a mean squared error (MSE) of only 0.00011, highlighting its precision in fitting the dataset. Furthermore, in the testing set, the logistic regression model exhibited the best performance with an MSE value of 0.00080, showcasing its strong generalization ability on independent datasets. Figure 4 and Table 3 reflect the model's prediction accuracy performance for the two cohorts.

Through DCA, we assessed the net benefit performance of the models across various threshold probabilities. In the analysis of the training set, the ridge regression model exhibited a net benefit exceeding the extreme curve, with the broadest range of threshold probabilities. At the optimal threshold, this model achieved the maximum net benefit of 0.628. Similarly, in the testing set, the random forest model's net benefit surpassed the extreme curve, with the widest interval of threshold probabilities, reaching the highest value of 0.520 at the optimal threshold probability point. Overall, most models demonstrated significant net benefits in practical decision support, except for classical decision tree and conditional decision tree models. Figure 5 and Table 4 illustrate the models' value for clinical applications.

4 Discussion

SLE is a potentially life-threatening autoimmune disease, with PLN being one of its most severe clinical manifestations,

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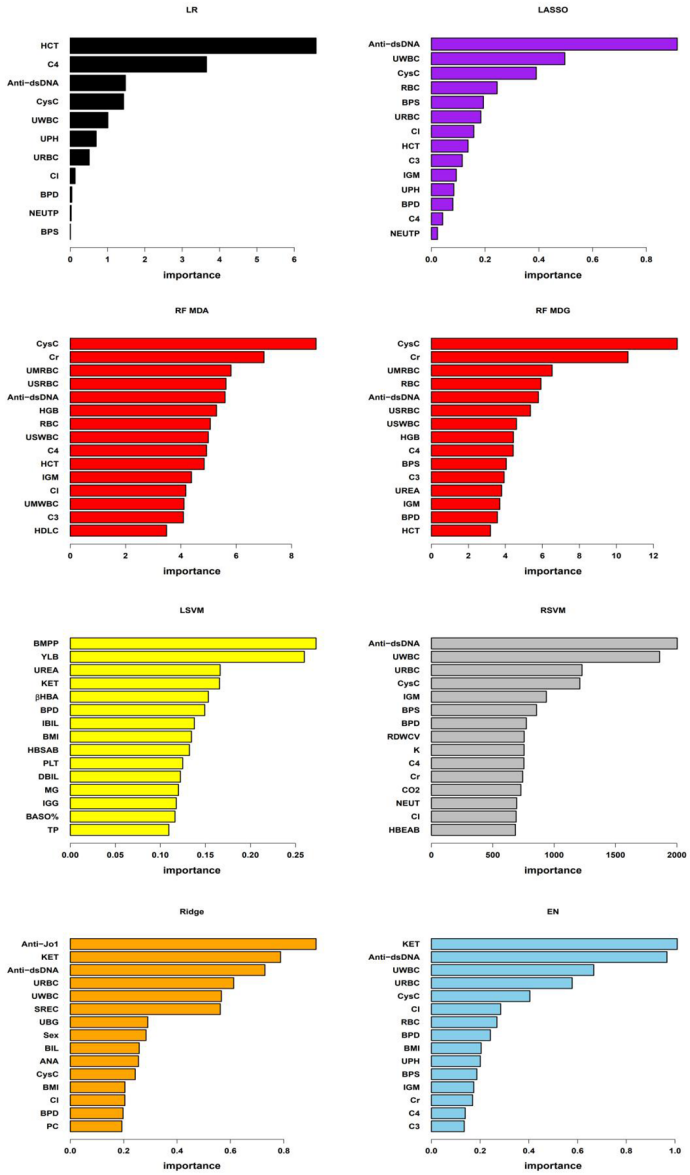

FIGURE 2
Important features of the models As shown in the figure, “LR” denotes logistic regression model, “RF MDA” and “RF MDG” represent random forest model’s variable importance assessed by MeanDecreaseAccuracy and MeanDecreaseGini respectively, “LSVM” stands for linear kernel Support Vector Machine model, “RSVM” denotes radial kernel Support Vector Machine model, “LASSO”, “Ridge”, and “EN” respectively represent Least Absolute Shrinkage and Selection Operator, Ridge regression, and Elastic Net regression models. In the LR, LSVM, LASSO, Ridge, and EN models, variable importance is assessed based on the coefficients of each variable within the models. For the RSVM model, the importance of each feature is determined by the average contribution of that feature across all support vectors. In RF model, variable importance is evaluated using MeanDecreaseAccuracy and MeanDecreaseGini. Due to the differing importance of features across various models and the different methods used to assess this importance, the specific importance values of each feature vary between models in the figure. In the figure, BPS and BPD represent systolic blood pressure and diastolic blood pressure, respectively; RBC, HCT, HGB, PLT, BASO%, RDWCV, NEUT and NEUTP represent red blood cells, hematocrit, hemoglobin, platelet count, basophil percentage, red blood cell distribution width CV, neutrophil absolute count and the percentage of neutrophils in whole blood, respectively; C3, C4, IgM, Cl, CysC, Cr, HDLC, UREA, BMPP, βHBA, IBIL, HBSAB, DBIL, MG, IGG, TP, K, CO2, HBEAB, Anti-Jo1, ANA and Anti-dsDNA represent serum levels of complement 3, complement 4, immunoglobulin M, chloride, cystatin C, creatinine, high-density lipoprotein cholesterol, urea, bactericidal membrane permeability protein, beta-hydroxybutyrate, indirect bilirubin, hepatitis B surface antibody, direct bilirubin, magnesium, immunoglobulin G, total protein, potassium, carbon dioxide binding, hepatitis B e antibody, Anti-Jo1 antibody, antinuclear antibody and anti-double-stranded DNA antibodies, respectively; UPH, URBC, UWBC, KET, SREC,UBG, BIL and PC represent PH, red blood cells, white blood cells, ketones, small round epithelial cells, urobilinogen, bilirubin and pus cells in urine, respectively. UMRBC, USRBC, USWBC, and UMWBC represent urinary sediment microscopy red blood cells, urinary sediment red blood cells, urinary sediment white blood cells, and urinary sediment microscopy white blood cells, respectively.

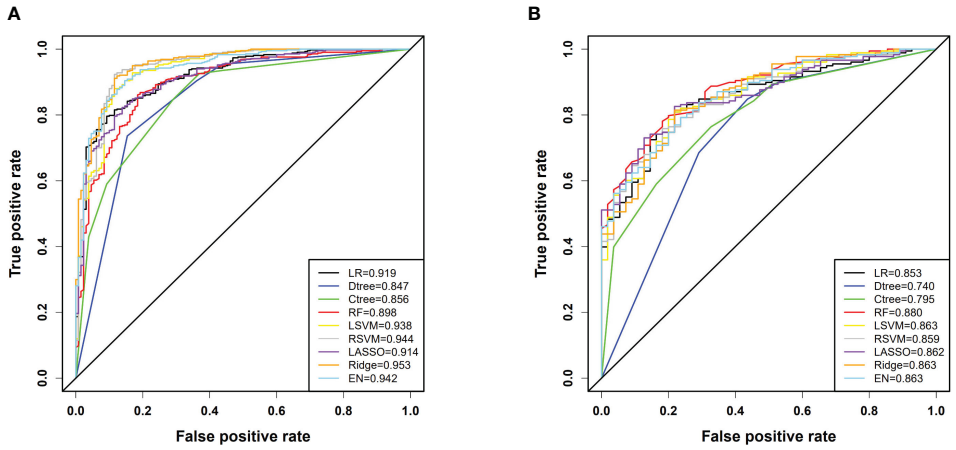

FIGURE 3
(A) ROC curves for each model in the training cohort; **(B)** ROC curves for each model in the testing cohort. In the figure, LR denotes Logistic Regression, Dtree represents Classic Decision Tree, Ctree stands for Conditional Inference Tree, RF is Random Forest, LSVM indicates Linear Kernel Support Vector Machine, RSVM denotes Radial Kernel Support Vector Machine, LASSO stands for Least Absolute Shrinkage and Selection Operator, Ridge refers to Ridge Regression, and EN signifies Elastic Net.

TABLE 2 Comparison of each model's performance in terms of AUC, sensitivity, specificity, and accuracy in the training and testing cohorts.

Training Cohort	Threshold	Sensitivity[95CI]	Specificity[95CI]	Accuracy[95CI]	AUC[95CI]
LR	0.808	0.796[0.755,0.832]	0.908[0.844,0.948]	0.823[0.788,0.852]	0.919[0.894,0.945]
Dtree	0.830	0.736[0.692,0.776]	0.846[0.774,0.899]	0.762[0.725,0.796]	0.847[0.808,0.887]
Ctree	0.784	0.849[0.811,0.880]	0.708[0.624,0.779]	0.815[0.781,0.846]	0.856[0.820,0.892]
RF	0.662	0.861[0.824,0.891]	0.815[0.739,0.873]	0.850[0.818,0.878]	0.898[0.867,0.929]
LSVM	0.752	0.880[0.845,0.908]	0.885[0.817,0.930]	0.881[0.851,0.906]	0.938[0.912, 0.963]
RSVM	0.704	0.923[0.893,0.945]	0.885[0.817,0.930]	0.914[0.887,0.935]	0.944[0.920,0.969]
LASSO	0.726	0.823[0.783,0.856]	0.862[0.791,0.911]	0.832[0.798,0.861]	0.914[0.887,0.940]
Ridge	0.684	0.911[0.880,0.935]	0.885[0.817,0.930]	0.905[0.877,0.927]	0.953[0.933,0.973]
EN	0.677	0.904[0.872,0.929]	0.854[0.782,0.905]	0.892[0.863,0.916]	0.942[0.920,0.964]
Test Cohort	**Threshold**	**Sensitivity**	**Specificity**	**Accuracy**	**AUC[95CI]**
LR	0.808	0.747[0.678,0.806]	0.818[0.695,0.900]	0.764[0.705,0.814]	0.853[0.801,0.904]
Dtree	0.830	0.685[0.614,0.749]	0.709[0.578,0.813]	0.691[0.629,0.747]	0.740[0.664,0.815]
Ctree	0.784	0.764[0.696,0.821]	0.673[0.541,0.782]	0.742[0.683,0.795]	0.795[0.733,0.858]
RF	0.662	0.815[0.751,0.865]	0.709[0.578,0.813]	0.790[0.733,0.837]	0.880[0.835,0.926]
LSVM	0.752	0.764[0.696,0.821]	0.800[0.675,0.886]	0.773[0.714,0.822]	0.863[0.813,0.913]
RSVM	0.704	0.803[0.738,0.855]	0.727[0.597,0.828]	0.785[0.728,0.833]	0.859[0.809,0.910]
LASSO	0.726	0.775[0.708,0.831]	0.782[0.655,0.872]	0.777[0.719,0.826]	0.862[0.814,0.910]
Ridge	0.684	0.837[0.775,0.885]	0.691[0.559,0.798]	0.803[0.747,0.849]	0.863[0.811, 0.914]
EN	0.677	0.820[0.757,0.870]	0.709[0.578,0.813]	0.794[0.737,0.841]	0.863[0.814,0.912]

LR denotes Logistic Regression, Dtree represents Classic Decision Tree, Ctree stands for Conditional Inference Tree, RF is Random Forest, LSVM indicates Linear Kernel Support Vector Machine, RSVM denotes Radial Kernel Support Vector Machine, LASSO stands for Least Absolute Shrinkage and Selection Operator, Ridge refers to Ridge Regression, and EN signifies Elastic Net. Threshold represents the optimal cutoff value determined based on the Youden's index, and sensitivity, specificity, and accuracy are determined based on this Threshold.

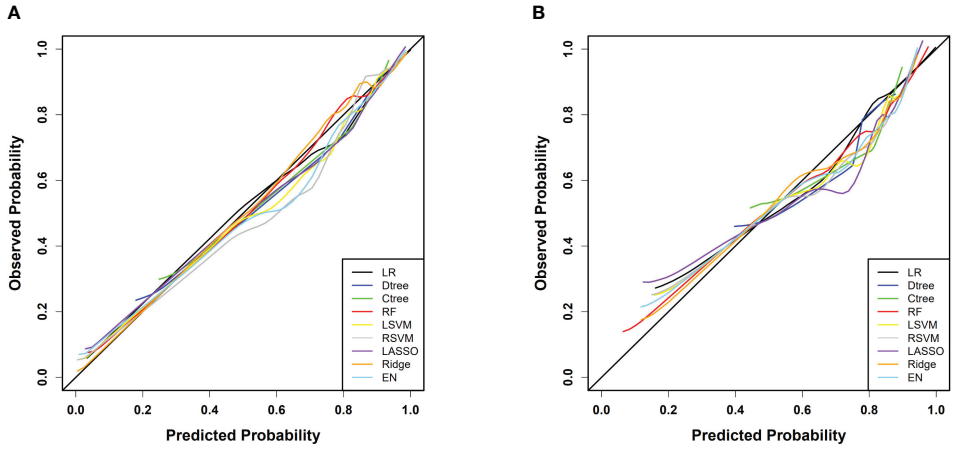

FIGURE 4
(A) Calibration curves for each model in the training cohort; **(B)** Calibration curves for each model in the testing cohort.

significantly increasing the risk of patient mortality and renal failure (13, 14). While renal biopsy remains the gold standard for diagnosing PLN, its invasiveness, potential risks, and inapplicability in specific circumstances limit its widespread use, particularly for certain special conditions or contraindicated patients. This limitation underscores the urgent need for a non-invasive diagnostic approach. An exhaustive search of the PubMed database reveals a scarcity of studies using machine learning to predict the risk of PLN. Consequently, this study aimed to harness high-dimensional feature data to construct and validate a series of machine learning models, aiming to accurately predict the risk of PLN occurrence.

In this study, we observed a stable overall prevalence rate of 76% for PLN. To our knowledge, only two previous studies attempted to construct predictive models for PLN. In these two studies, one model achieved a maximum AUC of 0.84 in the training set and 0.82 in the validation set (23), while the other study reported a lower predictive accuracy of only 0.637 (24). In comparison, our study utilized a larger dataset to build models, and the results demonstrate that our models achieved a maximum AUC of 0.953 in the training set and AUCs exceeding 0.850 in the testing set for all models except classical decision tree and conditional inference tree. Regarding predictive accuracy, our training set performance ranged from 0.823 to 0.914, while the testing set ranged from 0.764 to 0.803. Although the performance of models may be influenced by the selection of predictive variables, considering the scale of data and number of predictive variables used in our study surpass previous research, our models outperform those constructed in previous studies in all aspects. Furthermore, among the various machine learning models we developed, they all demonstrated high consistency and predictive accuracy. In clinical decision-making, except for classical and conditional decision tree models, all other models showed significant net benefits, validating not only the efficacy of the models but also enhancing their practical value in assisting clinical decision-making. Furthermore, the study observed a statistical difference in Anti-dsDNA between the training and testing cohorts. First, since the data was randomly split, we cannot guarantee identical distributions between the training and testing sets, making such differences possible. Second, the testing data is used to evaluate the model's performance. In real-world

TABLE 3 Comparison of calibration performance of each model in the training and testing cohorts.

Training Cohort	MAE	MSE	0.9 QAE	Test Cohort	MAE	MSE	0.9 QAE
LR	0.014	0.00043	0.034	LR	0.018	0.00080	0.051
Dtree	0.029	0.00148	0.063	Dtree	0.027	0.00142	0.062
Ctree	0.037	0.00163	0.050	Ctree	0.057	0.00420	0.084
RF	0.008	0.00014	0.019	RF	0.027	0.00096	0.046
LSVM	0.014	0.00051	0.038	LSVM	0.036	0.00207	0.070
RSVM	0.016	0.00083	0.041	RSVM	0.045	0.00274	0.082
LASSO	0.024	0.00093	0.062	LASSO	0.052	0.00418	0.106
Ridge	0.007	0.00011	0.018	Ridge	0.031	0.00136	0.064
EN	0.019	0.00080	0.043	EN	0.042	0.00235	0.067

MAE is the model's mean absolute error of prediction, MSE is the model's mean squared error of prediction, and 0.9 QAE is the 90% of Quantile of Absolute Error for the model.

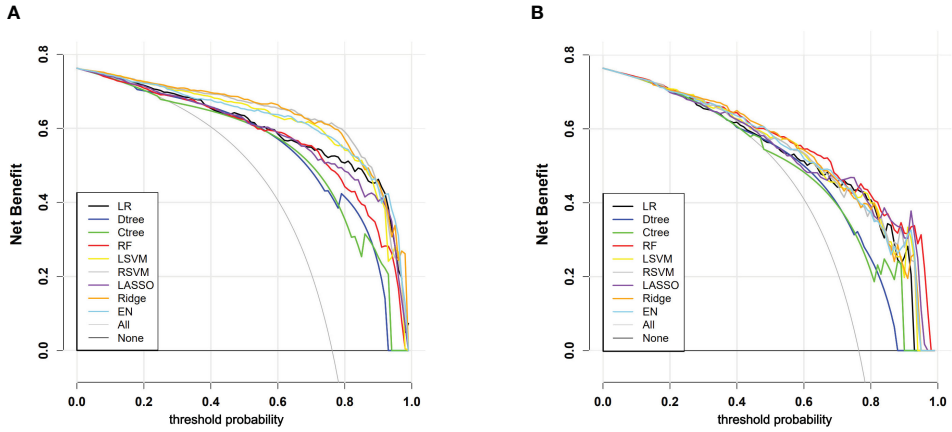

FIGURE 5
(A) DCA curves for each model in the training cohort; **(B)** DCA curves for each model in the testing cohort. "All" signifies that all patients have PLN and have all received an intervention, which resulted in a net benefit for the patients; "None" means that all patients have NPLN, none have received an intervention, and the net benefit is zero.

applications, the testing cohort represents the patients we aim to predict, and it is unlikely to find a dataset with a distribution identical to that of the training cohort. Lastly, the AUC for all seven models in the testing set is greater than 0.85, indicating that the models perform well even with discrepancies in data distribution, further demonstrating their strong generalization ability. Additionally, in both cohorts, the positive rate of Anti-dsDNA in PLN is significantly higher than in NPLN, which is consistent with the model's conclusions. Therefore, our model is not affected by this factor.

In this study, we evaluated seven predictive models with AUC values exceeding 0.85 in the testing set. The results showed that among these high-performing models, at least three models consistently identified 16 key predictive factors. These factors cover various physiological and biochemical indicators, specifically including BPS, diastolic blood pressure (BPD), serum chloride (Cl), neutrophil percentage (NEUTP), CysC, HCT, complement 4 (C4), urine pH (UPH), URBC, urinary white blood cells (UWBC), Anti-dsDNA, serum creatinine (Cr), red blood cell count in the blood (RBC), immunoglobulin M (IGM), complement 3 (C3), and BMI. The majority of shared features had a data missing rate of less than 5%, with blood pressure data missing rates of 8.59% and 8.72%, and C4 missing rate of 6.28%, all within the range of 5%-10%. However, the missing rate for IGM reached 11.79%, and the BMI's missing rate was significantly higher than other variables at 43.21%. This suggests that although BMI as a research indicator has certain potential value, its high data missing rate requires further exploration and validation in future studies. All seven models consistently demonstrated the importance of blood pressure; six models highlighted the significance of CysC, URBC, UWBC and Anti-dsDNA; C4 was considered a significant factor in five models; while IGM was identified as a key variable in four models. It is noteworthy that blood pressure, URBC, UWBC, Anti-dsDNA, C3 and C4, and Cr are not only traditionally used laboratory markers for predicting LN but also demonstrated their ability to distinguish between PLN and NPLN in this study. Furthermore, these biomarkers predicting PLN are consistent with those identified in previous studies (23, 24), further validating the stability and reliability of these indicators.

Although previous studies have revealed a significant correlation between CysC levels and the severity and pathological grades of LN (25, 26), the specific connection between it and PLN

TABLE 4 Comparison of DCA performance of each model in the training and testing cohorts.

Training Cohort	LR	Dtree	Ctree	RF	LRVM	RSVM	LASSO	Ridge	EN
Probability Range	0.03-0.99	0.18-0.92	0.25-0.93	0.03-0.98	0.01-0.98	0.01-0.98	0.03-0.98	0.01-0.98	0.01-0.98
Threshold NB	0.512	0.383	0.395	0.567	0.576	0.623	0.517	0.628	0.614
Test Cohort	**LR**	**Dtree**	**Ctree**	**RF**	**LRVM**	**RSVM**	**LASSO**	**Ridge**	**EN**
Probability Range	0.19-0.92	0.40-0.87	0.45-0.89	0.04-0.97	0.13-0.93	0.11-0.94	0.10-0.96	0.10-0.94	0.10-0.94
Threshold NB	0.390	0.188	0.263	0.520	0.429	0.452	0.465	0.478	0.488

Within this Probability Range, the net benefit of the model exceeds that of the extreme curves. Threshold NB is the net benefit of the model when the threshold probability is set to the value determined by Youden's index.

remains insufficiently supported by empirical evidence. The exact association between neutrophils and PLN is also subject to controversy (27, 28). Anemia symptoms in LN patients may be related to renal damage and the generation of autoantibodies (29, 30), however, there is currently no in-depth research indicating a direct link between anemia symptoms and PLN. Recent research indicates that IgM deposited in LN glomeruli can activate the complement system, driving disease progression, and lower IgM levels in LN patients' serum may be associated with more severe manifestations of the disease (31). LN patients may experience electrolyte and acid-base balance disturbances due to renal impairment (32), manifested by elevated serum Cl levels and decreased UPH. This study further clarifies some previously disputed or less widely used indicators, such as CysC, NEUTP, HCT, RBC, IGM, UPH, and Cl, indicating their potential importance in predicting PLN. These findings underscore the need for greater attention to these indicators in clinical practice. The identification of consensus indicators in this study not only highlights their crucial role in predicting PLN but also provides strong clues for future research on PLN biomarkers. Additionally, the correlation analysis of common features indicates a strong positive correlation between serum cystatin C and creatinine, systolic and diastolic blood pressure, red blood cells and hematocrit, as well as complement 3 and complement 4. Conversely, cystatin C or creatinine show a strong negative correlation with red blood cells or hematocrit (Figure 6). These findings are consistent with the clinical presentations of the patients and the characteristics listed in Table 1.

While our study has certain significance, there are limitations. It is a single-center retrospective study, and the results have not been validated through multicenter studies due to the relative rarity of lupus nephritis patients and limitations in research resources. Therefore, before translating the models into clinical practice, it is necessary to further validate and refine our models using multicenter data from different ethnic backgrounds. Additionally, considering data integrity, the study excluded non-routine testing variables with a missing rate exceeding 30%, which may result in the models not fully capturing all potential important explanatory features.

Our study pioneers the analysis of detailed, high-dimensional data from lupus nephritis patients over the past decade, encompassing comprehensive clinical and laboratory examination data. Multiple machine learning models were developed and comprehensively evaluated, affirming their discriminative ability, accuracy calibration, and potential clinical application. Beyond classical decision tree and conditional inference tree models, the other models demonstrate strong overall performance, offering innovative non-invasive methods for diagnosing and predicting PLN. Moreover, they show promise as reliable supplements or even alternatives to renal biopsy, especially in LN stratified management, crucial for patients ineligible for renal biopsy. Additionally, by identifying common features, this study suggests considering a more comprehensive panel of biomarkers for PLN diagnosis and prediction. At the clinical level, physicians can select the most suitable model based on patient-specific conditions and treatment needs, enhancing the accuracy of early detection and intervention for PLN. Our research significantly enhances the technical capabilities for early PLN diagnosis and treatment, providing clinicians with more robust and refined auxiliary tools.

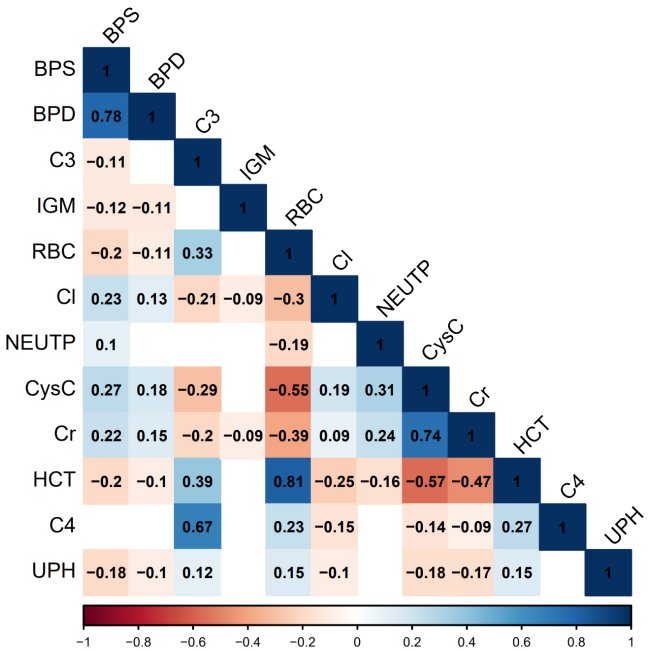

FIGURE 6
Correlation graph of common important features. Statistical significance is determined at P<0.05. In the figure, non-significant correlations are represented as blank spaces. Significant correlations are displayed in blue or red, with specific correlation values shown. Blue indicates positive correlations, red indicates negative correlations, and the color intensity reflects the strength of the correlation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Ethics statement

This study was approved by the biomedical research ethics committee of West China Hospital (2022-239). The informed content was waived. The study conformed to the Declaration of Helsinki. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because This was a retrospective study using only historical clinical data from patients.

Author contributions

PY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. ZL: Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft. FL: Conceptualization, Formal analysis, Writing – original draft. YS: Conceptualization, Formal analysis, Writing – original draft. PL: Formal analysis, Writing – original draft. QZ: Formal analysis, Writing – original draft. KW: Methodology, Writing – original draft. XZh: Methodology, Writing – original draft. XZe: Formal analysis, Project administration, Resources, Supervision, Validation, Writing – review & editing. YW: Data curation, Methodology, Project administration, Resources, 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1413569/full#supplementary-material

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A guideline on biomarkers in the diagnosis and evaluation in axial spondyloarthritis

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Objective: To develop a guideline for selecting biomarkers in the diagnosis and assessment in patients with axial spondyloarthritis (axSpA).

Method: A joint effort was carried out by the core team, the literature review team and the multidisciplinary voting panel to formulate recommendations regarding biomarkers in axSpA, using an evidence-based and consensus-based strategy. Certainty of evidence and strength of recommendation were determined, and levels of agreement within the voting panel were calculated.

Results: A total of 20 recommendations were formulated in this guideline, with levels of agreement ranging from 6.48 to 9.71. The two strong recommendations, HLA-B27 testing in patients suspected of axSpA and regular-interval monitoring of CRP/ESR represent the status quo of axSpA evaluation, while the 13 conditional recommendations represent the promising biomarkers with clinical utility in diagnosis, disease activity assessment, prediction of radiographic progression and therapeutic responses. This guideline does not dictate clinical choices of tests on axSpA, and decisions should be made based on comprehensive consideration of costs, accessibility, patients' values and willingness as well as the objective of testing in the local context.

Conclusion: This guideline addresses the interpretation of the clinical significance of biomarkers in axSpA, and the biomarkers endorsed in this guideline composed a clinical toolkit for healthcare professionals to choose from.

KEYWORDS

axial spondyloarthritis, biomarker, guideline, HLA-B27, C-reactive protein

1 Introduction

Axial spondyloarthritis (axSpA) is a disorder predominantly involving the axial skeleton, characterized by inflammation at the sacroiliac joint and spine, often with involvements of the peripheral joints and entheses, as well as extra-articular structures such as the anterior uvea and gastrointestinal tract (1, 2). It could potentially impose significant disease burden on the patients, which could derive from the pain caused by active disease, and functional disability caused by new bone formation and joint ankylosis subsequent to persistent inflammation (3, 4). Timely institution of appropriate treatment is critical to the remission of active disease and precluding radiographic progression. An early and correct diagnosis is important in this process, which often relies on both imaging examinations and laboratory findings, notably HLA-B27. However, even the combination of MRI and HLA-B27 testing does not guarantee complete accuracy of axSpA diagnosis (5); more efforts are still made to identify biomarkers that could potentially assist in the diagnosis of axSpA. Moreover, the concept of precision medicine has put forth new requirements to the medical community (6), even more so in the context of axSpA diagnosis and treatment. The taxonomy of axSpA encompasses various groups of diseases, with differing tendencies of radiographic progression with various clinical outcomes (1). Rheumatologists have to choose wisely from the toolkit of myriad biomarkers, properly interpret their clinical significance, stratify the patients based on disease activity and tendency of radiographic progression, tailor treatment and monitor therapeutic responses. Much research has been devoted to the identification and interpretation and the biomarkers associated with axSpA (7, 8). The translation of these biomarkers from research to clinical practice is, alas, still much lacked. Based on such observations, the objective of this guideline is to examine recent advances of biomarkers in axSpA and verify their reliability, formulating recommendations for rheumatologists about what biomarkers to choose in clinical practice.

2 Methods

This guideline was developed using the framework of the Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology to assess the certainty of evidence and develop recommendations (9–11). The detailed description of the methodology is explained in Supplementary Appendix 1 in Supplementary Table 1. The Core Team, the Literature Review Team and the Voting Panel led a joint effort to devise a preliminary set of biomarkers associated with axSpA. The Core Team and the Voting Panel comprised experts in rheumatology, orthopedic surgery and GRADE methodology. The complete list of participants could be seen in Supplementary Appendix 2 in Supplementary Table 2. Biomarkers discussed in this project were defined as molecules, genetic variants or other indicators which could be measured using blood, fecal or urine sample. To explore the significance and clinical relevance of each potential biomarker, assignments were handed out to each member of the Literature Review Team to conduct systemic literature reviews (SLRs). Search strategies and study inclusion process could be seen in Supplementary Appendices 3 and 4 in

Supplementary Tables 3, 4. This guideline was registered under the registration number of IPGRP-2020CN093.

Moving from evidence to recommendations, a recommendation is formulated under the comprehensive consideration of costs, accessibility, clinical significance and certainty of evidence of each biomarker. The rationale of developing recommendations is that a biomarker has to provide information which is helpful in the diagnosis or stratification of axSpA patients and ultimately could assist in optimizing treatment options. To this end, the clinical significance of each biomarker is stratified to four levels: a) diagnostic utility; b) indication of disease activity; c) prediction of radiographic progression; d) prediction or evaluation of therapeutic responses. A mere up-regulation or down-regulation does not suffice to make a recommendation. The Literature Review Team has to gather evidence regarding the four levels of clinical significance and prove that a certain biomarker could provide significant incremental information which could help rheumatologists or physicians form a better understanding of the axSpA patients. The strengths of each recommendation were classified as strong or conditional. A strong recommendation indicates that this biomarker should be considered in daily clinical practice given its significance in the four aspects, while a conditional recommendation indicates that this biomarker provides potentially helpful information to a certain extent and could be considered by the clinician.

Recommendation statements were written based on the evidence reports. An online meeting was held, during which the recommendation statements and the evidence reports were presented to the Voting Panel. Having reviewed the evidence reports and the recommendation statements, each member of the Voting Panel voted for or against the recommendations and rated the level of agreement. At least a consensus of 70% of the Voting Panel was required to include the preliminary recommendations in the final guideline.

It should be clarified that biomarkers discussed in this project only applies to patients suspected of axSpA or already diagnosed as axSpA. Since there is no preventive therapy, we do not recommend any of these biomarkers in the screening of the general population, unless an individual is at great risk.

3 Results

The recommendations of this guideline were summarized in Table 1, and the clinical significance of each biomarker was stratified in Table 2. The process of biomarker selection was presented in Table 3. A brief executive summary could be seen in Supplementary Appendix 8.

3.1 We strongly recommend HLA-B27 testing in patients suspected of axSpA

It has long been established that HLA-B27 is of critical significance to the diagnosis of axSpA, even more so to its prototypical type, namely ankylosing spondylitis (AS) (7). About 85% of AS patients are HLA-B27 positive, while only about 8% of the general population carry this gene (12). It serves as an indispensable component of the clinical arm in the Assessment of Spondyloarthritis International Society (ASAS) classification criteria of axSpA (13). However, being HLA-B27 positive does not necessarily equate with a diagnosis of axSpA, since the majority of HLA-B27 positive individuals do not develop axSpA (14). Diagnosis of axSpA should be based on clinical characteristics, HLA-B27 status, MRI and sometimes other biomarkers. The voting panel unanimously agreed that HLA-B27 should be tested in patients suspected of axSpA, more specifically, in patients with chronic lower back pain for over 3 months and the onset is before 45 years old.

Another intriguing observation is the association between HLA-B27 and radiographic progression, especially in the sacroiliac joint. It has been observed that HLA-B27-positive patients were more likely to develop from non-radiographic axSpA (nr-axSpA) to AS (15); however, HLA-B27 positivity has no value in the prediction of the radiographic progression or syndesmophyte formation in the spine (16). One argument is that HLA-B27 positivity can merely be associated with the high probability of true inflammation, while HLA-B27 per se does not participate in the process of new bone formation (7).

Some studies reported that HLA-B27-positive patients were more likely to respond to TNF-α inhibitors (17, 18). However, we believe this finding must be interpreted with caveat. Such observation could be attributed to the fact that HLA-B27-positive patients were more likely to receive early diagnosis and appropriate treatment. On the other hand, efficacy of secukinumab seemed to be not influenced by HLA-B27 status (19).

3.2 We conditionally recommend testing of HLA-B27 subtypes in patients with difficulties in diagnosis of axSpA

The heterogeneity of phenotypes and clinical outcomes in axSpA arise in part from the various subtypes of HLA-B27. To date, over 200 subtypes of HLA-B27 have been identified, but only a few were proved to be associated with the increased risk of axSpA (20). Our systemic literature review and meta-analysis concluded that HLA-B27*04 and 05 were significantly associated with an increased risk of axSpA (OR=1.91, 95% CI 1.08-3.39; OR=1.65, 95% CI 1.34-2.05) (Supplementary Appendix 6 in Supplementary Table 6), while carriers of HLA-B27*06 and 07 were less likely to develop axSpA. (OR=0.13, 95% CI 0.05-0.29; OR=0.30, 95% CI 0.17-0.54) Moreover, previous studies reported that peripheral arthritis was more prevalent in patients with HLA-B27*04 (21). The voting panel agreed on this recommendation that testing of HLA-B27 subtypes could increase the confidence of diagnosis, but it should only be considered in cases where imaging examinations and other laboratory tests returned ambiguous results. In terms of methodology, this guideline endorses DNA microarray or PCR-SSP in HLA-B27 subtype testing.

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TABLE 1 Recommendations on biomarkers pertinent to the diagnosis and evaluation of patients with axSpA.

	Recommendations	Certainty of evidence	Approval rate	Level of agreement
Biomarkers pertinent to diagnosis				
1	We strongly recommend HLA-B27 testing in patients suspected of axSpA.	High	100.00%	9.71
2	We conditionally recommend testing of HLA-B27 subtypes in patients with difficulties in diagnosis of axSpA.	Medium	100.00%	8.10
3	We conditionally recommend testing of polygenic risk score (PRS) in patients suspected of axSpA.	Low	90.48%	7.05
4	We strongly recommend against testing of antibodies in patients with axSpA in daily practice, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies against microbial targets.	Low	90.48%	8.10
Biomarkers pertinent to inflammatory status				
5	We strongly recommend monitoring of CRP and ESR concentrations in patients with axSpA over usual care without CRP or ESR monitoring.	High	100.00%	9.71
6	We conditionally recommend regular-interval monitoring of SAA in patients with axSpA	Medium	95.24%	7.14
7	We conditionally recommend the testing of leptin and HMW-APN in patients with axSpA.	Low	90.48%	6.48
8	We conditionally recommended against testing of VEGF in patients with axSpA.	Very low	95.24%	7.62
9	We conditionally recommend the testing of calprotectin in patients with axSpA, especially using the fecal sample to monitor gut inflammation.	Medium	85.71%	6.95
10	We conditionally recommend the testing of IL-6, IL-17 and TNF-α in the monitoring of disease activity in patients with axSpA.	Low	90.48%	7.24
11	We conditionally recommend the analysis of peripheral lymphocyte subsets in patients with axSpA.	Medium	95.24%	7.52
12	We strongly recommend against testing of non-coding RNAs in patients with axSpA in daily practice.	Very low	71.43%	7.52
Biomarkers pertinent to bone destruction and formation				
13	We conditionally recommend testing of bone turnover markers, including CTX-I and PINP, in patients with axSpA.	Low	90.48%	7.05
14	We conditionally recommend testing of sclerostin in patients with axSpA.	Low	95.24%	7.24
15	We conditionally recommend testing of DKK-1 in patients with axSpA.	Low	85.71%	7.14
16	We conditionally recommend against testing of OPG/RANKL/RANK in patients with axSpA.	Low	85.71%	7.14
17	We conditionally recommend against testing of MMP-3 in patients with axSpA.	Very low	95.24%	7.05
Biomarkers pertinent to prediction of therapeutic safety and efficacy				
18	We conditionally recommend genotyping of CYP2C9 alleles before axSpA patients start medication of NSAIDs metabolized by CYP2C9, such as diclofenac, meloxicam and celecoxib.	Low	95.24%	7.33
19	We conditionally recommend genetyping of NAT2 alleles before axSpA patients start medication of sulfasalazine.	Low	90.48%	7.33
20	We conditionally recommend measurement of antidrug antibodies in patients receiving medication of TNF-α inhibitors at the time of clinical non-responses.	Medium	95.24%	8.57

Deep red indicates that this guideline strongly recommends against the testing of this biomarker in patients with axSpA in clinical practice, while light red indicates that this guideline conditionally recommends against testing of this biomarker. Deep green indicates that this guideline strongly recommends the testing of the biomarker for the corresponding purposes in clinical practice, while light green indicates conditional recommendation, which should also take into consideration the costs, accessibility and patients' willingness.

3.3 We conditionally recommend testing of polygenic risk score (PRS) in patients suspected of axSpA

Despite its significant association with axSpA, HLA-B27 only contributes to ~20% of the heritability of axSpA (22). Genomic-wide association studies have identified numerous genetic loci which were associated with the genetic risks of axSpA (22–24). Among these genetic loci, MHC genes confer more significant genetics risks than non-MHC genes (25). Our meta-analysis confirmed that HLA-DRB1, especially the allele HLA-DRB*12, as well as HLA-B60 was associated with a higher risk of axSpA. (Supplementary Appendix 6 in Supplementary Table 6). Researchers aggregated from 110 to thousands of the most relevant single nucleotide polymorphisms and devised polygenic risk score (PRS) to assist in the diagnosis of axSpA. Results showed that the overall PRS (AUC=0.924), which included MHC and non-MHC single nucleotide polymorphisms, outperformed HLA-B27

TABLE 2 Stratification of the clinical significance of the biomarkers discussed in this guideline.

Biomarkers	Diagnosis	Assessment of disease activity	Prediction of radiographic progression	Prediction/monitoring of therapeutic responses
HLA-B27	√			
HLA-B27 subtypes	√			
Polygenic risk score	√			
Antibodies, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies againts microbial targets				
CRP	√	√	√	√
ESR		√	√	√
SAA		√		
Leptin and HMW-APN		√	√	
VEGF				
Calprotectin		√		
Inflammatory cytokines including IL-6, IL-17 and TNF-α		√		
Peripheral lymphocyte subsets		√		
Non-coding RNAs				
Bone turnover markers, including sCTX and PINP			√	
Sclerostin			√	
DKK-1			√	
OPG/RANKL/RANK				
MMP-3				
NSAIDs-related genes				√
SSZ-related genes				√
Anti-drug antibodies				√

The symbol √ indicates that results of the systemic literature review supports that this biomarker bears clinical significance in this field.

(AUC=0.869), MRI (AUC=0.885) or CRP (AUC=0.700) in diagnostic utility (25). 90.48% of the voting panel agreed on the recommendation of PRS for patients suspected of axSpA.

3.4 We strongly recommend against testing of antibodies in patients with axSpA in daily practice, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies against microbial targets

There is not sufficient evidence to prove the diagnostic values of antibodies in axSpA. Despite earlier studies showing that anti-CD74 antibodies and anti-CLIP antibodies could be detected in 69% and 85.1% of axSpA patients (26, 27), subsequent studies showed high inconsistency regarding their diagnostic capacity. The SPACE cohort showed that the positive predictive value (PPV) and negative predictive value (NPV) of anti-CD74 antibodies were only 58.8% and 59.1% (28), and its diagnostic capacity in East Asians population was also limited (29). Anti-sclerostin and anti-noggin antibodies could be implicated in signaling pathways regulating new bone formation (30), but there is no reliable evidence proving that these antibodies could be predictors of new bone formation. Antibodies against microbial targets such Klebsiella pneumonia, Salmonella, Saccharomyces cerevisiae could also be detected in axSpA (31), but their diagnostic values remained unclear. 90.48% of the voting panel agreed on the recommendation against testing of antibodies in axSpA in daily practice.

3.5 We strongly recommend monitoring of CRP and ESR concentrations in patients with axSpA over usual care without CRP or ESR monitoring

As an acute phase reactant, C-reactive protein (CRP) is a long-established biomarker of disease activity in axSpA patients, while

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TABLE 3 The selection process of biomarkers in this guideline.

Preliminary set		Systemic literature review	Preliminary recommendations	Final recommendations
HLA-B27	DKK-1	HLA-B27	HLA-B27	HLA-B27
HLA-B27 subtypes	OPG/ RANKL/ RANK	HLA-B27 subtypes	HLA-B27 subtypes	HLA-B27 subtypes
Genes	MMP-3	Genes	PRS score	PRS score
Antibodies, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies againts microbial targets	MMP-8	Antibodies, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies againts microbial targets	Antibodies, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies againts microbial targets	Antibodies, including anti-CD74 antibodies, anti-sclerostin and anti-noggin antibodies, antibodies againts microbial targets
CRP	MMP-9	CRP	CRP and ESR	CRP and ESR
ESR	BMP-2	ESR	SAA	SAA
SAA	TNC	SAA	Leptin and HMW-APN	Leptin and HMW-APN
Adipokines, including leptin, adiponectin and resistin	Fetuin A	Adipokines, including leptin, adiponectin and resistin	VEGF	VEGF
VEGF	YKL-40	VEGF	Calprotectin	Calprotectin
CXCL8	MIF	Calprotectin	Inflammatory cytokines including IL-6, IL-17 and TNF-α	Inflammatory cytokines including IL-6, IL-17 and TNF-α
HGF	Vitamin D	non-coding RNA	Peripheral lymphocyte subsets	Peripheral lymphocyte subsets
Calprotectin	Gut microbiota	Inflammatory cytokines including IL-6, IL-17 and TNF-α	non-coding RNA	non-coding RNA
Pentraxin 3	Metabolomics signature	Peripheral lymphocyte subsets	Bone turnover markers, including β-CTX and PINP	Bone turnover markers, including β-CTX and PINP
non-coding RNA	NSAIDs-related genes	Bone turnover markers, including β-CTX and PINP	Sclerostin	Sclerostin
Inflammatory cytokines including IL-6, IL-17 and TNF-α	SSZ-related genes	C1M, C2M, C3M, C6M and VICM	DKK-1	DKK-1
TL1A	Anti-drug antibodies	Sclerostin	OPG/RANKL/RANK	OPG/RANKL/RANK
Peripheral lymphocyte subsets		DKK-1	MMP-3	MMP-3
Neutrophil-to-lymphocyte ratio		OPG/RANKL/RANK	NSAIDs-related genes (CYP2C9)	NSAIDs-related genes (CYP2C9)
Fibrinogen-to-albumin ratio		MMP-3	SSZ-related genes (NAT2)	SSZ-related genes (NAT2)
Bone turnover markers, including β-CTX and PINP		BMP-2	Anti-drug antibodies	Anti-drug antibodies
C1M, C2M, C3M, C6M and VICM		TNC		
COMP		Gut microbiota		
Aggrecan		Metabolomics signature		
Osteocalcin		NSAIDs-related genes		
RBP4		SSZ-related genes		
Sclerostin		Anti-drug antibodies		

Deep red indicates that this guideline strongly recommends against the testing of this biomarker in patients with axSpA in clinical practice, while light red indicates that this guideline conditionally recommends against testing of this biomarker. Deep green indicates that this guideline strongly recommends the testing of the biomarker for the corresponding purposes in clinical practice, while light green indicates conditional recommendation, which should also take into consideration the costs, accessibility and patients' willingness.

erythrocyte sedimentation rate (ESR) is another important indicator of inflammation. Serum CRP level above the upper limit has only a sensitivity of 50% and a specificity of 80% (25), but it was included as a SpA feature in the ASAS classification criteria for axSpA (13). Regarding their association with radiographic progression, our meta-analyses concluded that the baseline levels of CRP and ESR were both significant predictors of radiographic progression of the spine, more specifically the mSASSS score increase. (OR=1.02, 95%CI 1.00-1.03; OR=1.02, 95% 1.01-1.03) (Supplementary Appendix 6 in Supplementary Table 6) Patients with elevated CRP levels seemed to respond better to TNF-α inhibitors such as etanercept (32) and adalimumab (33), as well as IL-17 inhibitors such as bimekizumab (34) and secukinumab (19). The voting panel unanimously agreed on the recommendation of regular -interval monitoring of CRP and ESR over usual care without CRP or ESR monitoring. More specifically, CRP/ESR levels should be monitored at 0 week, 3 weeks, 6 weeks, 12 weeks and every 3 months during follow-up visits. This recommendation was in line with the 2019 ACR recommendations for the treatment of radiographic and non-radiographic axial spondyloarthritis, which conditionally recommended regular-interval use and monitoring of CRP concentrations or ESR over usual care without regular CRP or ESR monitoring (35). The recommended assay for CRP is immunoturbidimetry.

3.6 We conditionally recommend regular-interval monitoring of SAA in patients with axSpA

Serum amyloid A (SAA) is another acute phase reactant indicative of active inflammation, and multiple studies have established the strong positive correlation between SAA and other indices of disease activity, such as BASDAI and CRP (36–38). SAA could be an addition to other inflammatory markers, and baseline levels of CRP and SAA combined could be predictors of ASAS response for patients receiving treatment of TNF-α inhibitors (38). SAA should be tested at first visits and follow-up visits to monitor disease activity. Moreover, serum SAA levels could be a potential biomarker of amyloid A amyloidosis, a known complication in radiographic axial spondyloarthritis (36). 95.24% of the voting panel agreed on this recommendation.

3.7 We conditionally recommend the testing of leptin and HMW-APN in patients with axSpA

Adipokines are mostly secreted by adipocytes and participate in multiple metabolic processes. The most researched adipokines include leptin, adiponectin and resistin (39). Leptin is also considered a pro-inflammatory cytokine given its capacity of stimulating T cell proliferation and enhancing T cell activation (40), while adiponectin is considered an anti-inflammatory cytokine since it could inhibit the production of inflammatory cytokines (41). Meta-analysis showed that leptin was up-regulated in the serum of AS patients <40 years old, while AS patients ≥ 40 years old had significantly higher serum adiponectin levels (42). Several studies have investigated the association between the adipokines and radiographic progression, and results showed that both higher baseline levels of leptin and lower baseline levels of high-molecular-weight adiponectin (HMW-APN) were predictors of radiographic progression in axSpA (43, 44). Given their relevance in disease activity and radiographic progression, this guideline recommends testing of leptin and HMW-APN with an approval rate of 90.48%, but costs and accessibility should also be considered before ordering a test. We also conducted a systemic literature review on resistin (Supplementary Appendix 6 in Supplementary Table 6), which also belongs in adipokines, but it was decided that resistin could not provide incremental values to leptin and HMW-APN.

3.8 We conditionally recommended against testing of VEGF in patients with axSpA

Vascular endothelial growth factor (VEGF) is a critical mediator in angiogenesis, and it is also implicated in the inflammatory process by increasing the vascular permeability and promoting infiltration of inflammatory cells (45). Although meta-analysis showed that serum levels of VEGF were significantly higher in patients with axSpA than healthy controls, it also showed that VEGF levels were poorly correlated with disease activity (30). Moreover, baseline levels of VEGF could predict neither spinal inflammation nor syndesmophyte formation (46). There is no sufficient evidence to build a case for the recommendation of VEGF, and 95.24% of the voting panel agreed on the recommendation against testing of VEGF in clinical practice.

3.9 We conditionally recommend the testing of calprotectin in patients with axSpA, especially using the fecal sample to monitor gut inflammation

Calprotectin is a cytosolic protein complex comprising S100A8 and S100A9. When excreted, it could combine with Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE), followed by activation of innate immune responses and inflammation (47). It has been acknowledged that fecal calprotectin is a sensitive biomarker of inflammatory bowel disease (IBD) and has been applied in the clinical practice (48). Meta-analysis confirmed that both serum and fecal calprotectin were significantly elevated in spondyloarthritis patients and associated with disease activity (49). Previous epidemiological study showed that 46.2% of SpA patients exhibited microscopic gut inflammation, and axSpA was often complicated with IBD (50). The value of calprotectin lies in its ability of monitoring gut inflammation, since there is still a lack of non-invasive approaches of monitoring gut inflammation apart from endoscopy. We believe that calprotectin, especially when tested with fecal sample, could close that gap and provide critical information about inflammation in the gastrointestinal tract. This is increasingly relevant since IL-17 inhibitors should be used with caution in patients with susceptibility to IBD (51).

3.10 We conditionally recommend the testing of IL-6, IL-17 and TNF-α in the monitoring of disease activity in patients with axSpA

1. Meta-analysis confirmed that the serum interleukin-6 (IL-6) levels were significantly elevated in patients with axSpA (52), and multiple studies have confirmed the association between IL-6 and CRP as well as ESR (53, 54). One study reported that baseline levels of IL-6 could predict changes of mSASSS.

2. Interleukin-17 (IL-17) plays an important role both in the inflammatory process and in the ossification process. IL-17 is significantly elevated in the serum of axSpA patients (52).

3. Tumor necrosis factor α (TNF-α) is also a cytokine reflecting inflammatory status, with potential correlation with other inflammatory indicators such as ESR and IL-6 (53). Evidence is still lacked regarding its capability in predicting radiographic progression and therapeutic responses.

90.48% of the voting panel agreed on this recommendation.

3.11 We conditionally recommend the analysis of peripheral lymphocyte subsets in patients with axSpA

Our meta-analysis showed that the proportions of Th17 cells as well as Th1/Th2 ratios in the peripheral blood is significantly elevated in patients with axSpA, while Tregs were down-regulated (Supplementary Appendix 6 in Supplementary Table 6). The Th17 cells are known as an important lymphocyte subset in the pathogenesis of axSpA, notably in the skin disease as well as enthesitis (55). Tregs possess immunomodulatory traits and lower proportions of Tregs could indicate active inflammation (55). Previous studies have showed that Th17 cells were positively correlated with disease activity, while Tregs were inversely correlated with disease activity (56, 57). However, costs and accessibility should be considered before a flow cytometric analysis is ordered.

3.12 We strongly recommend against testing of non-coding RNAs in patients with axSpA in daily practice

There have been extensive studies investigating roles of non-coding RNAs in the pathogenesis of axSpA, including microRNA, lncRNA and circRNA. Transcriptomic analysis revealed that the altered levels of some microRNAs could be implicated in the inflammatory processs, ossification process, dysregulation of T cells in axSpA, such as miR-29a, Let-7i and miR-16 (58). miR-29a could target DKK-1 and GSK3b and interfere with the bone formation process, with some studies reporting that levels of miR-29a were significantly elevated in peripheral blood mononuclear cells of AS patients and could result in increased activity of osteoblasts (59). One study reported that serum levels of TUG1 were negatively correlated with CRP in ankylosing spondylitis patients (60). Despite the numerous studies in this field, many of the results were rarely replicated by subsequent studies, and it came to our notice that an unusual number of articles in this field of research were retracted. Members of the literature review team expressed concern regarding the reliability of the evidence, and combined with the many challenges in non-coding RNA testing, such as instability of RNA, various subtypes of mononuclear cells and lack of validation studies (7), this guideline determined to strongly recommend against testing of non-coding RNAs in patients with axSpA, unless high quality evidence is brought forward. This recommendation triggered debate within the core team and the voting panel. Some members of the voting panel argued that such categorical denial of the merits of non-coding RNAs would be inappropriate and that we should not easily dismiss the evidence as unreliable. It was reiterated to the voting panel that this recommendation was not trying to negate the significance of non-coding RNA in the pathogenesis of axSpA, but given the current evidence we did not encourage routine testing of non-coding RNA in clinical practice. This recommendation was sustained with an approval rate of 71.43%.

3.13 We conditionally recommend testing of bone turnover markers, including CTX-I and PINP, in patients with axSpA

In terms of the osteoinflammatory process, axSpA is characterized by the paradoxical disequilibrium between bone resorption and bone formation (61). Both osteoporosis and new bone formation are prominent features in axSpA, and the prevalence of vertebral fractures could be as high as 30% (62). Bone turnover markers include markers of bone absorption and markers of bone formation. Our meta-analysis confirmed that both C-terminal telopeptide of type I collagen (CTX-I) levels in the serum and the deoxypyridinoline(DPD)/creatinine ratio in the urine were significantly elevated in patients with axSpA, suggesting excessive bone absorption (Supplementary Appendix 6 in Supplementary Table 6). Markers of bone formation, such as Procollagen I N-terminal peptide (PINP), could be indicators of therapeutic responses of anti-osteoporosis medication. Although evidence regarding the values of bone turnover markers in the management of axSpA was indirect, we still believe that such markers could help visualize which direction the balance of the osteoinflammatory process is tipping towards. However, matrix metalloproteinase-mediated degradation fragments of extracellular matrix, including C1M, C2M, C3M, C6M and VICM, was not included in this recommendation due to limited quality of evidence.

3.14 We conditionally recommend testing of sclerostin in patients with axSpA

Sclerostin is a glycoprotein produced and secreted mostly by mature osteocytes (63). It is an inhibitor of the Wnt signaling pathway, which could inhibit osteoblast-induced new bone

formation (64). Moreover, sclerostin can stimulated RANKL secretion by osteocytes, thereby promoting osteoclastogenesis and bone resorption (65). Despite the heterogeneity observed in the studies investigating serum levels of sclerostin in axSpA patients, the majority of studies could confirm that serum sclerostin levels could be an indicator of bone formation activity, and patients with lower sclerostin levels were more likely to exhibit radiographic progression (66–68). We believe that this heterogeneity could be derived from the different ossification activity of the included patients. It should be noted that sclerostin was not correlated with disease activity (Supplementary Appendix 6 in Supplementary Table 6). Based on the gathered evidence, this guideline conditionally recommended testing of sclerostin as an indicator of new bone formation, with an approval rate of 95.24%.

3.15 We conditionally recommend testing of DKK-1 in patients with axSpA

Dickkopf-1 (DKK-1) is another inhibitor of the Wnt/β-catenin signaling pathway, which could competitively combine with LRP5/6 and ultimately inhibit new bone formation (69). Previous meta-analysis concluded that lower serum DKK-1 levels could be observed in the subgroups of AS patients with increased CRP (CRP > 10 mg/L) and high mSASSS (mSASSS > 30), indicating an inverse correlation between DKK-1 and disease activity as well as radiographic progression (70). Lower DKK-1 levels could be interpreted as higher risks for radiographic progression and might require more advanced treatment. We conditionally recommend the testing of DKK-1 in patients with axSpA, and 85.71% of the voting panel agreed on this recommendation.

3.16 We conditionally recommend against testing of OPG/RANKL/RANK in patients with axSpA

Receptor activator of nuclear factor-kappa B ligand (RANKL) could combine with the receptor activator of nuclear factor-kappa B (RANK) on the cell surface of osteoclast precursors and mediate osteoclastogenesis, while osteoprotegerin is a soluble decoy RANKL receptor produced by osteoblasts and could inhibit bone resorption (71, 72). The OPG/RANKL/RANK system regulates the balance between bone resorption and bone formation, hence the speculation that these molecules could be potential biomarkers in axSpA. However, in the systemic literature review, despite pooled results that serum levels of OPG, RANKL, and RANKL/OPG ratio were significantly elevated in axSpA (73), we could not find evidence that the OPG/RANKL/RANK system could be a predictor of syndesmophyte formation, while studies investigating their correlation with disease activity were highly inconsistent (Supplementary Appendix 6 in Supplementary Table 6). In light of the limited quality of evidence, we decided to recommend against routine testing of OPG/RANKL/RANK in patients with axSpA until more substantial evidence is brought forward. 85.71% of the voting panel agreed on this recommendation.

3.17 We conditionally recommend against testing of MMP-3 in patients with axSpA

Matrix metalloproteinase 3 (MMP-3) could degrade the ECM and is associated with the destruction of articular cartilage and bone (74). It was hypothesized that the up-regulated activity of MMP-3 is correlated with increased disease activity and the extent of articular damage in axSpA. The systemic literature review examined its role in disease activity and radiographic progression, and results showed significant heterogeneity in its correlation with disease activity. (Supplementary Appendix 6 in Supplementary Table 6) Only two studies investigated its capacity as a predictor of radiographic progression (75, 76), while only one study found that baseline serum MMP-3 levels were significantly associated with 2-year progression of mSASSS, and MMP-3 was primarily contributory in patients who already had substantial baseline damage (76). Moreover, MMPs are also involved in the therapeutic implications in axSpA (77). It was unclear what incremental value MMP-3 could bring to the current panel of biomarkers. Based on this consideration, this guideline conditionally recommends against routine testing of MMP-3. The approval rate was 95.24%.

3.18 We conditionally recommend genotyping of CYP2C9 alleles before axSpA patients start medication of NSAIDs metabolized by CYP2C9, such as diclofenac, meloxicam and celecoxib

Multiple non-steroidal anti-inflammatory drugs (NSAIDs) were metabolized by CYP2C9, including diclofenac, meloxicam and celecoxib with the exception of aspirin (78). Dozens of alleles of the gene CYP2C9 have been identified, and most allelic variants of CYP2C9 would cause reductions in the enzymatic activity. Currently the most researched allelic variants include CYP2C9*2 and CYP2C9*3, which were slightly more common in Caucasians with prevalence of 12.68% and 6.88%, as compared with <1% and 3.38% in East Asians (79). In terms of pharmacokinetics, carriers of CYP2C9*2 or CYP2C9*3 were more likely to be slow metabolizers of NSAIDs with higher peak concentration and greater area under the curve (AUC) (79). Our meta-analysis confirmed that carriers of CYP2C9*2 or CYP2C9*3 were more likely to have gastrointestinal adverse reactions, more specifically upper gastrointestinal bleeding, compared with homozygotes of CYP2C9*1 (Supplementary Appendix 6 in Supplementary Table 6). There was not enough evidence to suggest that variants of CYP2C9 were associated with other adverse reactions of NSAIDs, such as cardiovascular events, despite a few reports. It was also hypothesized that the variants of PTGS2, which encodes cyclooxygenase 2 (COX-2), could have an impact on the efficacy and safety of NSAIDs, but evidence is limited (80). We conditionally recommend genotyping of CYP2C9 alleles before medication of NSAIDs, and for carriers of CYP2C9*2 or CYP2C9*3 as well as patients identified as slow metabolizers of NSAIDs based on previous medical history, it is advised to start with half the lowest dose. It should be taken into consideration that since genetic testing could be expensive in some areas, patients' values and willingness should also be considered.

3.19 We conditionally recommend genetyping of NAT2 alleles before axSpA patients start medication of sulfasalazine

Most of sulfasalazine is hydrolyzed in the colon into 5-aminosalicylic acid and sulfapyridine, and the latter is absorbed into blood and metabolized in the liver by N-acetyltransferase 2 (NAT2) (81). Individuals carrying the wild type gene of NAT2, namely NAT2*4 could be categorized as fast acetylator, while those carrying the mutated genes NAT2*5, 6, 7 could be categorized as slow acetylator (81). Our meta-analysis confirmed that the slow acetylators carrying the allelic variants NAT2*5, 6, 7 were at a significantly higher risk of dose-dependent adverse events, such as nausea, vomiting, dizziness, but slow acetylation was not associated with hypersensitivity-related adverse events, such as skin rash or granulocytopenia (Supplementary Appendix 6 in Supplementary Table 6). Interestingly, mutations of NAT2 are very prevalent across the general population (~50%) (82). Apart from NAT2, ABCG2 is another gene reported to be associated with the safety and efficacy of sulfasalazine, but evidence is still limited (83). Based on the evidence above, we conditionally recommend genotyping of NAT2 genetic variants before medication of sulfasalazine. For slow acetylators determined through genotyping or based on previous medical history, it is advised to start with half the lowest dose of sulfasalazine, or choose different kinds of medication. It should be noted that since the adverse events associated with slow acetylation are not life-threatening and genetic testing could be expensive, patients' willingness to avert possible adverse reactions through genetic testing should be considered.

3.20 We conditionally recommend measurement of antidrug antibodies in patients receiving medication of TNF-α inhibitors at the time of clinical non-responses

Measurement of antidrug antibodies (ADAbs) falls in the category of therapeutic drug monitoring (TDM). A recent clinical trial exhibited that among patients receiving maintenance therapy with infliximab, proactive TDM was more effective than treatment without TDM in sustaining disease control (84). EULAR also developed points-to-consider addressing the principles and clinical utility of TDM, pointing out that measurement of ADAbs should be considered to understand clinical non-response in the case of immunogenic biopharmaceuticals (85). Measurement of ADAbs should also be considered in the case of a hypersensitivity reaction, mainly related to infusions, but not injection-site reaction. Our meta-analysis concluded that ADAbs were significantly associated with lower drug concentrations of TNF-α inhibitors. (Supplementary Appendix 6 in Supplementary Table 6) On the other hand, IL-17 inhibitors generally exhibited good immunogenicity. The incidence rate of ADAbs in secukinumab was less than 1% (86, 87). For ixekizumab, the general incidence rate of ADAbs was 9-19.4%, yet such ADAbs were not neutralizing antibodies and could not predict treatment outcomes (88). It is currently believed that ADAbs to secukinumab, ixekizumab and bimekizumab were not associated with adverse events (89). Based on the evidence above, we formulated a recommendation of ADAbs measurement in patients receiving medication of TNF-α inhibitors, but not IL-17 inhibitors, at the time of clinical non-responses. In line with the EULAR points-to-consider, proactive testing of ADAbs is not recommended.

4 Other biomarkers to consider

4.1 Metabolomic signature

Metabolomics studies in patients with axSpA have revealed significant alterations in the metabolism of amino acids, fatty acids and choline. Diagnostic panels were formulated based on the metabolomic signature of axSpA patients, with AUC as high as 0.998, but such diagnostic panels did not undergo external validation or were not verified by subsequent studies (90, 91). It was also unclear what incremental value it could bring to the currently established biomarkers. With considerations of costs, accessibility and certainty of evidence, we decided not to formulate a recommendation, but the metabolomic signature shall be revisited in the future to determine its clinical utility.

4.2 Gut microbiota

The diversity and abundance of gut microbiota could be explored by means of metagenomic shotgun sequencing and 16S rRNA gene sequencing. Current studies have revealed changes of a-diversity and elevated abundance of dialister, actinobacteria and clostridium, which could be associated with disease activity (92, 93). Diagnostic panels were devised, but were not validated by subsequent studies (94). Considering the costs, accessibility and certainty of evidence, we decided not to formulate a recommendation, but the potential of gut microbiota as a biomarker in axSpA shall be revisited in the future.

5 Discussion

This guideline puts forward recommendations for choosing biomarkers in the diagnosis and assessment of axSpA, using an evidence-based and consensus-based methodology. A total of 20 recommendations were formulated in this project. The only two strong recommendations endorsed the testing of HLA-B27 in patients suspected of axSpA, and CRP/ESR as indices of disease activity. These two recommendations represent the status quo of the clinical practice of axSpA, yet the intention of this guideline was not to maintain the status quo; it sought to push the clinical practice further and expedite the process from bench to bedside, by pooling all the evidence of the utility of biomarkers in the diagnosis and assessment of patients with axSpA. We conducted an exhaustive examination of the biomarkers which have been studied in axSpA, in terms of diagnostic utility, disease activity, radiographic progression and predicting/monitoring therapeutic responses.

Based on the systemic literature review, these recommendations highlight the interpretations of the biomarkers in axSpA in the four dimensions mentioned above.

However, this guideline does not dictate clinical choices of tests on axSpA patients. Along with the two strong recommendations, the 13 conditional recommendations compose a toolkit for healthcare professionals to choose appropriate testing items from. In clinical practice, decisions should be made based on the pragmatic consideration of costs, accessibility, patients' values and willingness in the local context, and most importantly, the objective of the tests. Studies focusing on the economic evaluations of the biomarkers were sparse, thus limiting the certainty of evidence in this project. Another issue is that local laboratory conditions might vary, causing difficulties in establishing the reference range for the biomarkers, affecting the generalizability of these recommendations. Joint efforts should be carried out to standardize the testing methodology and the reference range.

This guideline only examined some of the biomarkers that have been extensively studied. In light of the advances in research, we believe that more promising biomarkers will keep emerging and ultimately complement the toolkit we propose. Considering that evidence is still sparse regarding certain biomarkers, we decided not to formulate recommendations for the time being, such as the metabolomic signature, gut microbiota and TNC, but these biomarkers shall be revisited in the future, when more evidence becomes available.

In conclusion, this guideline formulated recommendations on biomarkers in the diagnosis and assessment of axSpA patients advising on whether, in whom, when to choose the laboratory tests and how to interpret the alterations of these biomarkers. The ultimate goal of this guideline is to stratify patients based on the information provided by the biomarkers and facilitate personalized care to patients with axSpA.

Author contributions

DL: Formal Analysis, Investigation, Methodology, Writing – original draft. YX: Investigation, Methodology, Writing – review & editing. LT: Data curation, Investigation, Writing – review & editing. XWe: Data curation, Investigation, Writing – review & editing. BDL: Data curation, Investigation, Writing – review & editing. MY: Data curation, Investigation, Writing – review & editing. XYW: Data curation, Investigation, Writing – review & editing. XZ: Data curation, Investigation, Writing – review & editing. XL: Data curation, Investigation, Writing – review & editing. LZ: Data curation, Investigation, Writing – review & editing. JLW: Data curation, Investigation, Writing – review & editing. BL: Investigation, Writing – review & editing. KW: Investigation, Writing – review & editing. OJ: Investigation, Writing – review & editing. QL: Investigation, Writing – review & editing. XWa: Investigation, Writing – review & editing. JQ: Investigation, Writing – review & editing. LW: Investigation, Writing – review & editing. DBZ: Investigation, Writing – review & editing. DH: Investigation, Writing – review & editing. SH: Investigation, Writing – review & editing. WH: Investigation, Writing – review & editing. SY: Investigation, Writing – review & editing. HZ: Investigation, Writing – review & editing. JYW: Investigation, Writing – review & editing. YFW: Investigation, Writing – review & editing. SL: Investigation, Writing – review & editing. ZBL: Investigation, Writing – review & editing. ZT: Investigation, Writing – review & editing. CX: Investigation, Writing – review & editing. YLW: Investigation, Writing – review & editing. DHZ: Investigation, Writing – review & editing. FZ: Investigation, Writing – review & editing. LC: Investigation, Writing – review & editing. YW: Investigation, Writing – review & editing. JW: Data curation, Investigation, Writing – review & editing. SW: Investigation, Writing – review & editing. ZTL: Investigation, Writing – review & editing. YS: Investigation, Project administration, Writing – review & editing. KY: Methodology, Project administration, Supervision, Writing – review & editing. JG: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1394148/full#supplementary-material

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