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Autoantibodies for diagnostics, prognostics, and surveillance in autoimmune disease

Edited by
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Autoantibodies for diagnostics, prognostics, and surveillance in autoimmune disease

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Editorial: Autoantibodies for diagnostics, prognostics, and surveillance in autoimmune disease

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autoantibodies, autoimmunity, rheumatoid arthritis, autoimmune liver disease (ALD), myositis, antiphospholipid syndrome, antinuclear antibodies (ANA), type I IFN

Editorial on the Research Topic
Autoantibodies for diagnostics, prognostics, and surveillance in autoimmune disease

Breach of tolerance to self-antigens and serological autoimmunity, reportedly occurs long before onset of clinical autoimmune disease (1). Still, circulating disease-specific autoantibodies remain a hallmark of B-cell driven autoimmune disease although they may also be detected in healthy individuals and transiently in patients with infections. Over the years associations between specific autoantibodies and clinical disease have improved our understanding of mechanisms in autoimmunity as well as clinical diagnostics (2, 3).

Lack of immune tolerance and increased production of antibodies directed against self-antigens may induce or precipitate autoimmune disease as clearly demonstrated for the TSH receptor antibodies in Graves' disease. Beyond that, autoantibodies may represent an epiphenomenon in autoimmune disease without distinct pathophysiologic relevance; this is exemplified by smooth muscle antibodies (SMA) in autoimmune hepatitis (AIH). However, most autoimmune diseases, e.g., systemic lupus erythematosus (SLE), idiopathic inflammatory myopathies (IIM), and Sjögren's disease (SjD) are characterized by mixtures of autoantibodies with varying degrees of pathogenic potential. They can help physicians to make a correct diagnosis or to predict the severity of the disease or the response to treatments. In addition, "new" autoantibodies with clinical relevance are constantly discovered and new areas of application for "old" autoantibodies are emerging (4).

The Research Topic "*Autoantibodies for Diagnostics, Prognostics, and Surveillance in Autoimmune Disease*" collected original works, review articles and case reports offering new insights on clinical applications of new and established autoantibodies in autoimmune diseases. The clinical use of autoantibodies has the potential to facilitate early diagnosis,

informed selection of patients for targeted and personalized therapies which in turn can be expected to improve long-term outcomes for patients with autoimmune diseases. Furthermore, the discovery of (new) autoantibodies and their autoantigens may give clues to disease mechanisms and reveal new areas of research. We hereby summarize the final content of the Research Topic which covers a variety of autoimmune diseases and autoantibodies.

In a single center study by Marklund et al. the presence of myositis-specific autoantibodies (MSA) and myositis associated autoantibodies (MAA) were examined in relation to lung involvement in patients with myositis. Patients presenting with MAA, particularly anti-SSA/Ro52 antibodies were found to be at higher risk of developing interstitial lung disease (ILD). Interestingly, it was also noticed that patients presenting only with antibodies against Mi-2α, Mi-2β, NXP2, HMGCR, and TIF1γ, or no MSA/MAA, were all spared from ILD.

Zhang et al. contributed a case report describing a patient with anti-MDA5 positive amyopathic dermatomyositis, also positive for anti-SSA/Ro52. This patient, who suffered from rapidly progressive ILD was successfully treated with methylprednisolone pulses combined with cyclosporine A and hydroxychloroquine.

In a review, Antiochos and Casciola-Rosen thoughtfully described the autoantigens associated with the interferon (IFN) system. They included the type I IFN induced autoantigen SSA/Ro52, as well as MDA-5, a cytoplasmic dsRNA sensor that promote type I IFN production. The authors also describe autoantibodies against IFN types I, II and III themselves, and linked them to autoimmune diseases and immunodeficiencies.

A cross-sectional study by Sciascia et al. investigated autoantibodies recognizing high density lipoprotein (HDL) in antiphospholipid syndrome (APS). Patients with APS showed higher levels of anti-HDL when compared to healthy controls; the highest levels were found among patients with arterial, as compared to venous thrombosis, even when adjusting for total IgG. The results suggest that whether anti-HDL autoantibodies could serve a biomarker for thrombotic events in APS deserve further studies.

Andraos et al. assessed a large collection of sera from healthy blood donors ($n = 825$) for ANA using both HEp-2 cells and ALBIA. A considerable proportion of the sera contained autoantibodies, though without any clear association to self-reported symptoms. Importantly, the combination of ANA fine-specificities, relevant symptoms and high IFN-α levels identified a small proportion of blood donors with autoimmune disease (two cases with SjD).

In a case-control study by Liu et al., the presence, titer and pattern of antinuclear antibodies (ANA) among patients with newly diagnosed rheumatoid arthritis (RA) were investigated. ANA positivity, especially at high titers and homogenous staining pattern on Hep-2 cells, was associated with a higher probability of RA when compared with healthy individuals and non-RA subjects with arthritis.

Another study, by Martinsson et al., examined the relation between a periodontitis pathogen (*Aggregatibacter actinomycetemcomitans*; A.a.) and the development of RA in at-risk RA patients from two cohorts. Leukotoxin A (LtxA) is expressed by A.a. and has the indirect ability of citrullination, thereby providing a possible route for an increased load of citrullinated antigens. These may induce anti-citrullinated protein antibodies (ACPA), a hallmark of RA. An association between anti-LtxA antibodies with disease progression to RA was found in one of the two cohorts, but not in the other. The authors suggest that there might be population-dependent differences in the oral microbiome and the risk to develop RA, which should be further explored in future studies.

A review article by Wang et al. covered the area of Chinese herbal medicine and the beneficial effects of various herbs and herbal compounds in the treatment of RA. A structured overview of these compounds, their biological targets and potential mechanism of action were given. The focus was on anti-inflammatory and antioxidative effects. This review provides inspiration for further research into herbal remedies that, in the future, may expand treatment options for patients with RA.

Van den Beukel et al. a investigated autoantibodies against six post-translationally modified (PTM) proteins in patients with autoimmune liver disease. Anti-PTM were more frequent among patients compared to healthy controls and presence of multiple anti-PTM was more frequently found in the subgroup of patients with autoimmune hepatitis (AIH). These patients also displayed a higher rate of complete biochemical response to treatment, defined as normalized IgG and aminotransferases. In a commentary to this contribution, Taubert et al. described polyreactivity of IgG to multiple autoantigens highlighting the risk of false positive results when measuring autoantibodies in sera from patients with AIH (5). The polyreactivity includes bovine serum albumin a canonical ELISA blocking reagent (5, 6). Van den Beukel et al. responded to this commentary by assuring that appropriate control antigens have been used in their ELISA assays. It was exemplified by a deepened description of their detection of autoantibodies to carbamylated proteins.

In summary, our Research Topic provides valuable insights into autoantibodies and autoimmune diseases, with emphasis on novel applications of known autoantibodies. We are convinced that these contributions will aid in improving clinical diagnostics, prognostication and patient management as well as inspire further research.

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BRIEF RESEARCH REPORT
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IgG Anti-high-Density Lipoproteins Antibodies Discriminate Between Arterial and Venous Events in Thrombotic Antiphospholipid Syndrome Patients

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Introduction: Recurrent thrombotic events are a hallmark of Antiphospholipid Syndrome (APS). However, biomarkers to identify if a patient with antiphospholipid antibodies (aPL) is at higher risk to develop an arterial or a venous event are lacking. Recently, the pathogenic role of anti-high-density lipoproteins antibodies (anti-HDL) in the occurrence of cardiovascular disease (CVD) in autoimmunity has emerged. The aim of the present study was to evaluate the presence of IgG anti-HDL antibodies in a cohort of thrombotic APS patients and to investigate their association with clinical outcomes.

Methods: Serum levels of IgG anti-HDL antibodies, total IgG, and complete aPL profile were assessed in 60 APS patients and 80 healthy donors (HDs) by immunoassays.

Results: Higher levels of IgG anti-HDL were found in APS patients compared to HDs ($p < 0.001$), even after correcting for total IgG levels ($p < 0.001$). No associations with treatments or traditional cardiovascular risk factors, except for smoking habit ($p < 0.0001$), were found. Patients who experienced at least one arterial event ($n = 30$) had significantly higher levels of anti-HDL antibodies when compared to patients with venous thrombosis ($n = 30$, $p = 0.046$), this difference being stronger when adjusting for total IgG ($p = 0.007$). Additionally, patients tested positive for antiphosphatidylserine/prothrombin (IgG/IgM) antibodies had significantly higher levels of anti-HDL antibodies ($p = 0.045$).

Conclusions: Increased levels of IgG anti-HDL antibodies can be found in APS, mainly in patients with arterial thrombosis, independently of aPL antibodies and traditional risk factors. These findings point to a role of anti-HDL antibodies in APS and support their use as a potential biomarker for arterial thrombotic events.

Keywords: antiphospholipid syndrome, thrombosis, anti-HDL, autoimmunity, autoantibodies

INTRODUCTION

Antiphospholipid Syndrome (APS) is the most common acquired thrombophilia. At the clinical level, APS is defined by the occurrence of thrombotic events, with the peculiar trait of potentially involving both arteries and veins and/or pregnancy morbidity, in individuals found to be persistently positive for antiphospholipid antibodies (aPL), including: lupus anticoagulant (LA), anti-cardiolipin (aCL), and anti-β2glycoprotein I (anti-β2GPI) antibodies (1). Additionally, premature cardiovascular disease (CVD) and atherosclerotic development have been proven to be more prevalent in APS compared to the general population (2, 3).The mechanisms underlying thrombosis and CVD in APS patients are not completely understood, but recent evidences have brought to light the existence of a complex interplay between conventional cardiovascular risk factors and disease-specific features, such as the presence of autoantibodies (4).

Interestingly, several studies have reported that aPL might be able to cross-react with lipoproteins and their components, contributing to endothelial dysfunction, enhancing atherosclerosis, and ultimately leading to CVD progression (5, 6). However, the clinical relevance of such findings is unknown. In addition, recent studies have discovered the existence of a heterogeneous group of autoantibodies specifically directed against lipoproteins and their components, namely IgG anti-high-density lipoproteins antibodies (anti-HDL), which have been demonstrated to impair the anti-inflammatory and anti-oxidative roles exerted by HDL-cholesterol (7). Higher anti-HDL levels have been described in a broad range of autoimmune diseases (8–10). However, whether anti-HDL antibodies may be associated with clinical features in APS remains unclear. Critically, while it is known that the presence of aPL confers a high risk for thrombosis (11), biomarkers to if a patient is at higher risk to develop an arterial or a venous event are lacking. The aim of the present study was to evaluate the presence of IgG anti-HDL antibodies in a cohort of thrombotic APS patients and to investigate if these antibodies can discriminate between arterial and venous thrombosis.

METHODS

Ethics Statement

The study protocol, involving human samples, was performed in compliance with the Declaration of Helsinki and reviewed and approved by the Institutional Review Boards (IRBs) from the University of Turin and the University of Oviedo. All participants gave written informed consent prior enrolment.

Patients

This cross-sectional study included 60 APS patients attending the Giovanni Bosco Hospital, Turin, Italy. Inclusion criteria comprehended: patients with persistent aPL positivity and that fulfilled the Sydney criteria for thrombotic APS (venous and/or arterial) (1). A group of 80 age- and sex-matched healthy individuals from the same population was recruited as healthy donors (HDs). Medical records from APS were retrospectively revised in order to register clinical characteristics, including previous episodes of venous and/or arterial thrombosis. Blood samples were collected after the first thrombotic event.

Antiphosholipid Antibodies Testing

The aPL profile included LA, aCL, and anti-ß2GPI, and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies. The aCL, anti-ß2GPI, aPS/PT (IgG and IgM) were semi-quantitatively assayed using a commercial ELISA kit by Inova Diagnostics, Inc (San Diego, CA, United States).

Plasma samples were tested for the presence of LA according to the recommended criteria from the International Society on Thrombosis and Haemostasis Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies (12, 13).

IgG Anti-HDL Antibodies

IgG antibodies against HDL were measured in all serum samples by ELISA as previously described (9). ELISA plates (Maxisorp, Nunc) were coated overnight (4°C) with 20 μg/ml human HDL-cholesterol (Sigma) in 70% ethanol (test half) or ethanol alone (control half). Then, plates were blocked with PBS + 1% BSA (Sigma) for 1 h at room temperature and washed with PBS. Serum samples (1:50-diluted in PBS + 0.1% BSA), and standard curves from pooled sera (diluted 1:16 to 1:512) were incubated in both plate halves for 2 h at room temperature. Plates were then washed twice with TBS and alkaline phosphatase-conjugated anti-human IgG (1:1,000) (Immunostep) was added for 1 h. Finally, p-nitrophenylphosphate (Sigma) in diethanolamine buffer (pH 9.8) was added and absorbance at 405 nm was recorded. Anti-HDL Arbitrary Units (AU) were calculated for each sample according to the standard curves. Intra- and inter-assay reproducibility for our assay was 10 and 13%, respectively. Total IgG was quantified by conventional ELISA techniques and AU values obtained from the anti-HDL ELISA were corrected using total IgG levels (anti-HDL/IgG). The positivity to anti-HDL antibodies was evaluated using the 90th percentile of the anti-HDL/IgG in HDs (=12.94) as cut-off (9). This cut-off provided the following analytical estimates in the present study: sensitivity = 0.46, specificity = 0.90, and positive predictive value = 0.91.

Statistical Analysis

Categorical variables are presented as number (%) and continuous variables are presented as mean (S.D.). Differences were evaluated by the chi-squared test, Fisher's exact test or the unpaired *t*-tests (Mann–Witney or Kruskal–Wallis), as appropriate. Spearman rank's test was used to analyze correlations. ROC curves were performed to evaluate the association between anti-HDL positivity and thrombotic outcomes, and the corresponding area under the curve (AUC) and its 95% confidence intervals and *p*-values were computed. A two-sided $P < 0.050$ was considered as statistically significant. All statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA).

RESULTS

IgG Anti-HDL Antibodies in APS Patients

Sixty APS patients were enrolled in this study, 43 (71.6%) patients being primary APS patients (PAPS) and 17 (28.4%) patients having a concomitant diagnosis of SLE, according to the recently approved classification criteria of the European League Against Rheumatism and the American College of Rheumatology, which includes at least one positive antinuclear antibody test and the combination of a number of clinical and immunological criteria (14). All 60 patients had at least one thrombotic event: 30 (50%) previous arterial events and 37 (61.6%) venous events (seven patients experienced recurrent events, both venous and arterial). For the purpose of this study the analysis was performed taking into account the first thrombotic event, meaning that 30 patients were considered as arterial thrombotic APS patients and 30 patients as venous thrombotic APS patients. No differences have been found between PAPS and secondary APS patients (SAPS) in terms of age ($p = 0.190$), gender ($p = 0.21$), cardiovascular risk factors [including arterial hypertension ($p = 0.281$), hyperlipidemia ($p = 0.670$), and smoking habit ($p = 0.290$)], C-reactive protein values ($p = 0.540$) and HDL cholesterol levels ($p = 0.721$), and aPL profile (all $p > 0.050$). In addition, no differences have been found when looking at the levels of anti-HDL antibodies between these two groups ($p = 0.570$), even when correcting for total IgG levels ($p = 0.860$). One SAPS patient had undergone B-cell depletion therapy within 1 year prior to blood sample collection. Full demographics and clinical characteristics of the study cohort are detailed in **Table 1**.

Higher levels of IgG anti-HDL were found in APS patients compared to HDs [mean 46.1 (SD ±69.8) vs. mean 14.3 (SD ±14.5), respectively; $p < 0.001$] (**Figure 1A**). Anti-HDL levels were found to be increased even after correcting for total IgG levels [mean 12.6 (SD ±16.3) vs. mean 4.7 (SD ±5.5), respectively; $p < 0.001$] (**Figure 1B**).

The levels of anti-HDL antibodies were not associated with traditional CV risk factors, including arterial hypertension ($p = 0.800$), hyperlipidaemia ($p = 0.102$), diabetes ($p = 0.700$), and high body mass index ($p = 0.800$). Anti-HDL levels were not correlated to HDL levels in APS ($r = 0.090$, $p = 0.630$) nor in HDs ($r = 0.110$, $p = 0.370$). Similar results were retrieved when APS patients were analyzed separately as PAPS and SAPS ($r = 0.120$, $p = 0.561$; and $r = -0.050$, $p = 0.710$; respectively). On the contrary, higher levels of anti-HDL antibodies were observed in smokers compared with non-smokers [mean 112.42 (SD ±202.2) vs. mean 36.6 (SD ±38.7); $p < 0.0001$], even after adjusting for total IgG levels [anti-HDL/IgG: mean 16.2 (SD ±26.2) vs. mean 10.9 (SD ±11.1); $p = 0.012$]. Finally, levels of total IgG were found to be similar between APS patients and HDs [mean 382.23 (SD ±154.88) vs. mean 333.63 (SD ±115.37); $p = 0.262$] and not related to clinical parameters, thrombosis status or treatments (all $p > 0.050$).

Overall, an increased prevalence of IgG anti-HDL antibodies can be observed in APS patients. Traditional CV risk factors were not related to anti-HDL levels, with the exception of smoking habit.

TABLE 1 | Demographic, clinical, and laboratory characteristics of the APS patients and HDs included in the study.

Patients characteristics	HDs (*n* = 80)	APS patients (*n* = 60)
Female sex (*n*, %)	60 (75)	43 (71.6)
Age (mean, S.D.), years	48.6 ± 10.4	50 ± 10.8
Primary APS patients (*n*, %)	0 (0)	43 (71.6)
Concomitant diagnosis of SLE (*n*, %)	–	17 (28.3)
Disease duration since APS diagnosis (mean, S.D.), years	–	11.7 ± 7.5
Thrombosis (*n*, %)	0 (0)	60 (100)
Venous thrombosis (*n*, %)	–	37 (61.6)*
Arterial thrombosis (*n*, %)	–	30 (50)
Pregnancy morbidity (*n*, %)	0 (0)	1 (1.6)
Arterial hypertension (*n*, %)	4 (5)	22 (36.6)
Hyperlipidemia (*n*, %)	0 (0)	19 (31.6)
Smoking (*n*, %)	9 (11.2)	5 (8.3)
Obesity (BMI > 30) (*n*, %)	0 (0)	8 (13.3)
LA (*n*, %)	0 (0)	49 (81.6)
aCL IgG/M (*n*, %)	0 (0)	38 (63.3)
Anti-β2 GPI IgG/IgM (*n*, %)	0 (0)	29 (43)
aPS/PT IgG/IgM (*n*, %)	0 (0)	29 (48.3)
Triple aPL positivity (*n*, %)	–	20 (33.3)
Total cholesterol levels (mean, S.D.), mg/dl	145 ± 32.2	187.4 ± 51
HDL-cholesterol levels (mean, S.D.), mg/dl	72 ± 15.3	64 ± 9.1
Triglycerides levels (mean, S.D.), mg/dl	104 ± 26.2	98.7 ± 28.6
CRP levels (mean, S.D.), mg/dl	0.23 ± 0.2	0.45 ± 0.2
Statins (*n*, %)	0 (0)	20 (33.3)
Anti-hypertensive drugs (*n*, %)	3 (5)	19 (31.6)
HCQ (*n*, %)	0 (0)	15 (25)
Anticoagulant agents (*n*, %)	0 (0)	33 (55)
Antiaggregant agents (*n*, %)	0 (0)	36 (60)
B-cell depletion agent (Rituximab) (*n*, %)	0 (0)	1 (1.2)

**Seven patients experienced recurrent thrombotic events, both venous and arterial. For the purpose of this study the analysis was performed taking into account the first thrombotic event, meaning that 30 patients were considered as arterial thrombotic APS patients and 30 patients as venous thrombotic APS patients. APS, antiphospholipid syndrome; HDs, healthy donors; SLE, systemic lupus erythematosus; BMI, adult body mass index; LA, lupus anticoagulant; aCL, anti-cardiolipin antibodies; anti-β2 GP1, anti-β2glycoprotein I antibodies; aPS/PT, antiphosphatidylserine/prothrombin antibodies; aPL, anti-phospholipid antibodies; CRP, C-reactive protein; HCQ, hydroxychloroquine.*

IgG Anti-HDL Antibodies and Clinical Features in APS Patients

When separating for individual aPL positivities, patients tested positive for aPS/PT (IgG/IgM) antibodies had significantly higher levels of anti-HDL [mean 53.1 (SD ±81.1) vs. mean 20.7 (SD ±17.6); $p = 0.045$], which did not reach statistical significance after adjusting for total IgG ($p = 0.151$). No differences were observed with the rest of aPL tested. Similarly, no differences were observed when the different isotypes of aPL antibodies (IgG/IgM) were entered separately in the analyses (all $p > 0.050$). Additionally, no differences were observed between patient with primary APS and those with a concomitant autoimmune diagnose. Finally, IgG anti-HDL levels were not associated with treatments received (all $p > 0.050$).

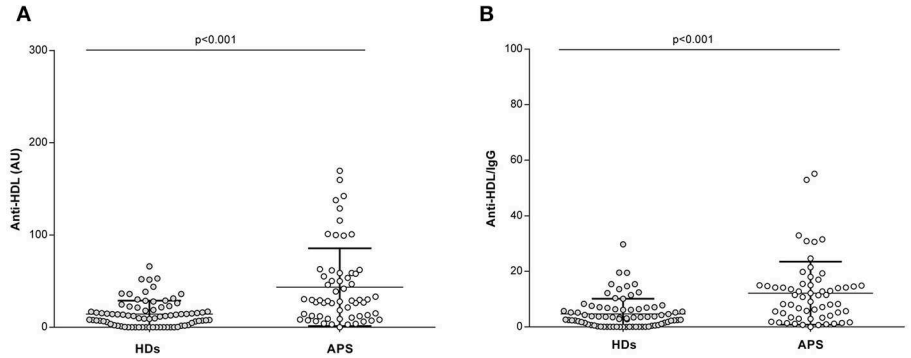

FIGURE 1 | Anti-HDL antibodies in APS patients. Levels of IgG anti-HDL antibodies measured as AU **(A)** or normalized after total IgG correction **(B)** in APS patients ($n = 60$) and HDs ($n = 80$). Bars indicate 25th, median and 75th percentiles. Differences were assessed by Mann–Whitney *U*-tests.

ROC analyses (**Supplementary Figure 1**) revealed a good discriminative power of the anti-HDL positivity to the presence of thrombosis (both arterial and venous) (AUC ROC [95% CI], *p*: 0.751 [0.633, 0.870], $p < 0.001$), hence strengthening their role as potential biomarker.

Finally, when separating patients for the different thrombotic manifestations of APS (arterial vs. venous), we observed that patients who experienced at least one arterial event had significantly higher levels of anti-HDL when compared to patients with venous thrombosis [mean 53.1 (SD ±94.1) vs. mean 34.3 (SD ±28.9), respectively; $p = 0.046$] (**Figure 2A**). This difference became stronger when adjusting for total IgG levels [anti-HDL/IgG: mean 13.1 (SD ±16.7) vs. mean 9.5 (SD ±6.6); $p = 0.007$] (**Figure 2B**, right panel). No significant difference was found in total IgG levels between patients who experienced an arterial or a venous thrombotic event [mean 378.2 (SD ±148) vs. mean 375.5 (SD ±136.3), respectively; $p = 0.950$]. Importantly, none of the aPL antibodies differ between arterial and venous thrombosis, and no differences were observed for clinical features and treatments received (all $p > 0.050$). Furthermore, the distribution of traditional CV risk factors was similar between both subsets of APS patients (all $p > 0.050$). Taken together, our results confirm that anti-HDL antibodies were associated with clinical outcomes in APS, as aPS/PT positivity and arterial thrombotic manifestation, independently of other clinical features, hence suggesting its potential use as biomarkers.

DISCUSSION

Autoimmune and rheumatic diseases are associated with a higher prevalence of CV morbidity and mortality, mainly due to an accelerated atherosclerotic process (15, 16). Far from the early conception of a natural, evolutive aging-related process, a compelling body of evidence supports that atherosclerosis and atherothombosis are dynamic and complex conditions resulting from an inextricable link of multiple pathogenic factors that trigger and perpetuate the vascular damage and impair its reparative mechanisms (17). Immune (systemic) mediators and autoantibodies emerge as crucial pathogenic players in this scenario. Therefore, atherosclerosis and atherothrombosis are now seen as the consequence of the interplay between traditional risk factors and autoimmune-related mechanisms (18). As a consequence, the presence of traditional CV risk factors alone cannot fully explain the increase CV morbidity (18). In addition, although the presence of chronic inflammation and the production of disease-specific antibodies, such as aPL, play a crucial role in the pathogenesis of endothelial dysfunction and thromboembolic manifestations, the exact mechanisms involved are largely unknown and recent evidence seem to point to the involvement of novel mediators. The results presented in this paper suggest the involvement of a new player, anti-HDL antibodies, in this scenario. If validated in larger prospective studies, our findings might also support the potential use anti-HDL antibodies as biomarkers for the early identification of patients with arterial thrombosis.

Despite previously being considered as mere bystanders, lipoproteins are important and active mediators of pathogenic processes such as inflammation, oxidative stress, and metabolic traits in autoimmunity. In fact, altered lipoprotein levels and/or functionality have been reported in several autoimmune conditions. In recent years, a growing body of evidence has highlighted the involvement of anti-HDL antibodies as a bridge between humoral immune-response, lipid dysfunction, oxidative status, and clinical outcomes in rheumatic disorders (8, 19, 20). To date, in the specific setting of APS, limited data are available regarding lipid profile and the association of anti-HDL with clinical features of the disease had been unexplored. Previous studies have demonstrated increased levels of anti-HDL in APS patients when compared to HDs and SLE patients without APS (6, 7, 21) In addition, increased levels of anti-HDL were found to be inversely correlated with the levels of paraoxonase-1 (PON1), accounting for the antioxidant effect of HDL (22). In line with the data available in the literature so far, our study showed that APS patients presented significantly increased levels of IgG anti-HDL when compared to HDs, hence confirming this result in the larger APS cohort analyzed until date. Interestingly, our results went further by confirming that this result remained after correcting for total IgG levels, thus suggesting that higher levels of anti-HDL antibodies cannot be attributed to a general over-activation of the immune system in the context of autoimmunity,

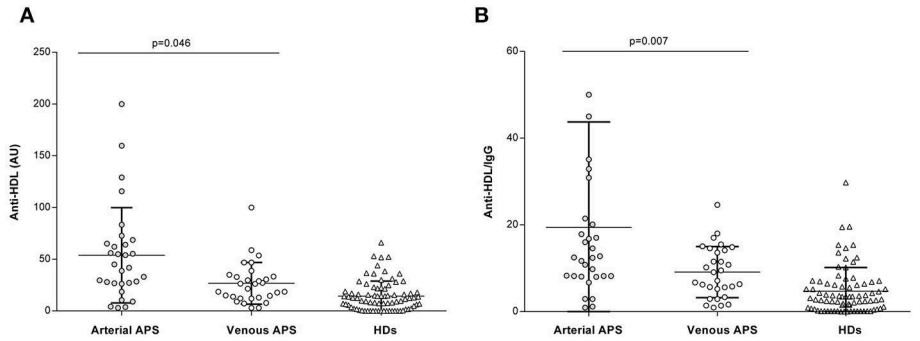

FIGURE 2 | Anti-HDL antibodies and clinical features in APS. Levels of IgG anti-HDL antibodies measured as AU **(A)** or normalized after total IgG correction **(B)** in APS patients with arterial thrombosis ($n = 30$, gray dots), venous thrombosis ($n = 30$, open dots) (**Table 1**) and HDs ($n = 80$, open triangles). Differences were assessed by Kruskal–Wallis tests and *p*-values indicated were derived from Dunn–Bonferroni tests for multiple comparisons.

but to the specific production of these antibodies. Additionally, the emergence of anti-HDL antibodies in APS was not linked to the positivity of other aPL antibodies or treatments. Moreover, anti-HDL were not related to traditional CV risk factors in APS patients, in line with previous findings in SLE and RA cohorts by our group and others (9, 21), thus strengthening their role as independent, complimentary biomarkers.

A remarkable finding from our study was the association with arterial thrombosis. Even if deep vein thrombosis represents the most common feature of APS, arterial events constitute the most dangerous and potentially life-threatening manifestations of the disease, affecting primarily the central nervous system and young adults <50 years old (3, 23, 24) Although some progresses have been made in order to identify those patients who are at higher risk for developing arterial events, it still represents an urgent unmet clinical need (25–27).

When analyzing the association between anti-HDL and clinical manifestation of APS in our study, we found statistically significant higher levels of IgG anti-HDL in those patients who have a history of arterial events. This result suggests that arterial APS patients might display a prominent impairment of the anti-atherogenic function of HDL, which represent a key step in endothelial dysfunction, oxidative stress, atherosclerotic plaque formation and progression, ultimately leading to atherothrombotic manifestations. Importantly, no differences in other clinical and laboratory parameters have been found between these groups of patients. If our observation were confirmed in larger prospective studies, IgG anti-HDL might represent an additional tool to CV risk factors profiling in the identification and management of "high-risk patients," which might guide the therapeutic strategies. In fact, the recent RAPs and TRAPs trials (28, 29) have reported different results when it comes to using new agents for venous and arterial events, hence strengthening the validity of our findings as biomarkers for the clinical setting. In this context, "high risk patients" might benefit of combined thrombo-prophylactic therapy as primary (e.g., anti-platelets and hydroxycholoroquine) or secondary prophylaxis (anti-platelets/hydroxycholoroquine associated to VKA) (30); similarly, they might be discouraged to the use of direct oral anticoagulants (31, 32). However, the real impact of IgG anti-HDL testing on therapeutic patients' management is not addressable in this study due to its cross-sectional design.

As mentioned above, our data showed that higher levels of anti-HDL were found in smokers. The link between smoke and autoimmunity has been already described in a wide range of pathologic conditions (33). Cigarette smoking exerts several pro-inflammatory effects, increasing oxidative stress, inducing the release of intracellular antigens, the augmentation of auto-reactive B-cells activity and an overall production of autoantibodies, including aPL (34). In this context, smoking might represent an important environmental trigger for anti-HDL production, and a potential mechanistic link between this risk factor and the occurrence of either thrombotic-embolism or atherosclerosis development, the main clinical outcomes related to anti-HDL in the literature. However, further analyses are needed in order to clarify this possible association from a mechanistic point of view and its clinical impact in APS.

Finally, our study shed new light into the associations between anti-HDL and disease-related autoantibodies. On the one hand, early studies from other groups reported certain degree of cross-reactivity between aCL and anti-HDL antibodies (6), although this was not confirmed in other studies (6). Importantly, previous analyses on these antibodies in APS were performed in low sample size populations. Our findings revealed no association between anti-HDL and aCL antibodies, challenging the previous notion. This is in line with previous studies from our group when analyzing other disease-related autoantibodies positivity (8–10). Recent advances have brought to light the existence of a heterogeneous group of pathogenic autoantibodies in APS. Among them, aPS/PT antibodies have been proven to have a clinical independent relevance in this setting (35), confirmed in international studies by our group and others (36, 37). In our analysis, despite anti-HDL levels not showing any association other aPL antibodies, patients tested positive for aPS/PT antibodies exhibited higher levels of anti-HDL. As the clinical role of aPS/PT is a rising topic, particularly when other aPL tests are negative, this association

could be of special interest and could make a case for anti-HDL as new potential biomarkers in this specific subset of patients.

This study has potential limitations, including mainly the cross-sectional retrospective design and the limited sample size. Indeed, prospective larger studies are needed to confirm these findings. However, our study documented, in line with the data available in the literature about the presence of IgG anti-HDL antibodies in APS and expanded the current knowledge about the emerging role of these autoantibodies in autoimmune-mediated diseases and CVD. Moreover, whether anti-HDL antibodies could be also associated with other surrogate markers of CVD (such as subclinical atherosclerosis), in addition to their association with thrombosis, remains to be elucidated. Similarly, patients exhibiting both arterial and venous events were not analyzed separately due to sample size concerns. Further studies may elucidate if these patients show a different/intermediate profile of anti-HDL antibodies. On the other hand, it may be interested to analyze whether anti-HDL antibodies may be linked to aPL-pregnancy related complications. However, due to sample size concerns and potential clinical differences (38), this was not explored in our study. Additionally, although differences in hyperlipidemia were observed between patients and controls, the lack of association between lipid profiles and anti-HDL levels, and the relatively small differences in lipid levels between these two groups lead us to think that this do not represent a major limitation for our findings. This point is also supported by the existing literature findings (8, 39–44). In summary, to the best of our knowledge, this is the first study informing an association between anti-HDL antibodies and thrombotic outcomes in APS patients. Our study warrants future pathogenic studies are needed to confirm such observations. Moreover, our findings support that anti-HDL might represent a promising tool for risk management and assessment and a reliable biomarker for the early identification of arterial thrombotic events. Despite some preliminary evidence (6, 45), exploring the role of autoantibodies against lipoprotein components is still intriguing in the APS setting, as their increased thrombotic and atherothrombotic profile might support the need of targeted specific approaches.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR'S NOTE

An interim version of this study has been presented as a poster at the 2018 American College of Rheumatology (ACR) annual meeting (Abstract Number 168). The authors have expanded the sample and analyses performed upon.

AUTHOR CONTRIBUTIONS

SS, IC, MR, and ER were in charge of patients' recruitment, clinical data collection, and analysis of the results. AS and DR contributed to the study conception, analysis, and discussion of the results. SS and JR-C conceived the study and designed the study protocol. SS, IC, and JR-C drafted the manuscript. JR-C performed the experimental procedures and edited the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2019.00211/full#supplementary-material

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REVIEW
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Classical Disease-Specific Autoantibodies in Systemic Sclerosis: Clinical Features, Gene Susceptibility, and Disease Stratification

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Systemic sclerosis (SSc) is an autoimmune disease characterized by abnormalities in microcirculation, extracellular matrix accumulation, and immune activation. Autoantibodies are markers of immune abnormalities and provide diagnostic and predictive value in SSc. Anti-topoisomerase antibodies (ATAs), anticentromere antibodies (ACAs), and anti-RNA polymerase antibodies (ARAs) are the three classical specific antibodies with the highest availability and stability. In this review, we provide an overview of the recent progress in SSc research with respect to ATAs, ACAs, and ARAs, focusing on their application in distinguishing clinical phenotypes, such as malignancy and organ involvement, identifying genetic background in human leukocyte antigen (HLA) or non-HLA alleles, and their potential roles in disease pathogenesis based on the effects of antigen–antibody binding. We finally summarized the novel analysis using ATAs, ACAs, and ARAs on more detailed disease clusters. Considering these advantages, this review emphasizes that classical SSc-specific autoantibodies are still practical and have the potential for patient and risk stratification with applications in precise medicine for SSc.

Keywords: anti-topoisomerase antibodies, anticentromere antibodies, anti-RNA polymerase antibodies, systemic sclerosis, clinical manifestations, gene, disease stratification

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INTRODUCTION

Systemic sclerosis (SSc) or scleroderma is a chronic multi-system disease with heterogeneous manifestations (1). There is still a lack of recommendations with strong evidence regarding the diagnosis and management of several SSc-specific complications (2), leading to a reduced quality of life and an enormous burden for patients. The mechanism underlying SSc is characterized by three manifestations: vascular injury, immune abnormality, and fibrosis. Vascular injury is identified as an initial factor, whereas fibrosis is considered a sign of the end stage. Furthermore, immune activation has been proposed as a bridge throughout the disease course. Autoantibodies, indicators of immune abnormality, are detected in >90% of patients with SSc (3). Anti-topoisomerase antibodies (ATAs), anticentromere antibodies (ACAs), and anti-RNA polymerase antibodies (ARAs), first described in the 1970–1990s (4, 5), are the classical disease-specific autoantibodies (1).

Because of the high validity and reliability of ATAs, ACAs, and ARAs for SSc (6), the 2013 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) SSc

classification criteria included disease-specific autoantibodies as a scoring item (1), and the 2018 Japanese Dermatological Association listed them as minor diagnostic criteria (7). SSc-specific antibodies were also listed in the very early diagnosis of SSc (8) or UCTD-risk-SSc criteria (9). In general, the presence of these three SSc-specific autoantibodies may be relevant to the different clinical manifestations of SSc, such as diffuse/limited cutaneous subtypes and pulmonary fibrosis. Recently, bioinformatics helped discover new roles of these autoantibodies; genetic susceptibility analysis revealed the intrinsic characteristics of patients in different autoantibody subgroups (10). Moreover, cytology studies suggested pathological roles for ACAs, ATAs, and ARAs beyond disease diagnosis (11). Thus, the detection of ACAs, ATAs, and ARAs may facilitate the development of precise medicine.

For a systemic understanding of classical SSc-specific autoantibodies, we have reviewed the general information on ATAs, ACAs, and ARAs in clinical manifestations, emphasizing their role in SSc-related cancer. Next, we have comprehensively summarized research breakthroughs describing the genetic features of these autoantibodies, illustrated the potential pathogenesis pathway, and identified the novel disease clusters related to these SSc-specific autoantibodies.

CLASSICAL DISEASE-SPECIFIC AUTOANTIBODIES IN CLINICAL MANIFESTATIONS

Epidemiology

Although several studies have reported a varying prevalence of classical disease-specific autoantibodies in SSc, their reported sensitivity and specificity remain relatively stable (12). The prevalence of ATAs in patients with SSc was reported to be 14–71%, with a sensitivity of 24% and a specificity of 99.6% (1). ARAs were detected in 4–20% of patients, with 16% sensitivity and 97.5% specificity (13). The prevalence of ACAs in patients with SSc was 20–57.8%, with a sensitivity and specificity of 33 and 93%, respectively (13, 14). However, unlike ATAs and ARAs that are rarely detected in other autoimmune diseases, ACAs may be produced in systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, and primary biliary cholangitis (15). Thus, the presence of ACAs in other disorders may help elucidate the occurrence trend of SSc overlap syndromes (16).

The levels of classical disease-specific autoantibodies reportedly vary in patients based on ethnicity. ACAs had a higher detection ratio in Hispanic and Caucasian patients compared with those belonging to African-American ($P < 0.0001$) and Asian ethnicities ($P < 0.001$) (14, 17). ATAs were mostly detected in Asian patients (17–19), whereas the prevalence levels of ARA were much higher in European (>10%) patients but lower in Asian (<6%) patients (14, 20).

Clinical Associations

Skin Involvement

Among the classical autoantibodies, ACAs are more specific for the limited cutaneous subset of SSc (lcSSc) or CREST syndrome than ATAs ($P = 0.005$, OR = 2.54, 95% CI = 0.05–0.44) (21) and ARAs ($P = 0.0005$, OR = 0.13, 95% CI = 0.04–0.41); a longer disease duration before diagnosis (22) is related to good prognosis in terms of survival (23). Increased levels of ATAs are mainly associated with diffuse cutaneous disease (dcSSc) ($P < 0.0001$, OR = 4.26) (22) and serious organ involvement (13, 24). Patients with ATAs had higher SSc-related mortality rate and poor prognosis (25). ARA presence indicates a high risk of rapidly progressive skin thickening ($P = 0.042$, OR = 3.24, 95% CI = 1.44–7.31), and changes in ARA levels may correspond to changes in modified Rodnan skin thickness score (26, 27). A recent study revealed ARAs to be more prevalent in patients with sine scleroderma (P = 0.03) (28), an SSc subtype without cutaneous manifestations but with visceral involvement and serologic abnormalities that is difficult to diagnose (29). Since skin involvement was confirmed related to disease severity, different autoantibody groups can provide a preliminary grouping of patients for disease management.

Organ Involvement

ACAs are used to determine disease specificity in consistent vessel dysfunction not only for long-standing Raynaud's Phenomenon (RP) ($P < 0.001$) but also in pulmonary hypertension (PAH) without fibrosis ($P < 0.001$), compared with ATAs. Other vessel abnormalities include digital ulcers ($P < 0.0001$, OR = 0.50, 95% CI = 0.36–0.71), and a possible early/active nailfold videocapillaroscopy pattern (30). Furthermore, prior to a definite diagnosis of pulmonary diseases, ACAs were associated with a relatively rapid rise in pulmonary arterial systolic pressure and pulmonary vascular resistance ($P < 0.001$) (31). Thus, ACAs play a crucial role in consistent vascular injury. The appearance of ACAs at an early stage of SSc, related to vascular disease, should be closely monitored in patients, especially in the cardiopulmonary system.

Studies have shown ATA association with a higher probability of interstitial lung disease (ILD) ($P < 0.0001$, OR = 4.76, 95% CI = 3.48–6.50), even in ATA-positive patients with lcSSc (22, 25, 32). Recent studies have indicated that ATAs may be related to disability in hand, oral manifestation (33, 34), and flexion contractures in metacarpophalangeal and proximal interphalangeal joints (35), indicating their specificity, to a certain degree, in organ fibrosis. Therefore, early screening for organ involvement is recommended in ATA-positive patients because organ fibrosis is indicative of an irreversible stage.

A higher prevalence of musculoskeletal involvement, gastric antral vascular ectasia, ILD, PAH, and scleroderma renal crisis

Abbreviations: SSc, systemic sclerosis; ATAs, anti-topoisomerase antibodies; ACAs, anticentromere antibodies; ARAs anti-RNA polymerase antibodies; ANA, antinuclear antibody; ECM, extracellular matrix; VEDOSS, very early diagnosis of SSc; UCTD, undifferentiated connective tissue disease; RP, Raynaud's phenomenon; CENP, centromere proteins; PAH, pulmonary hypertension; ILD, interstitial lung disease; SRC, scleroderma renal crisis; HLA, human leukocyte antigen; SNP, single-nucleotide polymorphism; STAT4, signal transducer and activator of transcription 4; PTP22, protein tyrosine phosphatase N22; BANK1, B-cell scaffold protein with ankyrin repeats gene; TNF, tumor necrosis factor; AIF1, allograft inflammatory factor 1; IRF, interferon regulatory transcription factor; PBMCs, peripheral blood mononuclear cells; IL, interleukin; TNFSF, tumor necrosis factor superfamily; EC, endothelial cells.

(SRC) has been reported in ARA-positive patients (26, 28, 36, 37). Notably, SRC was significantly more common in ARA-positive patients compared to ARA-negative ones ($P < 0.0001$). Moreover, ARAs showed high sensitivity (70.8%, 95% CI = 48.9–87.4), high specificity (87.8%, 95% CI = 84.3–90.8), and high negative predictive value (98.2%, 95% CI = 96.3–99.3) for patients with SRC. Interestingly, 16% of ARA-positive patients had a common history of silicone breast implants in a Japanese cohort (38, 39), suggesting a potential role of silicone in the development of disease with ARAs. In general, ARA measurement in patients with SSc is useful for diagnosis and risk stratification of severe manifestations, such as renal crisis and malignancy.

Malignancy

Similar to other autoimmune diseases, SSc is associated with malignancy in the lungs, breasts, liver, and hematologic systems. Although the role of autoantibodies is still under debate, ATAs, ACAs, or ARAs were barely detected in tumor-carrying patients without SSc (40).

ATAs were found to show higher risk of cancer after SSc diagnosis (HR = 1.4, 95% CI = 1.05–1.90, $P = 0.0224$) and have a significant negative impact on survival of the overall malignancy group (HR = 1.39, 95% CI = 1.08–01.80, $P = 0.0106$) (41). In a patient cohort with limited scleroderma/SSc overlap syndrome and mild organ involvement, ACAs correlated with a high risk of non-Hodgkin's lymphoma (42).

In contrast, ARAs are strongly associated with malignancy. Ami et al. first identified a strong association between RNAP I/III autoantibodies and malignancy contemporaneous with SSc ($P = 0.027$) (43). An Italian cohort study divided malignancy cases based on SSc onset: preceding (diagnosed >6 months before SSc onset), synchronous (6 months before to 12 months after), or metachronous (>12 months after); a significant association was observed between malignancies synchronous to SSc and ARA-positivity (OR = 7.38, 95% CI = 1.61–33.8) (44). Another large cohort study in the UK found breast cancer (>40%) to be the major malignancy subtype associated with SSc, and the frequency of cancer in ARA-positive patients was approximately twice that in the ATA- and ACA-positive groups (45). Similar findings (46–48) were reported in the Japanese and EUSTAR registries, further suggesting that ARA-positive patients with SSc shared similar pathological processes across different ethnicities. More recently, ARAs were shown to be an independent marker of coincident cancer and SSc irrespective of age (49). These results recommend a regular screening protocol for cancer in ARA-positive patients with SSc.

The relationship between these autoantibodies and malignancy provides new insights into cancer-risk stratification by clinical and serological phenotypes, thereby allowing targeted screening in this population.

Classical Disease–Specific Autoantibodies and Genetic Characteristics

A specific genetic background with a combination of environmental and stochastic factors apparently contributes to SSc development (5, 50, 51). Autoantibodies are an essential part of the immune response; their susceptibility genes are not restricted to the major histocompatibility complex (human leukocyte antigen, HLA), but also include antigen presentation, lymphocyte activation, and cytokines/chemokines secretion (**Tables 1**, **2**). Therefore, identifying the genetic background may provide a better understanding of SSc diagnosis, intrinsic classification, and therapeutic monitoring (73, 74).

HLA and Classical Disease-Specific Autoantibodies

HLA alleles encode specific antigen-binding sequences, and thus play an essential role in antigen presentation, lymphocyte activation, and autoantibody production. HLA-class II (DRB1, DQB1, DQA1, and DPB1) alleles associated with SSc-related antibodies vary among different ethnic groups (**Table 3**).

ATAs were associated with DRB1*11:01/*11:04 in North-American Caucasians ($P < 0.0001$, OR = 6.93, 95% CI = 3.9–12.2); DPB1*13:01 in both African American ($P < 0.001$, OR = 4.3); and European-American patients ($P = 1.47 \times 10^{-24}$, OR = 13.7) (78); DRB1*15:02-DRB5*01:02, DPB1*09:01 haplotypes in Japanese and DQB1*06:01 in Chinese patients (78–81). Although DRB1*08:04, DQA1*05:01, and DPB1*13:01 were associated with African subjects, DPB1*13:01 showed the highest odds ratio.

ACAs were found associated with DQB1*05:01/*26 alleles (82). In Chinese Han patients, the expression of DQB1*05:01 was significantly increased ($P = 1.6 \times 10^{-5}$, OR = 3.4, 95% CI = 1.8–6.4), whereas in the European-American population, DPB1*13:01 and DRB1*07:01 alleles were more strongly relevant ($P = 4.79 \times 10^{-20}$, OR = 0.1) (78–80). The available data on African subjects are lacking, perhaps because of the small number of samples studied. DQB1*02:01 was first shown to be associated with RNAP I-III by Kuwana et al. (76). Another study proved the association between anti-RNAP I/III antibodies and DRB1*04:05 ($P = 0.01$, OR = 6.0, 95% CI = 1.4–25.2), DRB4*01 ($P = 0.02$, OR = 10.1, 95% CI = 1.4–74.1), and DQB1*04:01 ($P = 0.01$, OR = 6.0, 95% CI = 1.4–25.2) in Japanese patients (81). Recent evidence found that DRB1*04:04 (OR = 5.13), DRB1*11 (OR = 1.55), and DQB1*03 (OR = 2.38) alleles were more present in Hispanic and Caucasian patients, whereas DRB1*08 allele (OR = 3.92) was more present in African patients with ARAs (78, 79).

These findings indicate that specific HLA-alleles may provide susceptibility to classical disease-specific autoantibodies in SSc. Although the HLA associations in SSc patients with classical disease-specific autoantibodies remains unclear, these findings provide insights for the individual recognition of antibody specificities.

Non-HLA Genes and Classical Disease-specific Autoantibodies

STAT4

Signal transducer and activator of transcription 4 (*STAT4*), a susceptibility gene for multiple autoimmune diseases, is associated with immune dysregulation, for example, in the imbalance of Th1/Th2 cytokine and the synthesis of the extracellular matrix across different ethnic groups (54, 83).

TABLE 1 | Publications of susceptible genes involved in lymphocyte activation in systemic sclerosis.

Gene	Author, Year [References]	Research type	Case/Control	Locus/SNPs	Associated autoantibodies	Population
STAT4	Krylov et al., 2017 (52)	Case–control	102/103	rs7574865 G/T	ATA	Russian
	Yi et al., 2013 (53)	Case–control	453/534	rs7574865 rs10168266	ATA	Han Chinese
	Dieudé et al., 2009 (54)	Case–control	440/485 (replication:445/485)	rs7574865 T	ATA	French Caucasian
PTPN22	Wipff et al., 2006 (55)	Case–control	121/103	PTPN22*R620W	No association	French Caucasian
	Balada et al., 2006 (56)	Case–control	54/55	PTPN22*R620W	No association	N/A
	Ramirez et al., 2012 (57)	Case–control	RA: 413 SLE: 94 SSc: 101 HC: 434	C1858T (rs2476601)	No association	Colombian
	Gourh et al., 2006 (58)	Case–control	White:850/430 Black:130/164 Hispanic:120/146 Choctaw Indian: 20/76	C1858T	ATA&ACA	US white, black, Hispanic, and Choctaw Indian individuals.
	Dieudé et al., 2008 (59)	Case–control & Meta–analysis	659/504	PTPN22 1858T	ATA	French Caucasian
	Diaz-Gallo et al., 2011 (60)	Meta–analysis	3422/3628	C1858T	ACA	Spain and 7 additional independent replication Caucasian
	Lee et al., 2012 (61)	Meta–analysis	4367/4771	C1858T	ACA	Multiple ethnicity
BANK1	Rueda et al., 2009 (62)	Case–control	2380/3270	rs10516487 G rs17266594 T rs3733197 G	ATA	Caucasian (American, Spanish, Dutch, German, Swedish and Italian)
	Dawidowicz et al., 2011 (63)	Case–control	900/1034	BANK1(N/A)	No association	European Caucasian

NA, not available.

Dieudé et al. first identified *STAT4* polymorphism rs7574865 in association with ANAs ($P = 0.01$, OR = 1.30, 95% CI = 1.11–1.53) in SSc, although the specificity for ACAs/ATAs/ARAs was not confirmed (54). Another study in a Russian population indicated a possible association between ATAs and *rs7574865* (52). A large-cohort study demonstrated that *rs7574865* ($P = 0.0012$, OR = 0.56, 95% CI = 0.38–0.81) and *rs10168266* ($P = 3.1 \times 10^{-4}$, OR = 0.51, 95% CI = 0.35–0.75) were strongly associated with ATA presence and pulmonary fibrosis in Chinese patients with SSc (53).

STAT4 is essential for the biological functions of various immune cells; however, its specific characteristics in SSc are unknown. Animal experiments have revealed that $STAT4^{-/-}$ mice were resistant to SSc (84). Thus, these autoantibodies may provide a basis for a better understanding of the disease.

PTPN22

Protein tyrosine phosphatase N22 (*PTP22*) encodes a phosphatase related to the T-cell signaling pathway and shares a definite association with multiple autoimmune diseases. However, conflicting findings are reported in SSc.

Wipff et al. and Balada et al. demonstrated that *PTPN22*620W* was not associated with autoantibody patterns in a cohort of French Caucasian patients with SSc (55, 56). In contrast, Gourh et al. indicated that *PTPN22 R620W* polymorphism was associated with ACA- and ATA-positive subsets and was considered a risk factor in both Caucasian and African patients (58). It was suggested that a variation of *PTPN22* expression in the autoantibodies (ACAs or ATAs) was based on differences in ethnicities and presence of single-nucleotide polymorphism (SNP) (57, 59–61, 85).

BANK1

B-cell scaffold protein with ankyrin repeat gene (*BANK1*) encodes the substrate of LYN tyrosine kinase and participates in phosphorylation of triphosphate receptors, that are specifically expressed in B lymphocytes (63, 86, 87). Recent evidence suggests that *BANK1*, *IRF5*, and *STAT4* risk alleles display a multiplicatively increased risk of dcSSc (58, 62, 88, 89).

The first study to significantly implicate *BANK1* in SSc was reported in 2009; in 2,380 Caucasian patients with SSc, *BANK1* polymorphisms—*rs10516487, rs17266594,* and *rs3733197*—were found to be restricted to ATA-carrying subgroups ($P = 0.03$,

TABLE 2 | Publications of susceptible genes involved in inflammatory factors in systemic sclerosis.

Gene	Author, Year [References]	Research	Case/Control	Locus/SNPs	Associated autoantibodies	Population
TNF	Sato et al., 2004 (64)	Case-control	214/354	TNF-863A	ACA	UK white
	Lomelí-Nieto et al., 2019 (65)	Case-control	53/115	TNFA-308G>A TNFA-238G>A	ARA	Southern Mexico
AIF1	Alkassab et al., 2007 (66)	Case-control	1015/893	rs2269475 (T and CT/TT)	ACA	Caucasian African American Hispanic
IRF7	Carmona et al., 2012 (67)	Case-control	2316/2347	rs1131665 rs4963128 rs702966 rs2246614	ACA	USA Caucasian USA Spain
Th17	Rueda et al., 2009 (68)	Case-control	143/246 (replication:365/515)	IL23R	No association	Dutch Replication: Spanish
	Agarwal et al., 2009 (69)	Case-control	1402/1038	IL23R: rs11209026 rs11465804	ATA	N/A
	Mellal et al., 2018 (70)	Case-control	136/317	IL-21: rs6822844	ARA	Algerian
TNFSF	Coustet et al., 2012 (71)	Case-control	1031/1014	TNFSF4: rs2205960	ACA	French white
		Genotype-phenotype association analysis and Meta-analysis	4989/4661	TNFSF4: rs2205960	ACA	European white
	González et al., 2018 (72)	Case-control	4584/5160	TNFSF13B: rs374039502	No association	European

NA, not available.

OR = 1.20, 95% CI = 1.02–1.41; $P = 0.01$, OR = 1.24, 95% CI = 1.05–1.46; $P = 0.004$, OR = 1.26, 95% CI = 1.07–1.47, respectively) (90).

Notably, *BANK1* is chiefly expressed in $CD19^+$ B cell-overexpressing patients with SSc (91). These findings may explain the role of abnormal B cells in SSc-specific autoantibody production.

TNF Alleles

Tumor necrosis factor (*TNF*), a key proinflammatory cytokine, plays an important role in SSc by upregulating Nuclear factor kappa B (92). Parks et al. first proposed that the *TNF-β +252* locus plays a crucial role in SSc etiopathogenesis (93). Other polymorphisms (*TNF-α* and *TNF receptor-II*) are also linked with autoantibodies in SSc (94). However, a linkage disequilibrium exists between *TNF* and HLA genes; therefore, the phenomenon may reflect the situation already described for HLA.

Several studies have attempted to elucidate this relationship. Extensive research has identified a strong primary association of *TNF-863A* and *TNF-1031C* alleles with ACA-positivity as well as *TNF-857T* allele with ATAs in SSc (64). Recent evidence indicated that *TNFA* polymorphisms, associated with higher sTNF-α levels, positively correlate with ARAs levels (65).

TNFSF

TNF (*TNFSF*) superfamily members *TNFSF13B*, encoding BAFF, and *TNFSF4*, encoding OX40 antigen ligand, are reportedly involved in SSc. Both play crucial roles in the interaction between T cells/antigen presentation and T- and B-cell activation (71, 72). Genotype–phenotype association analysis and meta-analysis confirmed *TNFSF4* as an SSc susceptibility gene and *rs2205960* as a putative causal variant with a preferential association with the ACA-positive SSc subtype ($P = 0.0015$, OR = 1.37, 95% CI = 1.12–1.66) (71).

TNFSF4 rs1234214 is significantly associated with ACA-positivity ($P = 0.005$, OR = 1.33, 95% CI = 1.1–1.6) and ATA-positivity ($P = 0.026$, *OR* = 1.31, 95% CI = 1.02–1.7) (95). The association of *rs844648* with ARAs ($P = 0.004$, OR = 1.4, 95% CI = 1.1–1.8) was also confirmed (95).

Thus, *TNFSF4* may be involved in autoimmunity for the development of SSc.

AIF1

Allograft inflammatory factor 1 (*AIF1*) encodes a cytoplasmic calcium-binding protein that is present in damaged vessels of the lungs and skin lesions of patients with SSc, thereby presumably playing a role in vascular pathology (96–99).

TABLE 3 | Antigen, prevalence, clinical features, and susceptible genotype of classical specific antibodies in systemic sclerosis.

Autoantibody	Antigen			Prevalence (%)			Clinical features		Susceptible genotypes	
	Designation	Major	Location	Function	General	Early SSc (1)	VEDOSS (16)	Cutaneous subset	Special features	HLA alleles	Genes involved in pathways
Anticentromere (ACAs)	Centromere proteins (CENP)	CENP -A, -B, -C	Around kinetochore	Constituent of the primary constriction of metaphase chromosomes	20–57.8 (13, 14, 75)	42.5–67.5	53.6	lcSSc (CREST syndrome)	Long-standing Raynaud's phenomenon PAH	DQB1*05:01/*26 DPB1*13:01 DRB1*07:01	TNF-863A AIF1 IRF7 TNFSF4 PTPN22
Anti-topoisomerase (ATAs)	DNA topoisomerase (Topo)	Topo I	Chromatin	Relaxation of supercoiled DNA	14–71 (20, 26, 76)	12.3–22.5	19.1–22	dcSSc	Cardiomyopathy IPF	DRB1*11:01/*11:04 DPB1*13:01, DRB1*15:02-DRB5*01:02 DPB1*09:01 DQB1*06:01 DRB1*08:04/DQA1*05:01	IL23R STAT4 PTPN22 BANK1 RXRB
Anti-RNA polymerase (ARAs)	RNA polymerase (RNAP)	RNAP I/III	Nucleoli nucleoplasm	Synthesis of ribosomal RNA precursors Synthesis of small RNAs	4–20 (13, 77)	0–31.3	N/A	dcSSc	Rapidly progressive skin thickening Musculoskeletal involvement, Gastric antral vascular ectasia, Tendon friction rubs, Synovitis, Myositis, Malignancy	DQB1*02:01 DRB1*04:05 DRB4*01 DQB1*04:01 DRB1*04:04 DRB1*11 DQB1*03 DRB1*08	TNFA-308G>A TNFA-238G>A IL-21

NA, not available.

Moreover, genetic association between *AIF1* polymorphism and the ACA-positive subset of SSc was confirmed (P = 0.006/0.002 in Caucasians/combined group, OR = 1.53/1.56 in Caucasians/combined group, 95% CI = 1.11–2.11/1.18–2.07 in Caucasians/combined group) (66). Limited by the absence of adequate data, confirmation of its potential biological relevance remains a significant challenge.

IRF7

Interferon regulatory factor 7 (*IRF7*), a member of the interferon regulatory transcription factor family and a key molecular determinant in interferon pathway, can activate type I interferon genes in response to viral agents or DNA/RNA-containing immune complex, first described by Carmona et al. (67).

IRF7 mRNA expression was significantly upregulated in the bleomycin-induced and tight-skin mouse models as well as in peripheral blood mononuclear cells and dermal fibroblasts from patients (100). Moreover, patients with different *IRF7* SNPs (rs1131665: $P = 6.14 \times 10^{-4}$, OR = 0.78; rs4963128: $P = 6.14 \times 10^{-4}$, OR = 0.79; rs702966: $P = 3.83 \times 10^{-3}$, OR = 0.82; and rs2246614: $P = 3.83 \times 10^{-3}$, OR = 0.83) were mostly related to ACA-positivity (67, 100, 101), thus supporting the fact that the *IRF7* locus represents a common risk factor for ACA production.

Genes Associated With T-helper 17 Cell Pathway

Recent findings indicated the role of Th17 pathway in SSc, which is promoted by several factors including interleukin (IL)-17A, IL-17F, IL-21, and IL-23R (68, 70).

IL23R polymorphisms (rs11209026, rs11465804) were associated with susceptibility to ATA-positive SSc ($P = 0.001$, $P = 0.0026$, respectively) and considered protective against the development of PAH in patients with SSc ($P = 3 \times 10^{-5}$, $P = 1 \times 10^{-5}$, respectively). Additionally, an association between *IL-21 SNP (rs6822844)* and ARA production as well as digestive involvement (69) was found, indicating that Th17 genes were associated with SSc-susceptibility and specific-organ involvement (70).

RXRB

A retinoid X receptor beta (*RXRB*) variant, *rs17847931,* is associated with antifibrotic activity in the skin and chromatin remodeling in ATA-positive patients with SSc (102). Since RXRB, a type of RXR, mediates the effects of retinoic acid that shows anti-fibrotic activity in skin tissues (103), the prospective therapeutic role of retinoic acid may be better applied in SSc groups with specific autoantibodies.

Applications of Classical Disease-Specific Autoantibodies as Predictors of SSc Development

RP exists in more than 90% of patients with SSc and could precede organ fibrosis by years or even decades (104). However, RP without specificity is also found in the early stages of other

autoimmune diseases. Importantly, patients with RP are at a risk of developing SSc.

SSc-specific autoantibodies independently predict definite SSc (105). Different autoantibodies were associated with a distinct time course of microvascular damage in a 20-year prospective study (105). ATAs were strongly predictive for SSc with a nine-fold probability of SSc occurrence in primary patients with RP (106). The presence of both ATAs and scleroderma patterns of

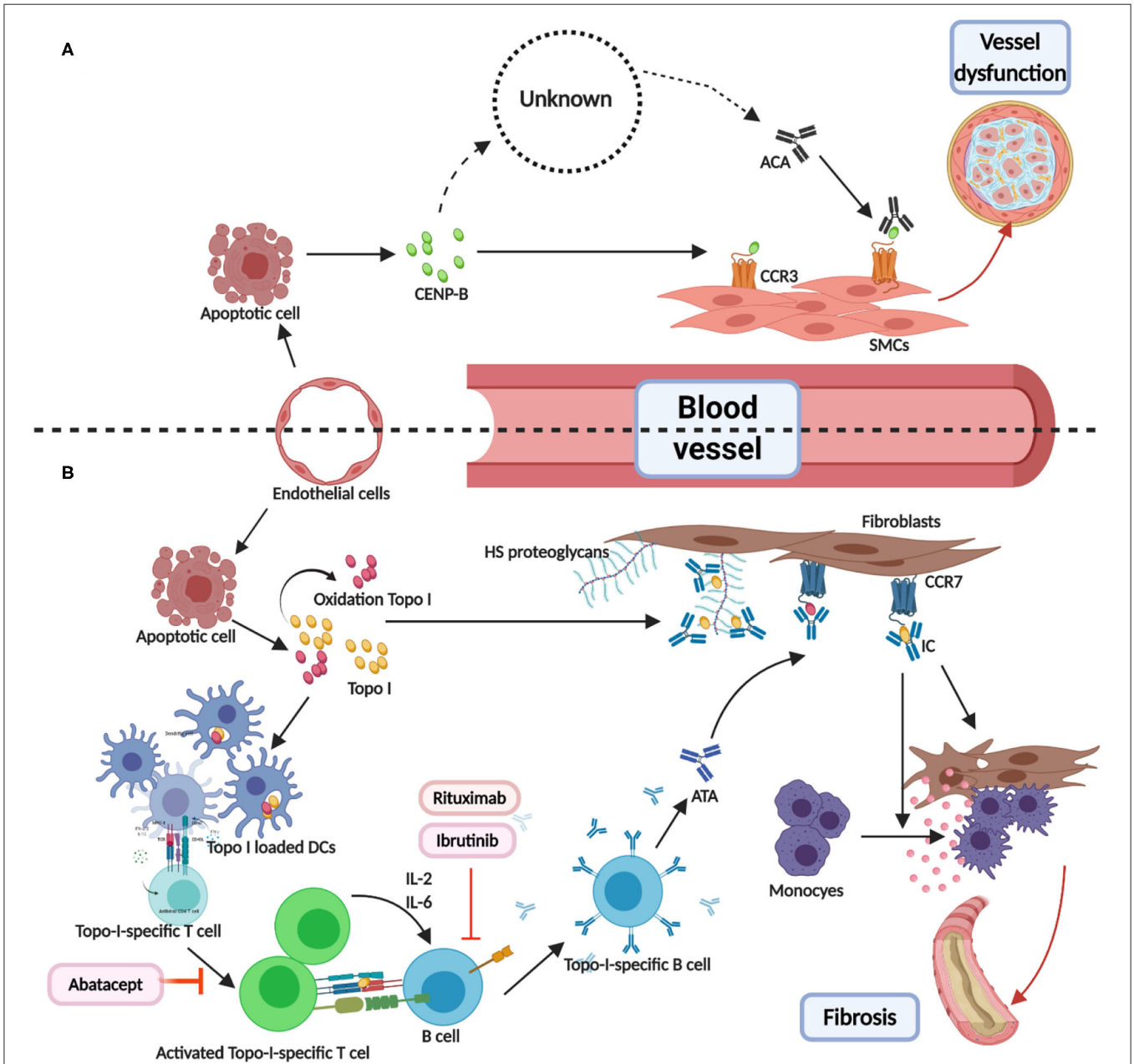

FIGURE 1 | Direct combination of antibodies and antigens in systemic sclerosis. **(A)** CENP-B were released from the apoptotic ECs. Then, the extracellular CENP-B bound to the contractile-type PASMCs via CCR3. Next, the binding of CENP-B to the contractile SMCs stimulated migration in the wound healing assays. The exact way of production of ATAs was known. When combined with CCR3-binding CENP-B, ATAs may abolish vascular self-repair, further leading to angiopathy. **(B)** TOPO I was released from apoptotic ECs and some of them were oxidized to AOPP. Then, TOPO I was bound to the bystander fibroblasts via CCR7 or HS proteoglycans. DCs loaded with selected TOPO I could activate the intrinsic TOPO I–specific T cells. The activated special T cells produced IL-2 or IL-6 and communicated with B cells through the interactions of MHC-TCR and CD40-CD40L. T cell–dependent B cells were activated, thereby becoming TOPO I–specific B cells and resulting in ATAs. Binding TOPO I recruited circulating ATAs and composed ICs, which could induce the adhesion and activation of circulating monocytes. Abatacept-regulated dysfunction T cells. Rituximab and ibrutinib may be used as B-cell depletion therapy. CENP-B, centromere proteins B; EC, endothelial cell; PASMC, Pulmonary artery smooth muscle cells; CCR, CC chemokine receptor; SMC, smooth muscle cell; AOPP, advanced oxidation protein products; HS, heparan sulfate.

nailfold capillaroscopy may increase the prediction accuracy and susceptibility (107–109).

Therefore, when patients present various clinical features and initial diagnosis is difficult, abnormal findings on these three SSc-specific autoantibodies could help distinguish SSc from early stages of other autoimmune diseases.

As Biomarkers of Disease Phenotypes

ACAs, ATAs, and ARAs remain the most common SSc-specific autoantibodies in the majority of real-world studies. The use of these autoantibodies to define novel clinical classifications or disease clusters has been demonstrated over the years.

Moinzadeh et al. (107) used them to define five patient clusters with different clinical features: ATAs, strong ARAs, weak ARAs, ATAs, and others. Moreover, the statistical difference between the five clusters indicated that their use was not restricted to classification of the cutaneous subsets alone as previously reported. Further, Srivastava et al. (110) found that organ involvement was more associated with antibody profiles, whereas joint and vascular dysfunction were more related to cutaneous subsets.

Interestingly, the combination of ATAs and ACAs with cutaneous subsets or more parameters may predict outcomes better than their individual use. Nihtyanova et al. proposed seven groups of patients with SSc, combining autoantibody specificity and skin involvement (ATA + lcSSc, ATA + dcSSc, ACA + lcSSc, ARA+, other antibodies + lcSSc, other antibodies + dcSSc) (111) while Sobanski et al. (112) characterized six clusters based on antibody profiles (cutaneous subsets, organ damage, and prognosis together), thereby achieving a more precise risk stratification of patients. Similarly, an increased risk of cancer was found in ACA-positive patients with ACAs (113). Additionally, cancer-specific risk varied in different cutaneous subtypes, and the ARA + dcSSc group tended to have a greater risk of breast cancer, whereas the ARA + lcSSc group had a high risk of lung cancer.

In summary, ACAs, ATAs, and ARAs could be cost-effective screening tools for disease subclassification and would improve the management of patients with SSc, progressive SSc, and those at risk of developing it.

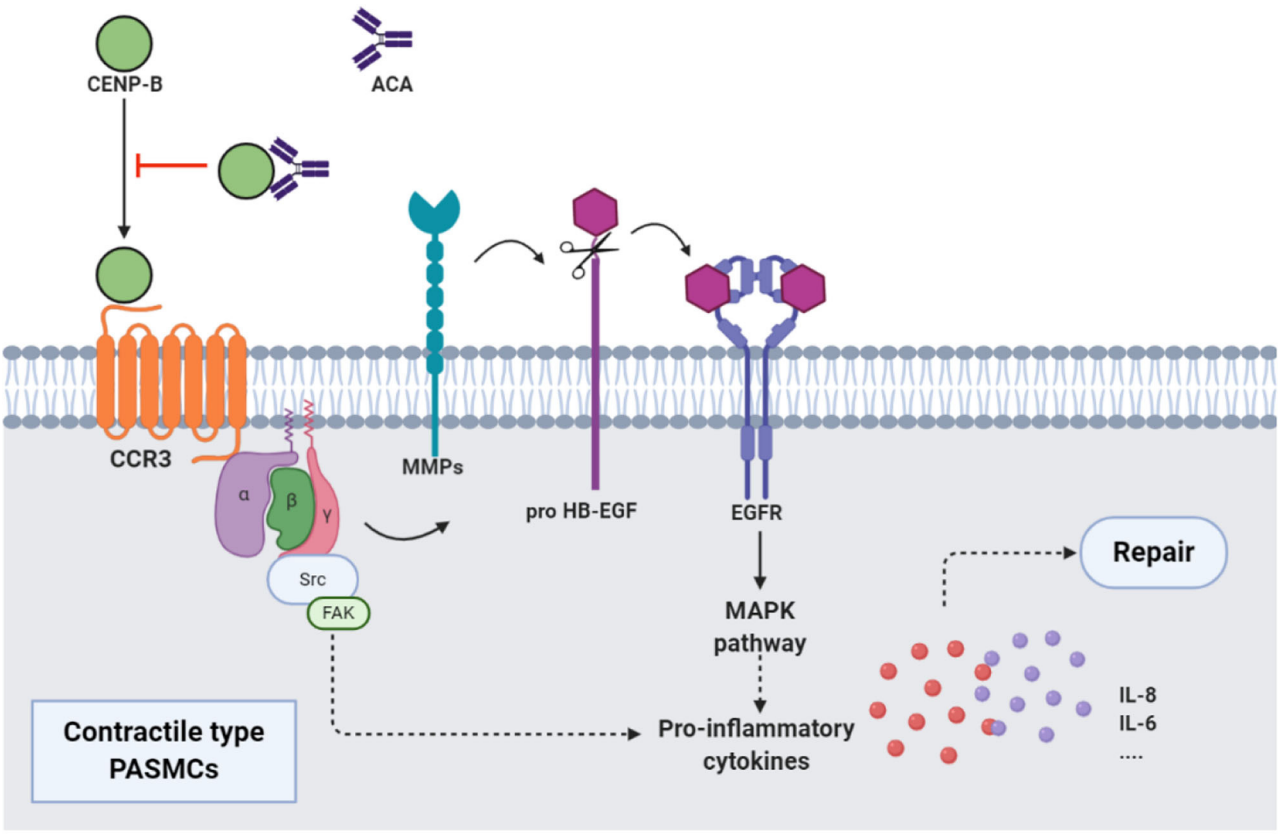

FIGURE 2 | ACAs and CENP-B: CENP-B bound to CCR3. Then, the cross-talk between CCR3 and EGFR, which was mediated by the MMPs-dependent processing of pro HB-EGF, activated MAPK pathway, and production of proinflammatory cytokines such as IL-8, and promoted the migration of contractile-type PASMCs, further leading to vascular self-repair. ATAs from patients with SSc, when combined with CCR3-binding CENP-B, abolished the abovementioned pathway and inhibited the vascular self-repair. EGFR, epidermal growth factor receptor; MMP, matrix metalloproteinase; HB-EGF, heparin-binding EGF-like growth factor; MAPK, mitogen activated protein kinase.

As Initiators of Pathogenesis

Considering the limited treatment options and unpleasant outcomes for patients with SSc, a better understanding of its pathogenesis is required. As a bridge between vascular injury and irreversible fibrosis, autoantibodies may act as the actual pathogenetic agents, secondary consequences of tissue injury, or pure footprints of etiological operators.

ATAs and ACAs were found to participate in a pathological pathway involving endothelial cells injury and antigen release and presentation (114–117). The antigens (centromere proteins, topoisomerase, and RNA polymerase) for ACAs, ATAs, and ARAs are distributed in and around the nucleus, and play important roles in cellular structure and function. Therefore, the release of antigens, combination of antigens, and cell surface receptors, T- and B-cell collaboration (32), and antigen–antibody binding are interlinked and involved in disease occurrence, with a central role for the binding of antigens (topo I and CENP-B) (118, 119) and cell surface receptors (Chemokine Receptor 7 and Chemokine Receptor 3) (120–122), illustrated in **Figure 1**. We hypothesized two effects of the formation of immune complexes (ATA-topo I and ACA-CENP-B): reinforcement of pathological functions and inhibition of physiological functions. **Figure 2** shows the pathway induced by the ACA-CENP-B complex and **Figure 3** displays the pathway leading by ATA-topo I complex.

Three immune models with underlying distinct autoantibody signatures using multilayer profiling were identified (123). The ATA cluster showed a vascular phenotype with disrupted angiogenesis reflected by imbalanced antiangiogenic factors and cytokines such as IL-21 and sFLT-1. The ACA cluster showed a follicular T helper–B cell phenotype, characterized by low expression of inflammatory markers, such as IL-21, and relatively limited and mild clinical features. The ARA cluster showed a fibrotic phenotype, with Th2/Th17-mediated fibrosis by cytokines such as IL-17 and IL-21.

With advances in the detection of autoantibodies and underlying pathological markers, more precise targeting

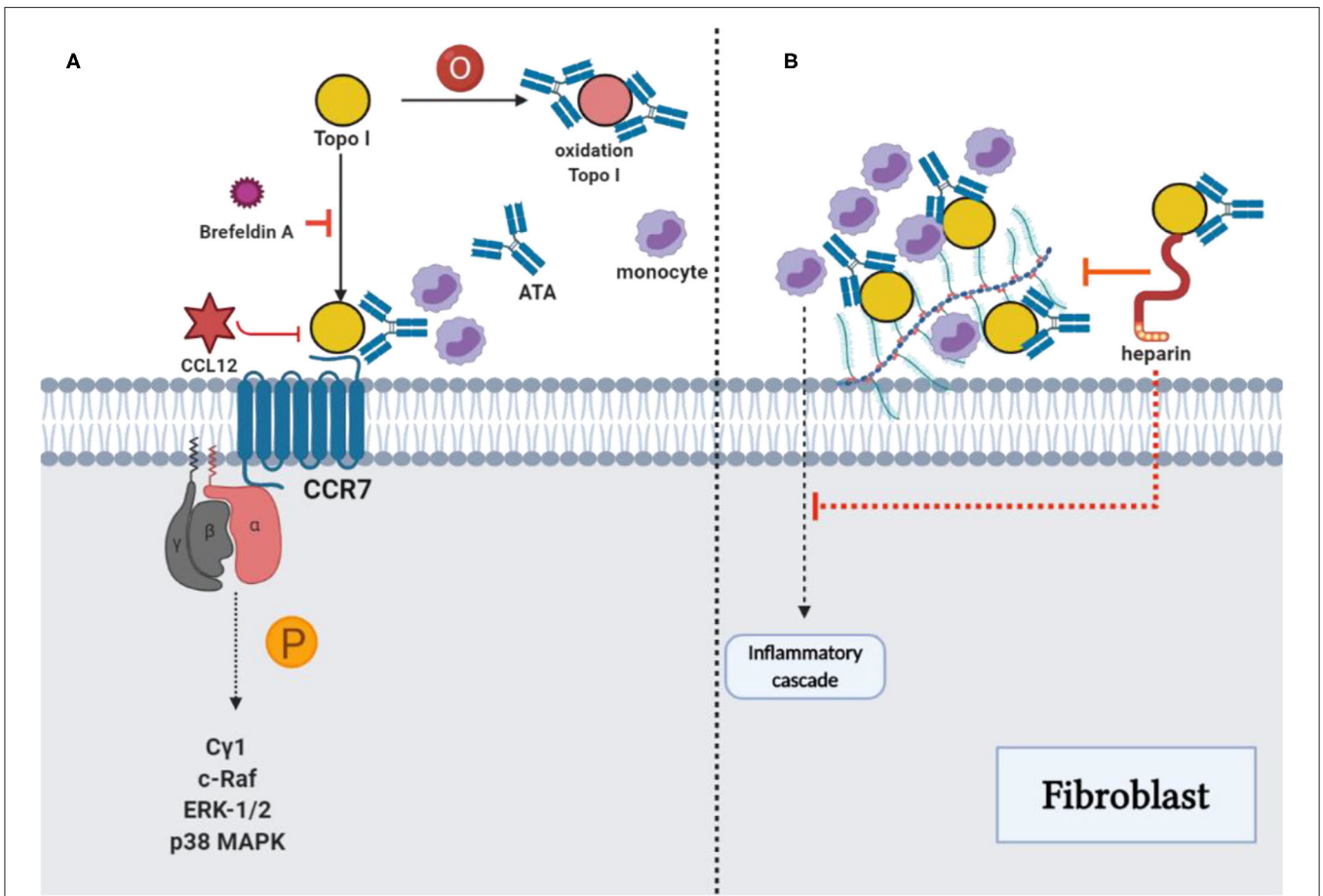

FIGURE 3 | ATAs and topo I: Reinforcement of pathological functions. **(A)** The combination of TOPO I and fibroblasts could be suppressed by using brefeldin A, and oxidized TOPO I may have increased the antigenicity. The potential intracellular signaling pathway stimulated by TOPO I was the phosphorylation of phospholipase Cγ1, c-Raf, ERK-1/2, and p38 MAPK, which stimulated the migration of fibroblast. Cytokine-like effects of TOPO I in the pathway could be inhibited by CCL21. **(B)** TOPO I bound to HS proteoglycans on the fibroblast surface, as well as the accumulation of TOPO I on cell surfaces by ATAs could contribute to the initiation of an inflammatory cascade stimulating the fibrosis. The effect could be inhibited by heparin through the interference with TOPO I binding and the consequent accumulation of TOPO I-ATA ICs could be restrained with decreased monocyte adhesion, proinflammatory factors, and fibrosis.

treatments, such as B-cell deletion, anti-cytokine antibodies, and vasodilators, may be developed for patients with different phenotypes.

CONCLUSIONS AND REMARKS

In summary, although several other antibodies are reportedly associated with SSc, classical disease-specific autoantibodies are still considered significant for the diagnosis with extensive applicability.

With an increase in cross-sectional and longitudinal studies over the past few years, more specific clinical features in different antibody groups were identified, providing new insights into the risk-stratification of patients; this allowed targeted screening of patients with not only different cutaneous manifestations (diffuse/limited or sine scleroderma), but also a high risk of vital organ involvement, such as PAH, IPF, and SRC, and malignancy.

Since ATAs, ACAs, and ARAs show high validity and reliability among SSc autoantibodies, their application should not be limited to diagnosis and basic clinical classification. Moreover, clinical features, genes, and intrinsic characteristics can reflect the distinct autoantibody subtypes and ultimately reveal the underlying pathogenic pathways. Studies on genetic characteristics provide new insights for identifying disease-specific autoantibodies that may precede clinical symptoms and signs.

Taken together, the next step in the study of SSc classical disease-specific autoantibodies should include a wider range of stratification and precision medicine, such as risk prediction, disease cluster, and mechanism. Furthermore, research on the classical disease-specific autoantibodies in patients with SSc should be combined with genomes, proteomes, and metabolomes, and should be applied clinically.

AUTHOR CONTRIBUTIONS

CY analyzed and interpreted the data regarding autoantibodies of systemic sclerosis and the data from gene research works, and was a major contributor in writing the first manuscript. ST collected statistical data of studies in the revision (*p*-value, OR value, as well as 95% CI value) and proofread all references. DZ contributed to the language polish and corrected the grammatical errors, making a great contribution in writing the revised manuscript. YD contributed to the conception of the study and helped perform the analysis with constructive discussions. JQ contributed significantly to improve the review structure. All authors read and approved the final manuscript.

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CASE REPORT
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Case Report: Treatment of Anti-MDA5-Positive Amyopathic Dermatomyositis Accompanied by a Rapidly Progressive Interstitial Lung Diseases With Methylprednisolone Pulse Therapy Combined With Cyclosporine A and Hydroxychloroquine

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Introduction: Patients with anti-melanoma differentiation-associated gene 5 (MDA5) antibody-positive amyopathic dermatomyositis (ADM) often develop rapidly progressive interstitial lung diseases (RP-ILD), with poor treatment success. Many studies have shown that this is the main cause of death in patients with anti-MDA5 antibody-positive ADM.

Case Presentation: A 37-years-old woman developed a cough, shortness of breath, and a rash on both hands, which resembled Gottron's signs. Upon laboratory examination, the results were as follows: antinuclear antibody (ANA) positive; anti-Ro52 antibody positive; and anti-MDA5 antibody positive. Pulmonary high-resolution CT (HRCT) scan showed pulmonary interstitial inflammatory changes, and mediastinal and subcutaneous emphysema. She was finally diagnosed with anti-MDA5 antibody-positive ADM accompanied by RP-ILD. She was first given high-dose-steroid pulse therapy with methylprednisolone (500 mg per day for 3 days) followed by methylprednisolone (40 mg, daily), cyclosporine A (100 mg, twice per day), and hydroxychloroquine (200 mg, twice per day). Since her discharge from our hospital in March of 2018, she has maintained the methylprednisolone therapy (tapered to 10 mg daily), cyclosporine A (100 mg, twice per day), and hydroxychloroquine (200 mg, twice per day).

Outcomes: Pulmonary HRCT scans taken on 4, 9, and 26 months after her discharge from our hospital showed that the interstitial pneumonitis had significantly improved and that mediastinal and subcutaneous emphysema had been gradually absorbed. The patient can now participate in regular work and activities of daily living.

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Conclusion: The treatment of methylprednisolone pulse therapy combined with cyclosporine A and hydroxychloroquine may be an option for the RP-ILD accompanied by anti-MDA-positive ADM. After the acute phase, this combination therapy strategy is helpful to the disease control of patients.

Keywords: combination treatment strategy, interstitial lung diseases, anti-MDA antibody, cyclosporine A, hydroxychloroquine, methylprednisolone pulse therapy, amyopathic dermatomyositis

INTRODUCTION

Idiopathic inflammatory myopathies (IIMs) are heterogeneous diseases characterized by symmetrical proximal muscle weakness, elevated muscle enzymes, and chronic skin or muscle inflammation (1). Since the 1970s, several sets of standards have been published for the classification and/or diagnosis of IIMs (2). In 1975, Bohan and Peter proposed several subgroups of IIMs: polymyositis (PM), dermatomyositis (DM), juvenile dermatomyositis, overlap myositis, and myositis associated with cancer (3, 4). In 2005, Troyanov et al. proposed a classification system based on clinical–serological definitions and introduced a new subgroup that was called clinicoserologic overlap myositis (5). Currently, IIM is most often classified into PM, DM, and inclusion body myositis (1). Amyopathic dermatomyositis (ADM) is a clinical subtype of DM, distinguished from other DM subtypes by presentation without symptoms of muscular disease. Euwer et al. first reported six cases of DM without evidence of muscle disease in 1991 (6). Sontheimer formally defined ADM in 2002 (7). According to the Classification and Diagnostic Criteria for IIMs released by the European Center for Neuromuscular Diseases and the American Myopathy Research Collaborative Group in 2004, ADM patients have typical rash manifestations of DM. ADM patients do not have objective muscle weakness, and their creatine kinase (CK) levels and electromyograms are normal.

ADM accompanied by rapidly progressive interstitial lung diseases (RP-ILD) has been reported mainly in Asia, with low treatment success (8). To our knowledge, there is no clinical trial for this disease, since the prevalence is too low and only a few cases are reported. The diagnosis of interstitial lung diseases (ILDs) is based on abnormal imaging findings with respiratory symptoms (9). Patients with ILDs often present with active dyspnea, restrictive ventilation disorder, decreased diffusion function, and hypoxemia. Pathologically, ILD is characterized by diffuse pulmonary parenchyma, alveolar inflammation, interstitial fibrosis, and diffuse shadow on chest X-rays. According to the clinical manifestations of ILDs, patients were divided into two types: acute/subacute type and chronic type (9). According to the International Consensus Statement on Idiopathic Pulmonary Fibrosis of the American Thoracic Society and the European Respiratory Society, RP-ILD is defined as a progressive ILD within 3 months after the onset of respiratory symptoms (10). Suda et al. reported that acute/subacute ILDs were generally resistant to drugs, while chronic ILDs responded well. At the same time, the mortality rate of acute/subacute ILDs was much higher than that of chronic ILDs (67 and 0%, respectively) (9).

There may be a link between ADM and ILDs, as postulated by Nakashima et al. in their study of DM specific autoantigens, and melanoma differentiation-associated gene 5 (MDA5) is a serological marker of both DM and ILDs (9). The 6-months and 5-years mortality rates of patients with anti-MDA5 antibody-positive ADM were significantly higher than those of patients with anti-MDA5 antibody-negative ADM (11). Patients with anti-MDA5 antibody-positive ADM often develop RP-ILDs with poor prognosis. Koga et al. reported that the death of all anti-MDA5 antibody-positive patients was attributed to RP-ILD respiratory failure (11). The research of Gono et al. pointed out that the cumulative 100-months survival rate was 66% for the entire anti-MDA-positive ADM patient group, and the cumulative 100-months survival rates were significantly lower in the RP-ILDs subset than in the non-RP-ILDs subset (log-rank test, $P = 0.039$). Fatal outcomes occurred remarkably often within the first 6 months (12). Anti-MDA5 antibody-positive ADM accompanied by RP-ILDs is generally treated via pharmacological methods. High-dose corticosteroids and immunosuppressants are commonly used treatments (13–16), but most of them have poor efficacy and prognosis.

In this study, we reported a case of anti-MDA5 antibody-positive ADM accompanied by RP-ILDs and performed a potential treatment that methylprednisolone pulse therapy combined with cyclosporine A and hydroxychloroquine.

CASE PRESENTATION

On October 2017, a 37-years-old woman developed polyarticular pain and swelling in her limbs, accompanied by morning stiffness and fever. The morning stiffness lasted a few minutes, and the highest body temperature was 39°C. At the onset of her fever, the skin around the joint was warm to touch. Upon a visit to another hospital, the details of the test results are as shown in **Table 1** (adult reference values in parentheses). She was tentatively diagnosed with connective tissue disease (CTD) and treated with prednisone (10 mg per day), hydroxychloroquine (200 mg per day), and loxoprofen sodium (60 mg per day).

Abbreviations: MDA5, melanoma differentiation-associated gene 5; ADM, amyopathic dermatomyositis; RP-ILDs, rapidly progressive interstitial lung diseases; ANA, antinuclear antibody; HRCT, high-resolution CT; IIMs, idiopathic inflammatory myopathies; PM, polymyositis; DM, dermatomyositis; CK, creatine kinase; ILDs, interstitial lung diseases; CTD, connective tissue disease; CADM, clinically amyopathic dermatomyositis; IVCY, intravenous cyclophosphamide; IVIg, intravenous immunoglobulin therapy.

After 2 weeks, the fever subsided. The polyarticular pain, swelling, and morning stiffness in her limbs improved but did not fully resolve. The patient subsequently developed a cough, shortness of breath, and Raynaud's phenomenon and was admitted to our hospital on November 2017. Our tests are shown in **Table 1** (adult reference values in parentheses). Pulmonary high-resolution CT (HRCT) scan showed interstitial inflammatory changes in the lungs. Indirect immunofluorescence was used to detect the expression of antinuclear antibody (ANA). Based on the above clinical evidence, we considered her symptoms consistent with mixed CTD. We treated her with prednisone (10 mg per day), hydroxychloroquine (200 mg per day), and mycophenolate mofetil (0.75 g, twice per day).

TABLE 1 | Laboratory test results.

	5 months before baseline	4 months before baseline	Baseline
PERIPHERAL BLOOD			
WBC (4.0–10.0 × 10e9/L)		7.8 × 10e9/L	10.21 × 10e9/L
NEU (2.0–7.5 × 10e9/L)		5.23 × 10e9/L	8.36 × 10e9/L
LYM (1.6–4.0 × 10e9/L)		1.51 × 10e9/L	0.94 × 10e9/L
IMMUNOLOGICAL TESTS			
Anti-CCP antibody	Negative	Negative	
ANA	Positive	Positive	Positive
Anti-dsDNA antibody	Negative		Negative
Anti-Ro52 antibody		Positive	Positive
Anti-MDA5 antibody			Positive
BLOOD GAS ANALYSIS			
PO_2 (83.0–108.0 mmHg)			63.5 mmHg
SaO_2 (95.0–99.0%)			91.4%
O_2Hb (94.0–98.0%)			90.3%
BLOOD CHEMISTRY			
CK		Normal	Normal
CK-MB		Normal	Normal
DDi (0–0.55 mg/L)			2.26 mg/L
LDH (120–250 U/L)			441 U/L
α-HBDH (72–182 U/L)			345 U/L
SEROLOGICAL TESTS			
ESR (0–20 mm/h)	43 mm/h	38 mm/h	36 mm/h
CRP (0–8 mg/L)	Negative	11.4 mg/L	24.1 mg/L
IgG (7.51–15.6g/L)			16.9 g/L

WBC, Leukocyte Count; NEU, Neutrophil count; LYM, Lymphocyte count; anti-CCP antibody, anti-cyclic citrullinated peptide antibody; ANA, antinuclear antibody; anti-ds-DNA antibody, anti-double-stranded DNA antibody; MDA5, melanoma differentiation-associated gene 5; PO_2, partial pressure of oxygen; SaO_2, Oxygen saturation; O_2Hb, Oxygenated hemoglobin fraction; CK, creatine kinase; CK-MB, Creatine Kinase, MB Form; DDi, D-dimer; LDH, lactate dehydrogenase; α-HBDH, α-hydroxybutyrate dehydrogenase; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein; IgG, immunoglobulin G.

However, her symptoms were not resolved. On March 2018, the patient was readmitted with aggravated cough and shortness of breath, as well as a rash on both hands, which resembled Gottron's signs (**Figure 1**). Laboratory test results are detailed in **Table 1** (adult reference values in parentheses). Western blot was used to detect the expression of anti-MDA5 antibody. The fluorescence pattern of ANA included nuclear dot (1:3,200), speckled (1:320), and cytoplasmic (1:100). The blood gas analysis was carried out under the condition of oxygen therapy (2 L/min, nasal catheter oxygen inhalation). The critical symptoms and tests of each visit are shown in **Table 2**. A second pulmonary HRCT scan showed that the pulmonary interstitial inflammatory changes had become more severe, and the patient developed mediastinal and subcutaneous emphysema. At this time, the subcutaneous crepitus was clearly detected in the patient's chest. She had to lie down and receive oxygen therapy (2 L/min, nasal catheter oxygen inhalation) to maintain proper SaO_2. Once she was active, her SaO_2 decreased, and her breathing difficulties increased. Her weak physical condition made it impossible for her to carry out her daily life and work, and she could not complete the pulmonary function test due to her poor condition. According to the ADM and RP-ILD diagnostic criteria, she was diagnosed with anti-MDA5 antibody-positive ADM accompanied by RP-ILDs. The patient was prescribed oxygen therapy (changed to 6 L/min, nasal catheter oxygen inhalation to maintain the finger pulse oxygen level over 95%) and methylprednisolone pulse therapy. She was first given high-dose-steroid pulse therapy with methylprednisolone (500 mg per day for 3 days) followed by methylprednisolone (40 mg daily), cyclosporine A (100 mg, twice per day), and

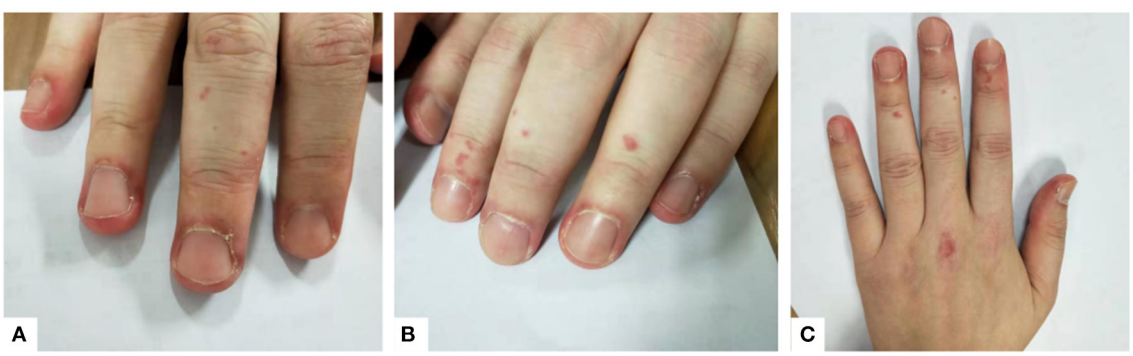

FIGURE 1 | (A–C) Erythema around the nail and insignificant Gottron's signs can be seen on the patient's hands.

TABLE 2 | Main symptoms and laboratory tests results of each stage.

	Main symptoms	Laboratory tests results
4 months before baseline	Polyarticular pain and swelling in limbs	ANA positive
	Morning stiffness	ESR 43 mm/h
	Fever	
5 months before baseline	Cough	Anti-Ro52 antibody positive
	Shortness of breath	CK and CK-MB normal
	Raynaud's phenomenon	
Baseline	Aggravated cough	Anti-Ro52 antibody positive
	Shortness of breath	Anti-MDA5 antibody positive
	Gottron's signs on both hands	PO2 63.5 mmHg

ANA, antinuclear antibody; ESR, Erythrocyte sedimentation rate; CK, creatine kinase; CK-MB, Creatine Kinase, MB Form; MDA5, melanoma differentiation-associated gene 5; PO_2, Partial pressure of oxygen.

hydroxychloroquine (200 mg, twice per day). Since there was no result of bacterial culture, we chose broad-spectrum antibiotics. She was simultaneously prescribed anti-infection treatment with moxifloxacin (400 mg per day for 7 days).

Since her discharge from our hospital on March 2018, she has maintained the methylprednisolone therapy (tapered to 10 mg daily), cyclosporine A (100 mg, twice per day), and hydroxychloroquine (200 mg, twice per day). Over the past 2 years, the patient has steadily improved. Pulmonary HRCT scans taken on 4, 9, and 26 months after her discharge from our hospital showed that the interstitial pneumonitis had significantly improved, and mediastinal and subcutaneous emphysema had been gradually absorbed (**Figure 2**). The patient is a professional translator, and now she can participate in regular work and activities of daily living. Now the patient returns to our hospital regularly for reexamination, and the new pulmonary HRCT scans are being booked.

DISCUSSION AND CONCLUSION

Relationship Between Amyopathic Dermatomyositis, Rapidly Progressive Interstitial Lung Diseases, and Anti-melanoma Differentiation-Associated Gene 5 Antibody

A study by Kawasumi et al. on the treatment of IIMs complicated with ILDs could demonstrate that an ILD was a prognostic factor for poor outcomes in DM (17). Huang et al. analyzed the clinical manifestations, serological examination, imaging features, treatment, and prognosis of 32 patients with positive anti-MDA5 antibody, and they concluded that anti-MDA5 antibody was closely related to ILDs and indicated poor prognosis (18). These cases serve as a reminder to treat the pulmonary symptoms of patients with ADM, in addition to the typical dermal and muscular symptoms. The prognosis of patients with ADM and ILD-related complications is poor, so more attention should be paid to the pulmonary changes of clinically ADM (CADM) patients. The question remains as to why ADM often presents with ILD complications. This may be related to the existence of anti-MDA5 antibodies.

Anti-MDA5 antibody is also known as anti-CADM-140 antibody. Sato et al. established an ELISA to detect anti-MDA5 antibody (19). In 2005, Sato et al. found that anti-MDA5 antibody is specific in patients with DM, especially in patients with CADM. In addition, the anti-MDA5 antibody is associated with RP-ILDs (20). A meta-analysis in 2013 showed that compared with anti-MDA5-negative patients, anti-MDA5-positive patients performed a higher prevalence of RP-ILDs ($P = 0.001$) (21). Another meta-analysis released in 2018 showed that the anti-MDA5 antibody was strongly associated with ADM and RP-ILDs (22). The anti-MDA5 antibody was linked to Gottron's sign and papules, mechanic's hand, V rash, skin ulcers, panniculitis, alopecia, arthritis/arthralgia, and pneumomediastinum and accompanied with low risk of muscle weakness, classic DM, and elevated CK (22). Sato et al. found that with the improvement of respiratory symptoms, the titer of anti-MDA5 antibody could be decreased below the critical value (23). Some reported cases have also shown that the titer of anti-MDA5 antibody is closely related to the course of RP-ILDs (20, 24). These suggest that quantitative detection of anti-MDA5 antibody may be helpful to monitor the disease activity of ADM patients with RP-ILDs (23).

Treatment of Anti-melanoma Differentiation-Associated Gene 5 Antibody-Positive Amyopathic Dermatomyositis Accompanied by Rapidly Progressive Interstitial Lung Diseases

The appropriate management of ILDs is essential to improving the prognosis of patients with DM (17). The treatment of patients who have ADM complicated by RP-ILDs is difficult, and there have been few effective treatment schemes reported. At present, most of the clinical studies on this disease are case reports. ILD is generally treated via either pharmacological or non-pharmacological methods. Common pharmacological treatments include glucocorticoids, immunosuppressants, anti-fibrosis drugs, and cysteine prodrugs, while non-pharmacological treatments consist of oxygen therapy, mechanical ventilation, and plasma exchange (PE). If pulmonary interstitial fibrosis occurs, lung transplantation is currently the most effective treatment. As for MDA, corticosteroids are the only pharmaceutical agents approved by the US Food and Drug Administration for treating myositis (1).

Sato et al. reported a case of RP-ILDs accompanied by anti-MDA-positive ADM. At first, they used the prednisolone pulse therapy (1 g per day for 3 days), and then the prednisolone was maintained at 50 mg daily, and cyclosporine A (100 mg per day) was added to relieve the symptoms of RP-ILDs. After

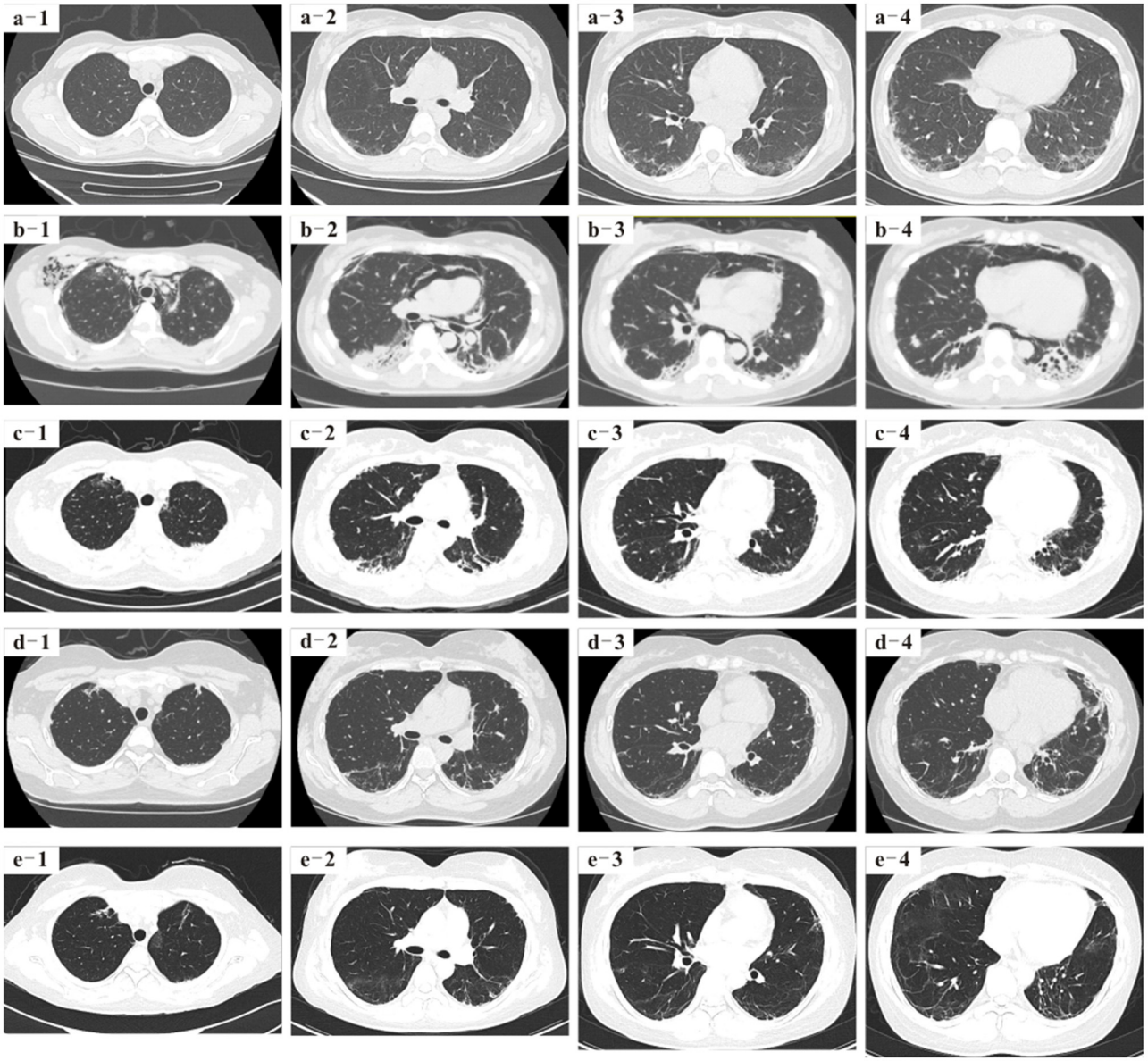

FIGURE 2 | (a1–a4) Pulmonary high-resolution CT (HRCT) 4 months before baseline showed interstitial inflammatory changes in the lungs. **(b1–b4)** Pulmonary HRCT of baseline showed that the pulmonary interstitial inflammatory changes were more serious than before, mediastinal and subcutaneous emphysema appeared, and diffuse ground-glass shadows were seen in both lung fields. **(c1–c4)** Pulmonary HRCT reexamination on 4 months after the patient was discharged from our hospital showed that the diffused ground-glass shadows were less than those on March 2018, and mediastinal and subcutaneous emphysema were significantly absorbed. **(d1–d4)** Pulmonary HRCT on 9 months after the patient was discharged from our hospital showed that the lungs were much better than on March 2018. **(e1–e4)** More than 2 years later after the patient was discharged from our hospital, pulmonary HRCT showed lesser ground-glass shadows, and her lungs were much better than before.

the improvement of clinical symptoms, low-dose prednisolone and cyclosporine A treatment was maintained; after that, no recurrence has occurred for 5 years. Their case supported the view that prednisolone combined with cyclosporine A is effective in the treatment of ADM with RP-ILDs and that using this treatment immediately before respiratory failure occurs can quickly reduce pulmonary symptoms (23). Hamada Ode et al. reported a case of anti-MDA5 antibody-positive ADM accompanied by RP-ILDs in 2015. After five courses of combination therapy with prednisolone, cyclosporine A, and intravenous cyclophosphamide (IVCY), the IVCY treatment was exchanged for high-dose intravenous immunoglobulin therapy (IVIg). Treatment with IVIg improved the symptoms of RP-ILDs and normalized anti-ADM antibody levels, suggesting that IVIg is a promising candidate for the treatment of anti-MDA5 antibody-positive ADM accompanied by RP-ILDs (25).

A study published in 2014 by Zou et al. found that basiliximab may improve the survival rate of RP-ILDs in patients with CADM accompanied by anti-MDA5 antibody (8). In 2018, Alqatari et al. reported a case report of MDA-5-associated RP-ILD recurrent pneumothorax. They treated the patient with intravenous steroids, rituximab, tacrolimus, intravenous immunoglobulins, and cyclophosphamide. Unfortunately, the patient died as a result of the rapid progression of the MDA-5-associated RP-ILD (16). In 2020, Abe et al. reported successful treatment of refractory ILDs with anti-MDA5 antibody-positive treated by PE therapy, although the mechanism of PE in the treatment of RP-ILDs is unclear (26).

CONCLUSION

Accurate diagnosis is the key to curative effect. At the initial visit, the patient did not exhibit any characteristic symptoms of anti-MDA5 antibody-positive ADM except polyarticular pain in her limbs. Although more Asian patients were reported to have as anti-MDA5 antibody-positive ADM accompanied with RP-ILDs (13, 21), we and the clinicians who treated the patient before did not realize that she might have this disease at that time, since the prevalence of this disease was rarely low. After the following immunological tests, she was diagnosed with CTD. Even the subsequent pulmonary interstitial inflammation was also thought to be a general type of ILD caused by CTD. At present, the treatment of CTD-ILDs is mainly a combination of immunosuppressive drugs, and these treatments depend on clinicians, which vary widely (27). With the Gottron's signs manifesting and the positive anti-MDA5 antibody, she was finally diagnosed as having anti-MDA5 antibody-positive ADM accompanied by RP-ILDs. It was a challenge to diagnose this disease because of its low prevalence.

According to the case reports in recent years, when ADM patients develop RP-ILDs, high-dose methylprednisolone therapy can control the inflammatory response of the lungs in a shorter time. We believe that the use of methylprednisolone pulse therapy creates conditions for the application of immunosuppressants, such as cyclosporine A and hydroxychloroquine. Although PE therapy is also considered as a treatment for anti-MDA5 antibody-positive ADM accompanied by RP-ILDs (26, 28), unfortunately, we did not prescribe the PE therapy for our patient due to the shortage of plasma resources at that time in our hospital. During the 2-years follow-up, we observed a significant improvement in pulmonary symptoms in the patient who was treated with reduced methylprednisolone therapy combined with cyclosporine A and hydroxychloroquine. We learned about the improvement of the patients' pulmonary symptoms through pulmonary HRCT scans but did not continue to carry out quantitative detection of anti-MDA5 antibody. Her condition is gradually under control, which gives her the ability to take care of herself and to do her job as well as she did when she was not sick in the past.

With the progress of medical research, the treatment of anti-MDA5 antibody-positive ADM is also improving. We believe that the treatment of methylprednisolone pulse therapy combined with cyclosporine A and hydroxychloroquine may be an option for the RP-ILDs accompanied by anti-MDA-positive ADM. After the acute phase, long-term low-dose methylprednisolone combined with cyclosporine A and hydroxychloroquine therapy is helpful in the disease control of patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. 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

Q-CZ and Z-XC contributed to the data collection and in drafting the manuscript. M-YL contributed to the data collection and revised the manuscript. YC reviewed the data and polished the paper. C-SL contributed to the reviewing, revising, and re-writing work. QX conceived of the study and participated in designing, writing, reviewing, and revising of this manuscript. All authors have read and approved the manuscript.

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Interferon and autoantigens: intersection in autoimmunity

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Interferon (IFN) is a key component of the innate immune response. For reasons that remain incompletely understood, the IFN system is upregulated in several rheumatic diseases, particularly those that feature autoantibody production, such as SLE, Sjögren's syndrome, myositis and systemic sclerosis. Interestingly, many of the autoantigens targeted in these diseases are components of the IFN system, representing IFN-stimulated genes (ISGs), pattern recognition receptors (PRRs), and modulators of the IFN response. In this review, we describe features of these IFN-linked proteins that may underlie their status as autoantigens. Note is also made of anti-IFN autoantibodies that have been described in immunodeficiency states.

KEYWORDS

autoantibody, autoimmunity, interferon, innate immunity, autoantigen

Introduction

Autoantibodies arise in a wide array of immune-mediated diseases, including both organ-limited and systemic forms of autoimmunity (1, 2). Some autoantigens are organ-specific molecules that are expressed preferentially or even uniquely in the affected organ [e.g., thyroid-specific proteins in autoimmune thyroid disease (3)]. In contrast, antigens targeted in systemic autoimmune rheumatic diseases are frequently ubiquitously expressed, and perform a variety of essential cellular functions (2). The antigens most commonly targeted by antibodies in systemic rheumatic diseases are nuclear antigens, including proteins, nucleic acids, and nucleoprotein complexes (2). The mechanisms responsible for targeting these broadly distributed autoantigens are incompletely characterized, but are likely numerous and overlapping. Here, we will review the relationship between autoantibodies and the IFN system, highlighting the enrichment of IFN-linked antigens in systemic autoimmune rheumatic diseases, and potential explanations for their targeting by autoantibodies.

The IFN system (Figure 1) is a molecular network that perform host defense functions. Three types of IFNs are found in humans: type I IFNs are expressed by and act on nearly every cell type, type II IFN is more specific for immune cells, and type III IFNs mainly act on epithelial and endothelial cells at mucosal surfaces (4–6). Cell-intrinsic IFN signaling constitutes a primordial layer of innate immunity, enabling resident tissue cells to recognize and respond to a variety of microbial pathogens and nonmicrobial threats. Thus IFN induction within activated cells leads to IFN signaling in neighboring cells via IFNs and second messengers, and in both cases ISG induction occurs. IFNs also perform important cell-extrinsic functions, and are able to shape the immune response by influencing the behavior of immune cells (5).

The upstream elements of the IFN system are innate pattern recognition receptors (PRRs), which recognize an array of Damage and Pathogen Associated Molecular Patterns (DAMPs and PAMPs, respectively) (7). Innate sensors are found in various compartments of the cell, including the endosome (e.g., TLR7), cell surface (e.g., TLR4), and cytoplasm [e.g., cyclic GMP-AMP synthase (cGAS) (8)]. The interaction of ligand and sensor leads

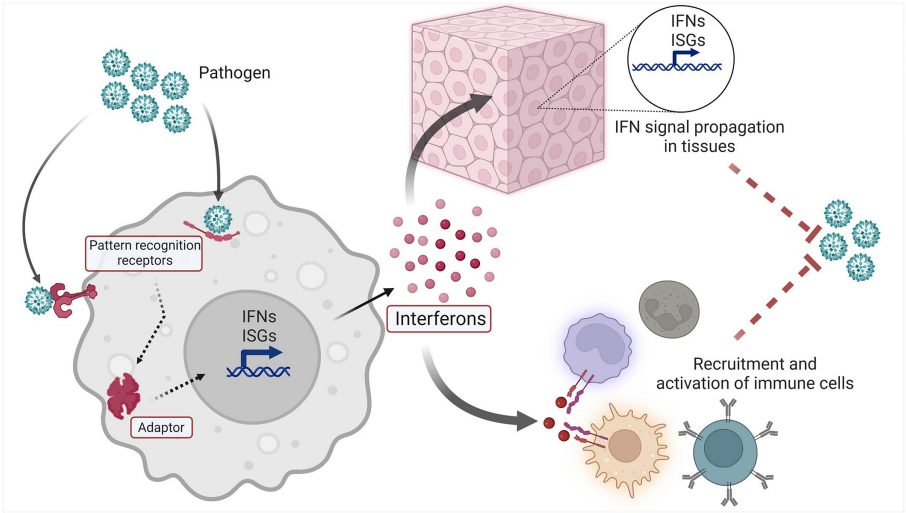

FIGURE 1
Schematic overview of the IFN system.

to subsequent activation of downstream signaling adaptors, which include molecules such as stimulator of interferon genes (STING), mitochondrial antiviral-signaling protein (MAVS) and MyD88 (8). Activated adaptors then promote signaling through various kinases and transcription factors, which ultimately trigger the expression of Interferon Stimulated Genes (ISGs) and IFNs themselves. This expression of IFNs and ISGs results in both autocrine and paracrine cellular effects. Secreted IFNs bind their cognate receptors on the cell surface, leading to intracellular signaling via the JAK/STAT pathway and expression of ISGs and IFNs in IFN-activated cells (9). In this manner, the IFN system propagates a danger signal rapidly throughout an affected tissue or organ, readying resident parenchymal cells for host defense functions, and influencing the cellular immune response that follows.

Dysregulation of the IFN system has long been recognized as a feature of many autoimmune rheumatic diseases, most notably systemic lupus erythematosus (SLE), Sjögren's syndrome (pSS), dermatomyositis (DM), and systemic sclerosis (SSc) (10–12). Upregulation of IFNs and ISGs has been observed both in the circulation and the target organs of patients with these diseases. In SLE, IFN I upregulation has been associated with increased markers of serologic and clinical disease activity. Interestingly, longitudinal studies have demonstrated that IFN expression is relatively stable despite changes in disease activity over time (13–17). In pSS, IFN expression has been linked to higher prevalence of autoantibodies and hypergammaglobulinemia, and increased lymphocytic infiltration of salivary tissues (18). Upregulation of IFN has been observed in many types of inflammatory myopathy, with IFN I upregulation particularly notable in DM muscle biopsies (19). Dysregulation of type I, II and III IFNs have all been observed, although the relative degree to which a specific IFN type is activated compared to others varies among individuals (19–23). In addition to the idiopathic rheumatic diseases, dysregulation of IFN has been identified as the driver of genetically-derived interferonopathy syndromes such as Aicardi–Goutières syndrome (AGS) and STING-associated vasculopathy with onset in infancy (SAVI); it is noteworthy that these genetic syndromes present with clinical features that often overlap with those of idiopathic rheumatic diseases (24). Taken together, these observations suggest that IFNs play a key role in the pathogenesis of the autoimmune rheumatic diseases. Consistent with this, therapeutic targeting of IFN has already shown promise in some patients, and is an area of ongoing research (25).

IFN-induced expression of autoantigens

An ISG is any gene whose expression is increased in response to IFN signaling; there are hundreds of such genes in human cells (26). Many autoantigens are included among the ISGs, suggesting that IFN-responsiveness may be involved in the development of this autoantibody subset. Notable among the IFN-induced autoantigens is Ro52 (encoded by the TRIM21 gene), which is targeted by autoantibodies in many rheumatic diseases, including pSS, SLE, DM, SSc and overlap syndromes (27). An important pathologic function of these antibodies has been defined - maternal anti-Ro52 antibodies demonstrate pathogenic function by mediating congenital heart block (28). In SLE, antibodies against Ro52 are associated with higher levels of circulating IFN I (29, 30). Ro52 is an IFN-induced E3 ligase that targets various substrates for removal via proteasomal degradation. In response to viral infection, Ro52 downregulates the innate immune response by enhancing clearance of the key transcription factor IRF3 (31, 32). Ro52 also promotes antiviral function by serving as a sensor of cytoplasmic IgG, marking intracellular viral-IgG immune complexes for proteasomal clearance (33, 34). Ro52 is therefore both a key regulator of the innate immune response and a functional component of the IFN pathway. It provides an important example of an antigen against which tolerance may be lost due to dysregulated IFN signaling. In this scenario, upregulated antigen expression in the setting of inflammation likely promotes the

frequency with which the induced protein is displayed by antigen presenting cells, thereby increasing the likelihood that autoreactivity might occur. Continued expression of IFN in the affected organs would ensure sustained elevated levels of ISG antigens, fueling the propagation phase of such an autoimmune response. It is noteworthy that ISG upregulation caused by interferogenic stimuli (e.g., viral infection) facilitates additional intermolecular interactions that may also lead to breaking of tolerance against Ro52 or other relevant ISGs.

DNA binding molecules

Several PRRs are included among the autoantigens targeted in rheumatic diseases. While some of these PRR antigens are also IFN-inducible, others are not. Among these non-IFN-induced antigens is Ku - a heterodimeric complex composed of Ku70 and Ku80 subunits that is targeted by autoantibodies in several autoimmune rheumatic diseases (35, 36). In SLE, anti-Ku antibodies have been reported at prevalence of 9.8–20.5% (35, 37). Anti-chromatin antibodies have been identified at greater prevalence among SLE patients with anti-Ku antibodies: anti-chromatin antibodies were found in 72.7% of anti-Ku positive versus 43.9% of anti-Ku negative patient sera in one study ($p < 0.0001$) (37). Anti-Ku antibodies have also been found in association with autoantibodies against additional DNA repair proteins, including DNA-PK, Mre11, WRN and PARP (35). While clearly implicated in DNA repair responses, the Ku complex has also been shown to serve as a cytoplasmic DNA sensor, translocating from the nucleus to the cytoplasm and binding dsDNA of various sorts (38, 39). Recently, Tao et al. demonstrated that cytoplasmic Ku interacts with cGAS to promote condensate formation and IFN signaling in response to cytoplasmic dsDNA (40). These findings raise the intriguing possibility that intermolecular interactions occurring in the context of DNA repair in the nucleus might underlie the targeting of Ku and related autoantigens in SLE, and that Ku may interact with other potential autoantigens in the cytoplasm.

A similar scenario has been observed in the case of poly(ADP-ribose) polymerase (PARP), an additional component of the cellular DNA damage response that is targeted as an autoantigen in SLE and other autoimmune conditions (41, 42). PARP1 translocates to the cytoplasm upon viral infection, where it PARylates cGAS. Interestingly, in contrast to the pro-IFN effect of Ku, this PARylation was reported to inhibit cGAS signaling (43). In addition, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), has also been identified as a sensor of cytoplasmic DNA (44), and recently demonstrated to negatively regulate cGAS via phosphorylation (45). It is noteworthy that DNA-PKcs, PARP and Ku are all translocated to the cytoplasm in the setting of dsDNA sensing, and together modulate the cell-intrinsic IFN I response generated by cGAS. Activation of the cytoplasmic dsDNA sensing pathway may therefore represent a stimulus that triggers antigenic changes in these proteins that are relevant to SLE pathogenesis.

RNA polymerase III (POL III) is a well-described autoantigen targeted in 15.3–26.6% of systemic sclerosis patients (46, 47). This enzyme transcribes a variety of noncoding RNA molecules required for routine cellular functions (48). However, its role in activating the IFN system is much less appreciated. A specific function for POL III in the innate immune response was reported by Chiu et al., who showed that POL III converts cytosolic dsDNA into 5′-ppp RNA, which is subsequently detected by RIG-like receptors (RLRs), generating a MAVS-dependent IFN response (49). Thus, Ku, PARP and POL III are all involved in the innate response to cytoplasmic dsDNA. The altered subcellular localization and interactions that occur in the setting of cytoplasmic dsDNA sensing therefore might represent additional mechanisms that could contribute to loss of tolerance against these antigens in autoimmune diseases characterized by an aberrant IFN I response.

Oligomerizing innate sensors

Several additional autoantigens combine the characteristics of IFN-induced expression, nucleic acid binding, and a third feature specific to their activation: oligomerization. Recent findings from this interesting autoantibody group are reviewed below.

Antibodies against a 140 kDa protein were first described in a Japanese cohort of patients with clinically amyopathic DM (50). The identity of this autoantigen was later demonstrated to be melanoma differentiation-associated protein 5 (MDA5) (51). The initial clinical phenotype described in association with MDA5 antibodies was that of mild muscle involvement, with severe pulmonary manifestations and a variety of cutaneous findings; additional cohort studies have yielded a broader spectrum of clinical manifestations (52). MDA5 is a member of the RIG-Like Receptor (RLR) group of cytoplasmic dsRNA sensors that promote antiviral IFN I production. Upon sensing long dsRNA, MDA5 assembles into filamentous oligomers that activate MAVS and trigger downstream IFN I signaling (53–55). Like Ro52, MDA5 expression is induced by IFN and interestingly, these two antibodies are often targeted together in this subset of DM patients. As MDA5 is both an IFN-inducible and an interferogenic protein, its dysregulation could readily contribute to sustained IFN signaling. Indeed, gain of function mutations in the gene encoding MDA5 (IFIH1) have been identified in patients with interferonopathy syndromes as well as SLE (56–58). Strong IFN I upregulation has been identified in anti-MDA5-associated DM (59), and some have proposed labeling this syndrome an acquired type I interferonopathy (60).

IFI16 is an IFN-inducible dsDNA sensor in the AIM-like receptor (ALR) family (61). Similar to Ku, IFI16 translocates from the nucleus to the cytoplasm upon dsDNA sensing, where it promotes IFN signaling through STING (61). Similar to MDA5, IFI16 also assembles into filamentous oligomers when activated by dsDNA (62). Anti-IFI16 antibodies have been identified in SLE and pSS patients, and are associated with more severe disease features (63–66).

Absent in melanoma 2 (AIM2), another IFN-inducible dsDNA sensor in the ALR family, activates apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) upon dsDNA sensing, triggering inflammasome assembly and IL-1/18 secretion (67). We recently identified anti-AIM2 autoantibodies in SLE. These frequently co-occurred with anti-IFI16 and anti-dsDNA antibodies, as well as disease activity markers (68). Autoantibodies targeting ASC (which is also IFN-inducible) have been identified in patients with inflammatory diseases, and anti-ASC antibodies demonstrated a pathogenic ability to enhance inflammasome activation in recipient phagocytes in mice (69).

A noteworthy feature common to the 3 autoantigens MDA5, IFI16 and AIM2 is that they are all IFN-inducible innate sensors of double stranded nucleic acids. Their activation leads to the generation of large, filamentous oligomers of protein and bound nucleic acid ligand. Sustained activation of these sensors at a disease site is one potential explanation for their targeting by autoantibodies. Indeed, our own observation of activated filamentous IFI16 present in the salivary tissues of some pSS patients supports this concept (70). In addition, cytoplasmic interaction of AIM2 and dsDNA has been detected in cell lines derived from pSS salivary tissue (71), and we observed both IFI16 and AIM2 bound to neutrophil extracellular trap DNA in SLE renal tissues (68). These findings provide compelling additional evidence that DNA-bound sensors are present at sites of disease activity.

The presence of oligomerized sensors coupled to nucleic acid ligands may lead to the generation of novel epitopes not found in the monomeric forms, or may increase the potential for autoreactivity via the increased valence present in oligomers that are conveyed to immune cells at sites of immune activation. These autoantigens may therefore represent key molecules whose activation causes pathogenic inflammatory signaling in affected organs, as well themselves being targets of the autoimmune response. Future studies are warranted to examine whether these and/or other autoantibodies serve as biomarkers that identify subsets of patients in whom such innate signaling pathways are especially relevant to disease initiation or propagation. Insights from such studies will likely inform the more effective use of IFN-specific therapies.

Interferons as autoantigens

In addition to the spectrum of intracellular autoantigens associated with systemic autoimmune rheumatic diseases, antibodies against extracellular antigens have also been described in a variety of scenarios. Anti-cytokine antibodies have been observed in patients with SLE and other rheumatic diseases, and also in viral infection and immunodeficiency states (72). In SLE, antibodies against type I, II and III IFNs have been observed (73). These authors found that antibodies against type I IFNs had a neutralizing function, and patients with blocking anti-type I IFN antibodies demonstrated normalized IFN expression levels. Conversely, SLE patients with anti-IFN II antibodies suffered from more severe disease manifestations, including upregulation of type I IFNs. Antibodies against IFNs were also measured in patients with pSS at a comparable prevalence, but were not observed as often in RA.

Nearly 20 years ago, neutralizing antibodies against type II IFN were recognized in patients suffering unusual, severe mycobacterial infections (74, 75). Since that time, several hundred cases of anti-IFN-gamma-autoantibodies (AIGA) have been reported in patients presenting with a variety of infections. In addition to mycobacterial disease, salmonella, varicella, and fungal species have also been recorded, making AIGA an antibody-mediated form of acquired immunodeficiency. Recent studies in SARS-CoV2 have strengthened the evidence that anti-IFN autoantibodies have functional consequences in the setting of infection, as antibodies directed against type I IFNs have been measured in patients who suffer severe disease outcomes from COVID19 (76–78). These observations suggest that, in the setting of infection, anti-IFN antibodies constitute a potentially treatable form of immunodeficiency that renders the host more susceptible to infection. Conversely, in the setting of autoimmune diseases such as SLE, it remains less clear whether anti-IFN antibodies contribute to disease pathogenesis, or serve as markers of aberrant IFN signaling.

Conclusion

Autoantibodies target a multitude of cellular antigens, and diverse mechanisms are likely responsible for their targeting through the humoral immune response. Several autoimmune rheumatic diseases feature upregulation of IFN signaling along with autoantibodies directed against components of the IFN system. These IFN-linked autoantigens include ISGs, DNA-binding proteins, and oligomerizing pattern recognition receptors. Pathogenic activation of these IFN system components may underlie their status as autoantigens, and these autoantibodies might therefore indicate patients in whom the antibody-targeted antigens play critical roles in driving IFN activation. Antibodies against IFNs themselves mediate increased susceptibility to some infections and represent a form of acquired immunodeficiency mediated by humoral autoimmunity.

Author contributions

BA and LC-R contributed to conceptualization and writing of the manuscript. 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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Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment

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Background: (Auto)immune mediated and cholestatic liver disease (AILD) includes autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Especially AIH is characterized by the presence of autoantibodies and elevated serum immunoglobulins. In rheumatoid arthritis, autoantibodies against post-translational modifications (PTMs) such as citrullination (Cit) and carbamylation (CarP) are used as diagnostic and prognostic markers, respectively. We studied the presence of six anti-PTM antibodies in patients with the three AILDs and non-AILD.

Methods: Antibodies against six PTMs (malondialdehyde–acetaldehyde adducts (MAA), advanced glycation end-products (AGE), CarP, acetylation (AL), Cit, and nitration (NT)) were tested in sera of patients with AILD (n=106), non-AILD (n=101) and compared with healthy controls (HC) (n=100). Levels and positivity were correlated with clinical and biochemical features in a well-defined cohort of untreated AIH patients.

Results: Anti-PTM antibodies were more often detectable in sera from AILD patients compared with HCs (anti-MAA: 67.9% vs. 2.0%, anti-AGE: 36.8% vs. 4.0%, anti-CarP: 47.2% vs. 5.0% and anti-AL: 18.9% vs. 5.0%). In untreated AIH, time to complete biochemical response (CBR) was associated with anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies. Significantly more patients with at least three anti-PTM antibodies attained CBR at 12 months of treatment (13 vs. 3 p=0.01).

Conclusion: Anti-PTM antibodies are frequently present in AILD. The presence of anti-MAA, anti-AGE and anti-CarP antibodies correlates with the presence of AIH within this cohort. In AIH, harboring at least three anti-PTM antibody responses is positively associated with CBR. Determination of anti-PTM antibodies in liver disease may have diagnostic and prognostic value.

KEYWORDS

autoantibodies, autoimmune hepatitis, autoimmune liver disease, post-translational modifications, epidemiology

Highligts

- Antibodies against post-translational modifications (anti-PTM antibodies) are used as diagnostic and prognostic markers in several autoimmune diseases, such as rheumatoid arthritis
- This study shows that these antibodies are often present in autoimmune mediated liver disease
- Compared to cholestatic liver disease, in autoimmune hepatitis most patients harbor antibodies against multiple post-translational modifications
- Such a multiple positivity was associated with complete biochemical response after 12 months of treatment in autoimmune hepatitis patients

1. Introduction

(Auto)immune mediated and cholestatic liver disease (AILD) is a heterogeneous group of both cholestatic and hepatocellular diseases, consisting of primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and overlap variants. AIH and PBC are characterized by the presence of autoantibodies and elevated total immunoglobulin (Ig) G and IgM, respectively (1). The presence of autoantibodies against for example smooth muscle (SMA) and mitochondria (AMA) play an important role in the diagnostic scoring of AIH and PBC, respectively (2, 3). Although testing for different autoantibodies is implemented in the standard diagnostic work-up for liver disease with an unknown origin, they are not disease specific (4).

In another autoimmune disease, namely rheumatoid arthritis (RA), autoantibodies are also present, but in this disease antibodies frequently target proteins that have undergone post-translational modifications (PTM) (5). In particular, antibodies that target citrullination (anti-citrullinated antibodies: ACPA) and anti-carbamylated protein (anti-CarP) antibodies are used as diagnostic and prognostic markers in RA, respectively (6, 7). During inflammation, peptidyl arginine deiminases and cyanate are formed resulting in extracellular citrullination of arginine and carbamylation of lysine amino acids, respectively (8, 9). More recently, we have discovered antibody responses against the modifications malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE) in patients with systemic lupus erythematosus (SLE), defining a group of patients with neuropsychiatric manifestations (10). Both MAA and AGE are a result of oxidative stress and modify lysine amino acids (11, 12). Additionally, under oxidative stress nitration (NT) of the tyrosine amino acids and acetylation (AL) of lysines occur as a result of a reaction with peroxynitrite species and dysregulation of acetylation and deacetylation pathways, respectively (13, 14).

Inflammation occurs in both AIH and cholestatic liver disease, albeit at different sites (hepatocytes versus biliary tract). Oxidative stress occurs more frequently in patients with AIH compared to patients with cholestatic liver disease (15, 16). PTMs that are the result of oxidative stress have been reported to be highly immunogenic which could therefore result in anti-PTM antibody production, also in the context of AILD (17–19). However, studies assessing anti-PTM antibody responses in AILD are limited. Antibodies against cyclic citrullinated peptide (CCP) have been studied and were found in 9–11% of patients with type 1 AIH (20, 21), commonly in the absence of RA (21). Additionally, MAA modifications have shown to induce liver damage and to cause an autoimmune like pathophysiology in mice (22).

Since AIH, PBC and PSC are often considered (auto)immune mediated diseases that, like RA and SLE, display a variety of autoantibodies, we hypothesized that anti-PTM antibodies may be present in AILD and could have diagnostic or prognostic associations.

Here we report that anti-PTM antibodies are present in AILD, allow discrimination between subgroups of AILD and are related to treatment response in AIH.

2. Materials and methods

2.1. Study design and population

Patients visiting the Department of Gastroenterology and Hepatology of the Leiden University Medical Centre (LUMC) between 1996 and 2020 who signed informed consent for the Biobanking facility were eligible for inclusion. Patients visiting the Department of Gastroenterology and Hepatology of Erasmus Medical Centre, Rotterdam, with no objection against the use of residual material, were also included. The biobank protocol (B21.032) was prospectively approved by the Medical Ethical Committee of the LUMC. For the purpose of this study, patients were divided into three groups: AILD, (i.e., AIH, PBC or PSC), miscellaneous chronic liver diseases (non-AILD) and healthy controls (HC). HC were preselected from a biobank containing serum from healthy individuals. They were matched based on sex and age to the AILD cohort. No data on medical history of medication use was available, mimicking the general population. Although clinical, biochemical and histological overlap can occur, patients with overlap variants (AIH-PBC or AIH-PSC) were not included in the AILD cohort. AIH was diagnosed using the *revised original* or *simplified* criteria for the diagnosis for AIH (2, 23, 24). Patients with AIH were included at diagnosis. Of all AILD patients, 66 were diagnosed with AIH. Of these patients 8 patients already started treatment before inclusion. PBC and PSC were diagnosed according to the diagnostic criteria in the European guidelines and were included during follow-up (25). As the AIH cohort was the largest cohort with complete data, this was the cohort in which the final analyses were done.

2.2. Patient characteristics

Demographics and patient characteristics were collected from electronic patient files at the time of visit to the outpatient clinic. This included: age, sex, comorbidities, disease duration, presence of liver cirrhosis, simplified criteria for the diagnosis of AIH (24), revised original criteria for AIH (23), presence of self-reported arthralgia (i.e., extrahepatic manifestation of AIH) and medication use. Follow-up data (i.e., time to complete biochemical response (CBR), treatment response, mortality and liver transplantation) was also collected.

CBR was defined as normalization of aminotransferases and IgG below the upper limit of normal (26). Time to CBR was defined as the time from treatment initiation until the first time CBR was reached.

In addition to routine laboratory assessments (aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), IgG, gamma-glutamyl transferase (GGT), alkaline phosphatase (AP) and presence of autoantibodies), serum samples from each patient were collected (Supplementary Table S1). For patients with cholestatic liver disease, data regarding cholangiographic findings and laboratory assessments (GGT, AP and autoantibodies) were also collected (Supplementary Table S1).

2.3. Generation of PTMs

Modified proteins and their corresponding control non-modified protein were produced by either enzymatic or chemical reactions as previously described (10).

2.4. Assessment of anti-PTM antibodies

Anti-PTM antibodies were detected using an in-house enzyme-linked immunosorbent assay (ELISA), based on modified fetal calf serum (FCS) as described previously (10). Briefly, modified and non-modified FCS were coated to a Nunc Maxisorp ELISA plate (430,341, Thermofisher). In between each sequential step plates were washed three times using Phosphate Buffered Saline (PBS)/0.05%Tween (Sigma, P1379). After blocking [PBS/1% Bovine Serum Albumin (BSA)] for 6 h at 4°C, plates were incubated overnight at 4°C with 1/50, 1/100 or 1/1000 diluted serum. Each plate contained a standard of anti-PTM antibody positive serum to calculate arbitrary units. After incubation, IgG levels were detected using horseradish peroxidase (HRP) labeled Rabbit-anti-Human IgG (Dako, P0214). Plates were developed by incubating with 2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS)/0.015% H_2O_2 (A1888 and 7,722-84-1, both from Merck) and absorbance at 415 nm was measured using a microplate reader (Bio-Rad iMark). The cut-off for positivity was set as the mean arbitrary units plus two times the standard deviation of 100 HCs, excluding values higher than 10x the mean.

2.5. Statistical analysis

Statistical analyses were performed using IBM SPSS 25.0 (IBM, Armonk, NY). Baseline characteristics were evaluated using descriptive statistics. Differences in levels of anti-PTM antibodies between HCs, AILD, and non-AILD were assessed using Kruskall-Wallis Test and Chi-2 test. Analyses of correlation between anti-PTM antibody levels and clinical variables were done using Spearman rank analyses for continuous clinical variables and point biserial correlation (i.e., mathematical equivalent of Pearson correlation) for dichotomous clinical variables. The anti-PTM antibody levels were transformed to natural logarithms to perform point-biserial correlations. Wilcoxon signed-rank test was used to compare anti-PTM antibody levels at baseline versus levels at the second visit.

Correlations between the difference in anti-PTM antibody levels at baseline versus the second visit and the change in levels of ALAT, ASAT and IgG were done using Spearman's rho (r_s). Landmark analysis was used for the evaluation of CBR, with pre-determined timepoints at 3, 6 and 12 months, to prevent immortal time bias. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Study cohort

We studied 207 patients with liver disease comprising an AILD cohort ($n = 106$) and a non-AILD cohort ($n = 101$). The AILD cohort consisted of patients with AIH ($n = 66$), PBC ($n = 10$) and PSC ($n = 30$) and was subsequently divided into two separate cohorts: AIH and cholestatic liver disease (CLD) (i.e., PBC and PSC). The non-AILD cohort consisted of patients with alcoholic liver disease (ALD) ($n = 29$), chronic hepatis B (HBV) ($n = 4$), chronic hepatitis C (HCV) ($n = 22$), non-alcoholic fatty liver disease (NAFLD) ($n = 30$), non-alcoholic steatohepatitis (NASH) ($n = 1$), or a combination of these ($n = 15$) (Table 1). In the AILD and non-AILD cohort 63.2 and 33.7% of the patients were female ($p < 0.001$) with a mean age of 48.2 ± 16.6 years and 54.0 ± 11.0 years, respectively ($p = 0.003$) (Table 1). Cirrhosis was present in 39.6% of the AILD cohort and in 56.4% of non-AILD patients ($p = 0.035$). In the AILD cohort, 96.7% of patients with PSC had large duct PSC on cholangiographic imaging. Eighty percent of PBC patients was AMA positive. The mean age of the HCs was 50.2 ± 10.5 years and 49% were female.

3.2. Anti-MAA, anti-AGE, anti-CarP, and anti-AL antibodies are more prevalent in AILD compared to HC and non-AILD and are more likely to be positive for more than one anti-PTM antibody

Anti-PTM IgG antibody levels directed against 6 PTMs were measured in 207 patients with liver disease and 100 HCs (Figure 1 *and* Supplementary Table S2). Anti-MAA, anti-AGE, anti-CarP and anti-AL antibody levels differed significantly between AILD and HCs (1036.0, 234.5, 352.5 and 13.3 aU/mL vs. 266.9, 88.9, 74.0 and 0.0 aU/mL respectively, all $p < 0.01$). Only anti-MAA and anti-CarP antibodies were significantly increased when comparing non-AILD to HCs (495.8 and 241.0 aU/mL vs. 266.9 and 74.0 aU/mL, respectively, both $p < 0.01$). Additionally, AILD showed significantly higher median levels of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies compared to non-AILD (anti-MAA, anti-AGE and anti-AL 1036.0, 234.5, 13.3 aU/mL vs. 495.8, 130.0, 6.4 aU/mL, respectively, $p < 0.01$

TABLE 1 Characteristics of study population with autoimmune mediated and cholestatic liver disease (AILD) and non-AILD at time of inclusion.

Patient characteristics	Auto-immune liver disease (AILD) (*n* =106)	Non-autoimmune liver disease (non-AILD) (*n* =101)	*p* value
Primary diagnosis			
AIH	66 (62.3)	-	
PBC	10 (9.4)	-	
PSC	30 (28.3)	-	
NAFLD	-	30 (29.7)	
ALD	-	29 (28.7)	
HCV	-	22 (21.8)	
HBV	-	4 (4.0)	
NASH	-	1 (1.0)	
Hemochromatosis	-	0 (0.0)	
Combination†	-	15 (14.9)	
Female sex	67 (63.2)	34 (33.7)	<0.001*
Age sample (years)	48.2 ± 16.6	54.0 ± 11.00	0.003*
Cirrhosis	42 (39.6)	57 (56.4)	0.035*
Yes, compensated	28 (26.4)	27 (26.7)	-
Yes, decompensated	14 (13.2)	30 (29.7)	-
No cirrhosis	59 (55.7)	44 (43.6)	-
Unknown	5 (4.7)	0 (0.0)	-

Results are presented as *n* (%), mean ± SD or median (IQR). $p < 0.05$ is considered statistically significant (*). AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ALD, alcoholic liver disease; HBV, chronic hepatis B; HCV, chronic hepatitis C; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis. † Combinations: ALD + HBV (*n* = 1), ALD + HCV + HBV (*n* = 1), ALD + HCV (*n* = 6), ALD and hemochromatosis (*n* = 2), ALD + NASH (*n* = 2), ALD + PSC (*n* = 1), HBV + HDV (*n* = 1), HCV + HIV (*n* = 1).

and anti-CarP 352.5aU/mL vs. 241.0 aU/mL, $p < 0.05$). Median levels of anti-NT and anti-Cit differed significantly between AILD and HCs (269.0 and 3.1 aU/mL vs. 108.0 and 1.3 aU/mL, $p < 0.01$ and $p < 0.05$, respectively) but did not differ significantly between non-AILD and HCs.

Comparing the frequency of positivity, AILD patients showed significantly increased positivity of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies compared to HCs (67.9, 36.8, 47.2 and 18.9% vs. 2.0, 4.0, 5.0, and 5.0%, all $p < 0.01$). Increased positivity for anti-MAA, anti-AGE and anti-CarP antibodies (28.7, 17.8 and 27.7% vs. 2.0, 4.0 and 5.0%, respectively, all $p < 0.01$) was observed when comparing non-AILD and HCs. Additionally, increased positivity between non-AILD and AILD was observed for anti-MAA, anti-AGE and anti-CarP antibodies (67.9, 36.8 and 47.2% vs. 26.7, 17.8 and 27.7%, respectively, all $p < 0.01$). Also when the non-AILD control group is limited to a more stringent set of conditions, excluding HBV, NASH and hemochromatosis, all statistical associations remain intact (data not shown). Anti-PTM antibody positivity for different anti-PTM antibodies were combined to calculate positivity for multiple anti-PTM antibodies (Figure 2). Patients with AILD more frequently harbored at least one type of anti-PTM antibody compared to non-AILD and HCs (AILD: 81.2%, non-AILD: 58.4% and HCs: 20%). The data in Figure 2 also indicate that AILD patients are more likely to be positive for multiple anti-PTM antibodies. Overall, these data indicate that anti-PTM antibodies are especially present in patients with AILD.

3.3. Within AILD, patients with AIH harbor anti-PTM antibodies more often and present with specific combinations of anti-PTM antibodies

Next, AILD was dissected into the three major immune liver disease subgroups, namely AIH, PBC and PSC. Presence of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies were assessed in these subgroups, or AIH alone, and compared to non-AILD (Figures 1, 2, and Supplementary Table S3). Interestingly, patients with AIH harbored significantly more of these antibodies compared to non-AILD patients (anti-MAA: 77.3% vs. 26.7%, anti-AGE: 48.5% vs. 17.8% and anti-CarP: 63.6% vs. 27.7%, all $p < 0.001$, respectively). Within the AILD cohort, predominantly patients with AIH harbored anti-PTM antibodies. We did however also see some patients with CLD who were positive for some. Subsequently, the AILD was divided into two cohorts: AIH and CLD. Patients with AIH were significantly more often positive for anti-MAA, anti-AGE, anti-CarP and anti-Cit (77.3, 48.5, 63.6 and 25.8% vs. 52.5, 17.5, 20.0 and 2.5% respectively, all $p < 0.01$) compared to patients with CLD (Table 2).

Analysis of different anti-PTM antibody combinations showed that AILD patients mostly harbored a combination of anti-MAA, anti-AGE and anti-CarP antibodies (15/85 = 17.6%) or anti-MAA and anti-CarP antibodies (7/85 = 8.2%) compared to non-AILD (anti-MAA/-AGE/-CarP: 5/58 = 8.6% and anti-MAA/-AGE/-CarP: 3/58 = 5.2%) (Supplementary Figures S1A,B). Strikingly, comparing AIH patients with total AILD, all double (anti-MAA/-AGE/-CarP), almost all (except 1) triple (anti-MAA/-AGE/-CarP) and all quintuple (anti-MAA/-AGE/-CarP/-AL/-Cit) positive patients from the AILD group belonged to the AIH group (Supplementary Figure S1C). Taken together, patients with AIH harbored anti-PTM antibodies more often compared to other subgroups of AILD.

3.4. There are no significant associations between anti-PTM antibody positivity, presence of ANA and SMA, cirrhosis and sex in AIH patients

In AIH several other antibodies have been described such as ANA and SMA. We have analyzed to what extent these antibodies occur together with the anti-PTM antibody responses or to what degree detectable anti-PTM antibody responses differ depending on the positivity status for ANA or SMA. We did not observe a significant difference in the presence of anti-PTM antibodies in patients positive or negative for ANA or SMA, with the exception of anti-MAA positivity and ANA positivity in patients with AIH (Chi-2 (1) > = 4.687, $p = 0.030$). We further analyzed the positivity for anti-PTM antibodies in patients with AIH who were negative for both ANA and SMA. Despite it being a small cohort (n = 11), we observed that the absolute percentages for positivity of anti-MAA, anti-AGE, anti-CarP, anti-AL, anti-Cit and anti-NT was in general higher in patients who were both ANA and SMA negative compared to patients who were either ANA negative of

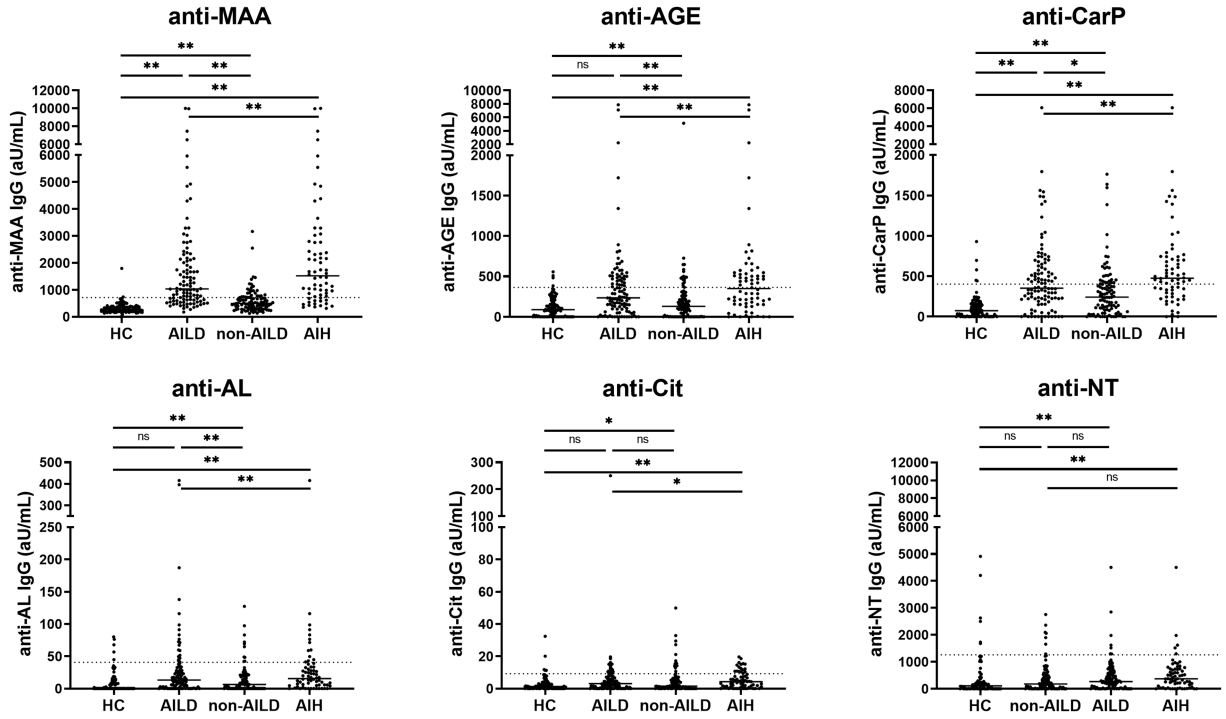

FIGURE 1
Anti-MAA, anti-AGE, anti-CarP, and anti-AL antibodies are increased in patients with AILD, and especially in patients with AIH. IgG antibody levels are presented as arbitrary units per milliliter (aU/mL) and cut-off for each PTM is indicated by the dashed line. *p<0.05, **p<0.01. Autoimmune Liver Disease: AIH, PBC and PSC; non-Autoimmune Liver Disease: NAFLD, HCV, HBV, ALD, Combination, NASH. AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde–acetaldehyde adduct; ns, not significant; NT, nitrated protein.

SMA negative (Table 3). This further supports the idea that anti-PTM antibodies provide different information compared to the already known antibodies ANA and SMA. Additionally, in the AIH cohort positivity for any of the anti-PTM antibodies did not show significant differences between patients when stratifying for cirrhosis. Furthermore, in the AILD cohort, we observed a significant association between anti-CarP and female sex (Chi-2 (1) >= 4.740, p = 0.029). In the AIH cohort however, none of the anti-PTM antibodies showed significant associations with sex.

3.5. Anti-MAA and anti-CarP antibodies significantly correlate with measures of biochemical treatment response

We investigated if increased anti-PTM antibodies correlated with commonly used serological and clinical markers in patients with AIH (Figures 3A–D and Supplementary Table S4). As treatment for AIH consists of immunomodulatory treatment, and might therefore influence biochemical markers, only patients with treatment naïve AIH were included in these analyses (Table 4). Both anti-MAA and anti-CarP correlated positively with serum IgG (p < 0.000/p = 0.001) and antinuclear antibodies (ANA) (p = 0.001). Anti-CarP correlated positively with ASAT (p = 0.009). We demonstrated correlations between anti-MAA, self-reported arthralgia and antibodies against soluble liver antigen (SLA) approaching statistical significance (p = 0.082 and 0.059 respectively). No significant correlations were found for anti-AGE and anti-AL.

3.6. Anti-MAA, anti-AGE, and anti-CarP antibodies positively correlate with CBR

Time to CBR negatively correlated with the presence of anti-PTM antibodies in patients with AIH, reaching significance for anti-AGE (p = 0.042) (Figures 3A–D). In line with these findings, anti-MAA and anti-AGE correlated positively with CBR at 3 months (p = 0.015 and 0.036, respectively). In addition, anti-MAA, anti-AGE, and anti-CarP positively correlated with CBR at 12 months (p = 0.014, 0.005, and 0.012, respectively) (Figures 3A-D). A trend toward significance was found for anti-AL and CBR at 12 months. No association between the presence of anti-PTM antibodies and long-term follow-up (i.e., liver transplantation or mortality) was found. A logistic regression was performed to analyze the effects of positivity for all six individual anti-PTM antibodies on the likelihood of reaching CBR at 3, 6 and 12 months. Positivity for any individual anti-PTM antibody was not independently associated with an increased or decreased likelihood of reaching CBR at 3, 6 or 12 months (data not shown).

3.7. Patients with AIH and positive for at least three anti-PTM antibodies reach CBR quicker after initiating treatment

Based on the discovery of multiple anti-PTM antibody positivity in patients with AIH, we attempted to discover the clinical relevance of harboring these multiple anti-PTM antibodies. The median follow-up was 8.7 years (4.6–15.3) (Supplementary Table S5).

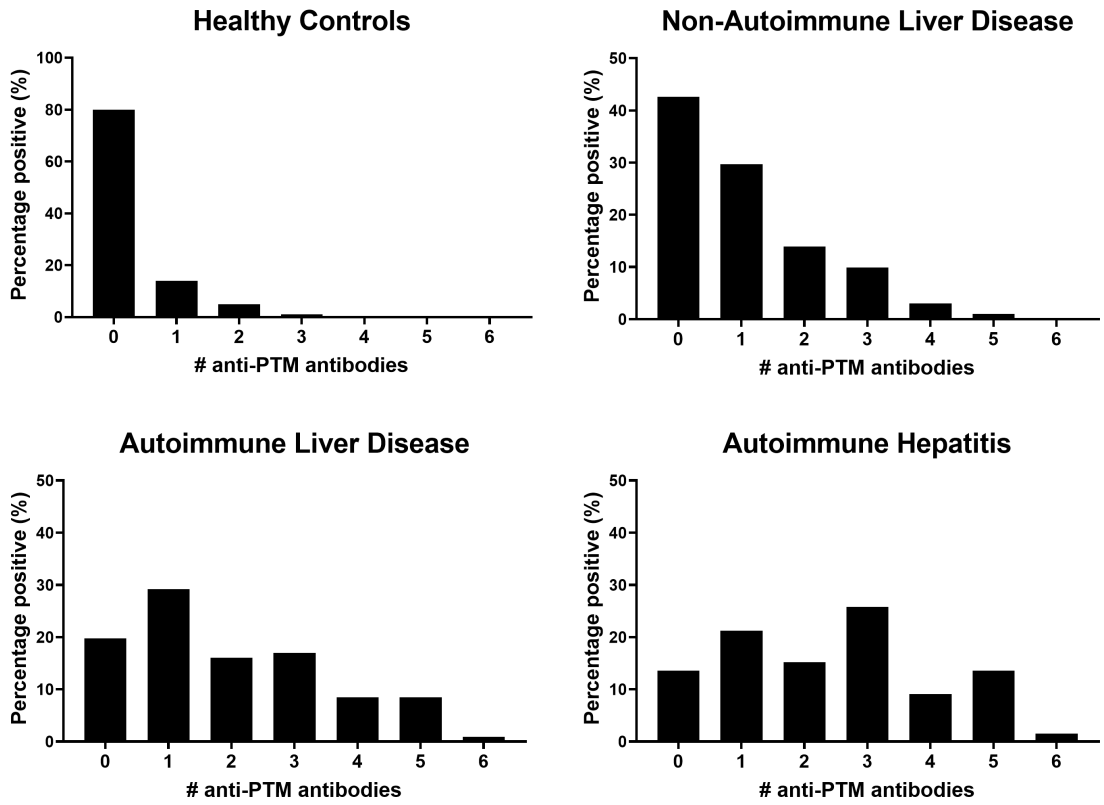

FIGURE 2
Patients with AILD, and especially AIH, are more likely to be positive for more than one anti-PTM antibody. Data is presented as percentage positive (%) patients for a number of anti-PTM antibodies in (from left to right) healthy controls, non-autoimmune liver disease autoimmune liver disease and autoimmune hepatitis.

TABLE 2 The association between the presence of anti-PTM antibodies in HC, non-AILD, AIH and cholestatic liver disease.

	Healthy controls *n* =100				Non-autoimmune liver disease *n* =101				Autoimmune hepatitis *n* =66				Cholestatic liver disease *n* =40			
	aU/mL [IQR]		*n* (% positive)		aU/mL [IQR]		*n* (% positive)		aU/mL [IQR]		*n* (% positive)		aU/mL [IQR]		*n* (% positive)	
Anti-MAA	266.9	[200.4–370.2]	2	(2.0)	495.8	[315.2–726.8]	27	(26.7)	1519.5	[760.0–2775.3]	51	(77.3)	771.5	[538.3–1247.7]**,#	21	(52.5)**,#,+
Anti-AGE	88.9	[0.0–182.5]	4	(4.0)	130.0	[4.2–261.2]	18	(17.8)	349.0	[156.0–537.0]	32	(48.5)	143.5	[27.0–304.0]+	7	(17.5)*,+
Anti-CarP	74.0	[1.5–157.9]	5	(5.0)	241.0	[83.5–422.0]	28	(27.7)	475.5	[293.2–741.8]	42	(63.6)	226.5	[9.8–328.5]**,++	8	(20.0)*,++
Anti-AL	0.0	[0.0–9.8]	5	(5.0)	6.4	[0.0–10.0]	10	(9.9)	15.4	[4.7–33.4]	13	(19.7)	10.8	[0.5–25.4]*	7	(17.5)*
Anti-Cit	1.3	[0.0–3.2]	6	(6.0)	1.6	[0.0–5.9]	15	(14.9)	4.3	[1.2–9.7]	17	(25.8)	1.8	[0.0–4.2]	1	(2.5)#,+
Anti-NT	108.0	[0.0–250]	6	(6.0)	179	[0.0–501.5]	7	(6.9)	369.0	[65.8–732.5]	5	(7.6)	212.0	[0.0–400.8]	2	(5.0)

Results are presented as median (IQR) of *n* (%). Chi-2-tests were used to assess the difference between the presence of the specific manifestations and non-AILD patients. HC versus cholestatic liver disease *$p < 0.005$, **$p < 0.001$; non-AILD versus cholestatic liver disease #$p < 0.005$, ##$p < 0.001$; and AIH versus Cholestatic liver disease +$p < 0.005$, ++$p < 0.001$. AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; Cit, citrullinated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein; non-AILD, non-autoimmune liver disease; PBC, Primary Biliary Cirrhosis; PSC, Primary Sclerosing Cholangitis.

TABLE 3 Percentage of positivity for anti-PTM antibodies in ANA positive, ANA negative, SMA positive, SMA negative and double negative (ANA and SMA) patients with AIH.

	AIH (*n*=66)				
	ANA positive (*n* =42)	ANA negative (*n* =24)	SMA positive (*n* =35)	SMA negative (*n* =31)	ANA negative / SMA negative (*n* =11)
Anti-MAA positive	85.7%	62.5%	71.4%	83.9%	81.8%
Anti-AGE positive	54.8%	37.5%	40.0%	58.1%	63.6%
Anti-CarP positive	69.0%	54.2%	62.9%	64.5%	72.7%
Anti-AL positive	23.8%	12.5%	20.0%	19.4%	27.3%
Anti-Cit positive	31.0%	16.7%	22.9%	29.0%	27.3%
Anti-NT positive	4.8%	12.5%	8.6%	6.5%	18.2%

AGE, advanced glycation end-product; AL, acetylated protein; ANA, anti-nuclear antibodies; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein; SMA, smooth muscle antibody.

Patients with at least three anti-PTM antibodies scored significantly higher on the revised original score for AIH and had significantly higher levels of IgG at time of diagnosis (Supplementary Table S5). Aminotransferase levels were higher in the group with at least three positive anti-PTM antibodies, albeit not significant. Anti-MAA and anti-CarP correlated positively with ASAT at baseline in the group with less than three anti-PTM antibodies present ($r_s = 0.37$ and $r_s = 0.45$, $p = 0.037$ and $p = 0.009$ respectively), but not in AIH patients with at least three anti-PTM antibodies. After 3 months treatment, significantly more AIH patients with at least three anti-PTM antibodies had reached CBR ($p = 0.03$). After 12 months of treatment, the difference was still significant ($p = 0.01$). Overall, a trend toward significance for time to CBR (in years) was found in favor of multiple anti-PTM antibody positivity.

3.8. Anti-PTM antibody responses decrease over time and show distinct associations with ALAT, ASAT or total IgG levels.

Clinical data of two different timepoints were available of 25 AIH patients and antibody responses over time was investigated (Figure 4). The first sample was taken before commencing treatment, the second sample during treatment. The median time interval between visit one and two was 65 months (6–138). Median ΔALAT, ΔASAT and ΔIgG were 352 IU/L (951–79), 324 IU/L (722–83) and 8 g/L (14–2) respectively. Levels of all four anti-PTM antibody responses decreased significantly over time (anti-MAA, anti-CarP, and anti-AL ($p \leq 0.0001$) and anti-AGE ($p = 0.024$)). Change in anti-AL antibody titers associated significantly with change in ASAT and ALAT (r_s: 0.46 and 0.40 $p = 0.02$ and 0.05 respectively) but did not associate with change in total IgG (r_s: 0.17 $p = 0.53$) (Supplementary Table S6). Change in anti-AGE antibody titers significantly associated with change in IgG (r_s: 0.63 $p = 0.007$). Change in anti-MAA and anti-CarP antibody levels was not associated with decrease in ALAT, ASAT and IgG. However, change in anti-CarP antibody levels did show a positive trend toward significant association with decrease of IgG (r_s: 0.48 $p = 0.052$) (Supplementary Table S6).

4. Discussion

To the best of our knowledge, this is the first report on the presence of anti-PTM antibodies in AILD. The presence of anti-PTM antibodies has been described in several other autoimmune diseases where they can serve as diagnostic or prognostic markers (6, 7, 27). Based on these results, we hypothesized that anti-PTM antibodies are also generated in patients with AILD. Additionally, we speculated that patterns in the presence of anti-PTM antibodies might serve diagnostic or prognostic purposes in AILD.

In this study there were five significant findings: First, four anti-PTM antibodies were more prevalent in patients with AILD compared to HCs and to non-AILD: anti-MAA, anti-AGE, anti-CarP, and anti-AL. Second, patients with AILD and particularly patients with AIH often harbored multiple types of anti-PTM antibodies. Third, anti-MAA and anti-CarP antibody positivity significantly correlated with markers for biochemical response in AIH. Fourth, AIH patients with at least three types of anti-PTM antibodies reached CBR at 12 months after initiating treatment more frequently. Lastly, after initiating immunosuppressive treatment next to aminotransferases and IgG also anti-AGE and anti-AL antibody titers decreased. These findings confirmed that anti-PTM antibodies are present in AILD and moreover multiple anti-PTM antibodies identify a group of AIH patients in which these anti-PTM antibodies associate with CBR. Interestingly, we observed that several anti-PTM antibodies are present and even more prevalent in AIH patients who were 'sero-negative' for the classical autoantibodies at diagnosis, compared to patients who were positive for either ANA or SMA. This is particularly captivating since conventional antibodies are not disease specific and may be expressed at a later stage of the disease in 'sero-negative' AIH patients. We suggest that anti-PTM antibodies may be present in patients with AIH before conventional antibodies can be detected. Therefore anti-PTM antibody assessment could especially be interesting in the diagnostic work-up for 'sero-negative' AIH patients. Future studies should further determine the possible implementation of anti-MAA, anti-AGE or anti-CarP assessment, all associated with CBR at 12 months, in the diagnostic algorithm for AIH.

The clinical presentation of AIH is very heterogeneous and can vary from asymptomatic disease to acute (on chronic) liver failure.

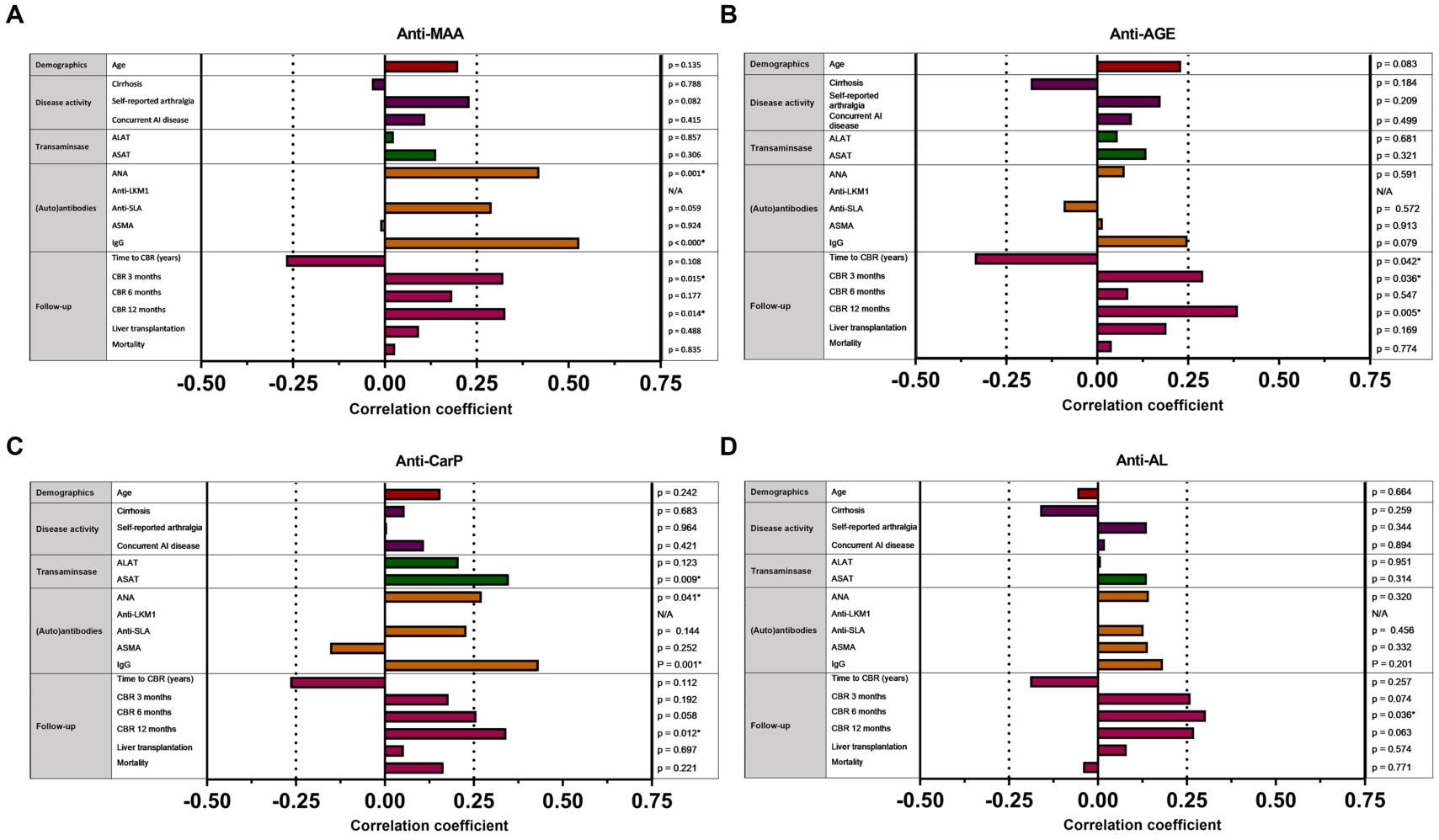

FIGURE 3
Correlation between **(A)** anti-MAA IgG, **(B)** anti-AGE IgG, **(C)** anti-CarP IgG and **(D)** anti-AL IgG antibodies and clinical and serological markers in patients with untreated auto-immune hepatitis (n=58). Correlation analyses are done using Spearman's rho correlation analysis and point-biserial correlation analysis. p<0.05 is considered statistically significant (*). AGE, advanced glycation end-product; ALAT, alanine aminotransferase; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, smooth muscle antibody; CBR, complete biochemical response; IgG, immunoglobulin gamma; LKM, Liver Kidney microsomal antibody; SLA, soluble liver antigen.

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TABLE 4 Characteristics of untreated AIH patients in the AILD cohort (*n*=58).

Patient characteristics	Autoimmune hepatitis (*n* =58)
Female sex	43 (74.1)
Age diagnosis (years)	46.4 ± 19.4
Simplified criteria for the diagnosis of AIH	8 (6–8)
Original revised criteria for AIH	16.7 ± 3.3
Positive antibodies	
ANA (n = 58)	38 (65.5)
SMA (n = 58)	29 (50.0)
Anti-LKM (n = 43)	0 (0.0)
Anti-SLA	2 (3.4)
*Others**	8 (13.8)
IgG (*n* = 57)	24.80 (19.85–32.65)
Histology	
Typical	45 (77.6)
Compatible	9 (15.5)
Atypical/biopsy not done	3 (5.2)
Negative viral hepatitis serology	57 (98.3)
Cirrhosis	23 (39.7)
Yes, compensated	14 (24.1)
Yes, decompensated	9 (15.5)
No cirrhosis	35 (60.3)
Unknown	0 (0.0)
Self-reported arthralgia	12 (20.7)
(More than one) concomitant auto immune disease**	17 (29.3)

Results are presented as *n* (%), mean ± SD or median (IQR). AIH, autoimmune hepatitis; ANA, anti-nuclear antibodies; SMA, smooth muscle antibody; IgG, immunoglobulin gamma; IQR, interquartile range; LKM, Liver Kidney microsomal antibody; SD, standard deviation; SLA, soluble liver antigen. *Others: pANCA (*n* = 8) **Other: auto-immune hemolysis (*n* = 1), celiac disease (*n* = 1), diabetes mellitus type 1 (*n* = 2), granulomatosis with polyangiitis (*n* = 1), Henloch-Schönlein purpura (*n* = 1), Hyperthyroidism (*n* = 3), hypothyroidism (*n* = 6), myastenia gravis (*n* = 1), sclerodermia (*n* = 2) ulcerative colitis (*n* = 1).

Occasionally, polyarthralgia without arthritis is present in patients with AIH (1, 28), and is considered an extra hepatic manifestation of AIH. However, this is often not recognized and is underreported. In clinical practice, reoccurrence of arthralgia is often seen during corticosteroid withdrawal (28). Next to arthralgia, RA is sometimes seen in AIH. We have previously reported that, in the context of RA, anti-CarP (29) and anti-Cit (30) antibodies in arthralgia predict development of RA. Additionally, anti-PTM antibodies have been described in the context of rheumatic disease (10, 31, 32). In this study only a trend was found for the correlation between self-reported arthralgia and anti-MAA antibodies in AIH. This could be a result of the small cohort size and would require further investigation.

Previous research showed that IgG levels are not associated with long-term outcomes in AIH, whereas normalization of aminotransferases is the main treatment goal in AIH, as this positively associates with survival in the first 12 months after diagnosis (33). Additionally, Hartl et al. found that patients with normal IgG levels showed a comparable treatment response to patients with elevated IgG (34). On the contrary, CBR is defined as normalization of ALAT, ASAT and IgG (26). The role of IgG remains a pivoting point in disease progression in AIH. The results of this study suggest that specific subsets of anti-PTM antibodies are associated with treatment response.

In this study, patients with AIH positive for at least three types of anti-PTM antibodies had significantly higher IgG levels at diagnosis and tended to reach CBR more often at 12 months of treatment than patients with AIH with less than three anti-PTM antibodies. By choosing more than three anti-PTM reactivities as a cut-off in this analysis we achieved an equal number of AIH patients in each group (26 with less and 32 with at least three anti-PTM antibodies). Larger studies could determine whether combinations of anti-PTM antibodies, also combined with serum levels of IgG, ALAT, and ASAT at baseline could be better predictors for the likelihood of treatment response. The anti-PTM antibody response is an IgG mediated response and is part of the significantly elevated IgG in this specific group of patients. Positivity for multiple autoantibodies has been reported to provide more reliable information than single biomarkers in for example diabetes (35) and pre-RA (36).

Our study has some limitations: the cohort is heterogeneous and has a limited size. We found that 40 % of AIH patients identify with specific combinations of anti-PTM antibodies (anti-MAA/-AGE/-CarP; anti-MAA/-AGE/-CarP; anti-MAA/-AGE/-CarP/-AL/-Cit) within the AILD group. These specific combinations might aid in the diagnostic work-up for AIH. Noteworthy is that anti-PTM antibodies are not solely found in AIH, but are also found in other liver diseases possibly as a result of breach in tolerance against PTMs that are formed during inflammation. For several autoimmune diseases it is well known that certain autoantibodies are already present many years before the patients develop clinically overt disease for example anti-Cit and anti-CarP Ab in the context of RA (6, 7). Whether this is also the case in these autoimmune liver diseases is currently unknown. PTMs formed as a consequence of inflammation together with impaired liver function may well accumulate and mediate a breach in tolerance, and in this setting anti-PTM antibodies can be formed as a consequence of liver disease. The same set of 6 anti-PTMs were studied in SLE, and anti-MAA, anti-AGE and anti-CarP antibodies were also most frequently found in patients with SLE compared to healthy controls (10). Interestingly, anti-MAA and anti-CarP associated with neuropsychiatric manifestations of SLE, a manifestation that lacked a biomarker. These findings are in the same range as anti-PTM antibody responses found in AILD. Anti-CarP and anti-Cit are well studied anti-PTM antibodies in RA and are found in approximately 50% of RA patients (6, 7). Discovery of new anti-PTM antibodies in RA helped in diagnosis and in following disease progression, and can potentially help to distinguish groups within so-called seronegative RA (5). In order to further validate these findings and prove the sensitivity and specificity of these anti-PTM antibody combinations in the diagnostic work-up of AIH, anti-PTM antibodies need to be assessed extensively in a larger cohort. This could provide the opportunity to set a cut-off titer level and perhaps even distinguish AIH from other liver diseases. In this limited cohort it was not possible to evaluate the prognostic value of anti-PTM antibodies for disease progression as 40% of patients

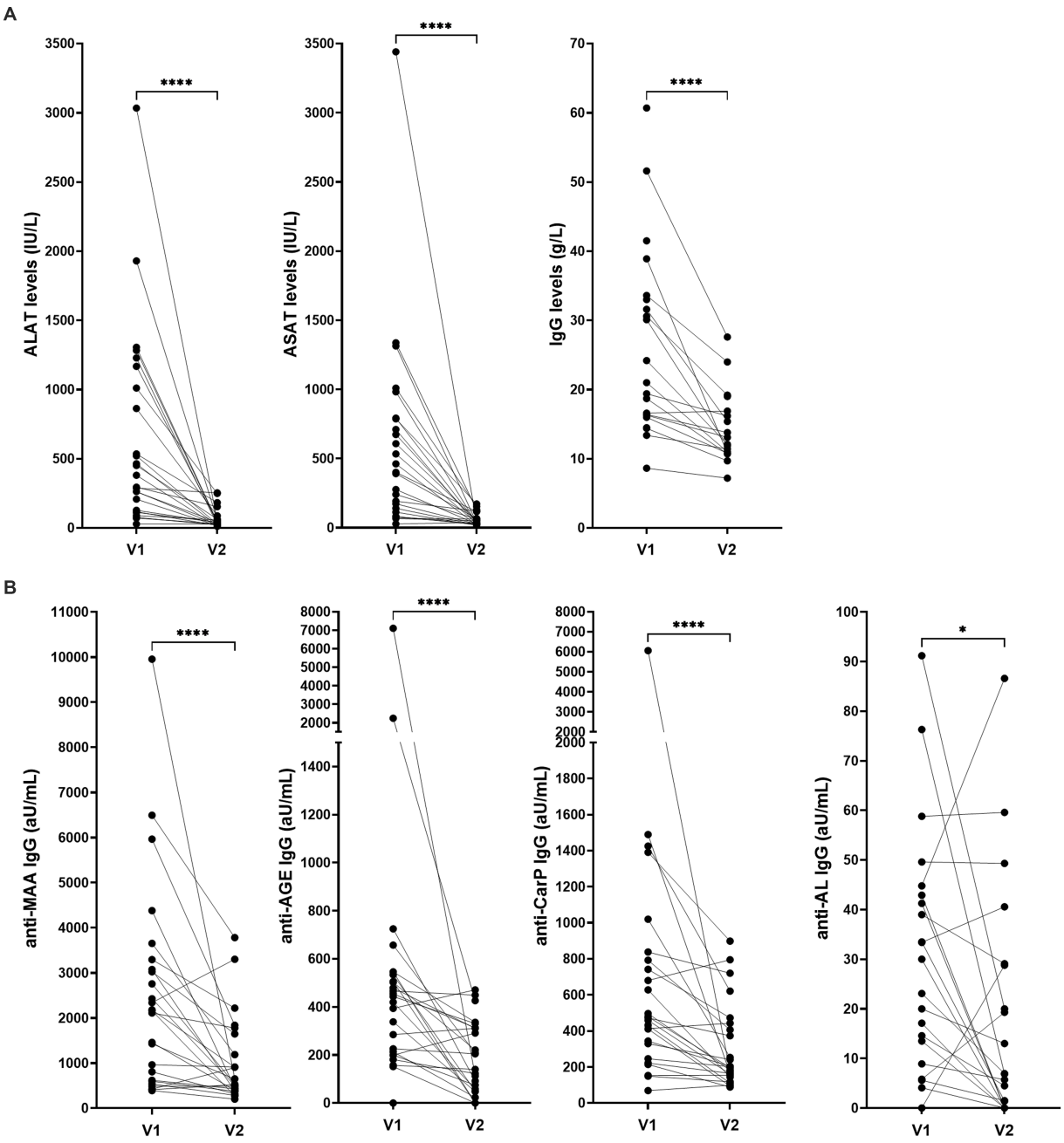

FIGURE 4
Levels of **(A)** ALAT, ASAT, IgG and **(B)** anti-PTM antibodies over time in patients with AIH (n=25). ALAT, ASAT and IgG levels were determined as standard procedure after inclusion. V1: before commencing treatment, V2: during treatment. Median time interval: 65months (6–138). Reactivity toward anti-PTM antibodies was determined using ELISA and is depicted as arbitrary units per milliliter (aU/mL). *p=0.0244 and ****$p\leq$0.0001. AGE, advanced glycation end-product; AL, acetylated protein; ALAT, Alanine aminotransferase; ASAT, Aspartate aminotransferase; CarP, carbamylated protein; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct; V1, first visit; V2, second visit.

already had cirrhosis at diagnosis. A larger cohort study should be conducted in patients with AILD and no cirrhosis at diagnosis with set follow-up timepoints.

Different clinical parameters are measured to monitor disease activity for AIH, PBC and PSC. As a result, the three groups within the AILD cohort are incomparable. However, in the AILD cohort we have included PBC and PSC, which are not pure auto-immune diseases (where immune injury results in cholestasis) (25, 37–43). We hoped to evaluate possible differences in anti-PTM antibodies patterns in AIH, PBC and PSC. When analyzing AIH patients, only untreated patients were included to prevent impact of treatment. One of the strengths of this study is that the group of interest, AILD, is well-defined according to simplified or original revised score for AIH. Therefore, we can state that the results found for these subgroups are representative.

Combining the prevalence data of all six anti-PTM antibodies tested we observe that approximately 20% of healthy controls harbored at least one anti-PTM antibody (Figure 2). PTM of proteins occurs in all individuals, these PTMs may represent neo-epitopes

toward which antibodies can be formed. Interestingly, this is apparently often not associated with disease, but is known to predispose to disease.

The standard therapy for patients with AIH consists of a combination of glucocorticoids and azathioprine (2). Most patients with AIH in this cohort were initially treated with this preferred treatment. Pape et al. demonstrated that a higher or lower initial predniso(lo)ne dose does not have impact on reaching CBR. In our study, stratification of the results by the initial steroid dose was not possible, as 42 patients (85.7%) received an initial predniso(lo)ne dose above 30 mg/day (44). When patients do not reach CBR, the treating physician may decide to intensify or adjust treatment regimens. The nature of the disease, characterized by intermittent loss of remission and flares, may give reason to frequently adjust therapy. The size of the studied cohort limited us to correct for change in therapy over time. Since it was not possible to obtain the necessary data we were not able to correct the correlation analyses regarding CBR for duration of steroid treatment, duration of tapering schemes, dose modification or drug withdrawal during follow-up. The median ASAT and ALAT did not differ between the patients who were prescribed budesonide compared to predniso(lo)ne, although this has been previously reported (45).

However, according to the guidelines and Delphi consensus on treatment response, treatment effect is first evaluated 6 months after commencing treatment (2, 26). We additionally did see more patients reaching CBR at 12 months of treatment if they had at least three positive anti-PTM antibodies. This may imply that having anti-PTM antibodies for at least three PTMs may be prognostically favorable regarding treatment response. Despite higher ALAT and ASAT levels at baseline in the AIH patients with at least three anti-PTM antibodies present, no association between transaminase levels and multiple positivity could be found. Only in patients with less than three anti-PTM antibodies present, a positive correlation between anti-MAA, anti-CarP and ASAT was found. This strengthens the implication that multiple positivity for at least three anti-PTM antibodies may be beneficial for treatment response and may guide treating physicians to earlier treatment intensification.

In conclusion, anti-PTM antibodies are present in patients with AILD. Some patients are positive for multiple anti-PTM antibodies. Having three or more anti-PTM antibody responses is associated with a favorable response to treatment in AIH.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Data, analytic methods, and study materials will be made available to other researchers upon any reasonable request. Requests to access these datasets should be directed to L.A.Trouw@lumc.nl.

Author contributions

MB, AS, AM, SM, LZ, MT, BH, and LT were involved in the design and interpretation of the study. Anti-PTM antibody analyses were performed by MB under the supervision of LT. Clinical data was collected by AS under the supervision of BH and MT. Statistical analysis and interpretation were performed by MB and AS under the supervision of LT, BH, and MT. AM kindly provided cohort sera samples. MB and AS drafted the manuscript, which was critically revised by LT, BH, MT, AM, SM, and LZ. 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1195747/full#supplementary-material

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Glossary

AILD	(auto)immune mediated and cholestatic liver disease
PBC	primary biliary cholangitis
PSC	primary sclerosing cholangitis
AIH	autoimmune hepatitis
Ig	immunoglobulin
SMA	smooth muscle antibodies
AMA	anti-mitochondrial antibody
RA	rheumatoid arthritis
PTM	post-translational modification
ACPA	anti-citrullinated antibodies
CarP	carbamylated protein
MAA	malondialdehyde-acetaldehyde adduct
AGE	advanced glycation end-products
SLE	systemic lupus erythematosus
NT	nitration
Anti-PTM antibody	anti-post-translationally modified protein antibody
CCP	cyclic citrullinated peptide
Non-AILD	non-autoimmune mediated and cholestatic liver disease
HC	healthy control
CBR	complete biochemical response
ASAT	aspartate aminotransferase
ALAT	alanine aminotransferase
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
PBS	phosphate buffered saline
BSA	bovine serum albumin
HRP	horseradish peroxidase
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AL	acetylation
ALD	alcoholic liver disease
HBV	chronic hepatis B
HCV	chronic hepatitis C
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
Cit	citrullination
ANA	antinuclear antibodies
SLA	soluble liver antigen

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Commentary: Commentary: Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment

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pIgG—polyreactive IgG, AIH—autoimmune hepatitis, PTM—post-translational modification, anti-CarP, antibodies against carbamylated proteins, FCS—fetal calf serum, BSA

A Commentary on

Commentary: Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment

by Taubert, R., Engel, B., and Campos-Murguia, A. (2023). *Front. Med.* 10:1275838. doi: 10.3389/fmed.2023.1275838

We read with great interest the commentary of Taubert et al. (1) on our article "Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment (2)." In their kind commentary the authors bring up the very important and relevant subject of polyreactive IgG (pIgG) as they have described to occur in autoimmune hepatitis (AIH) (3). The team of Taubert have identified such pIgG using an experimental set up roughly similar to the enzyme-linked immunosorbent assay (ELISA) setup as we have used for the detection of the antibodies against post-translationally modified proteins (anti-PTM). In their commentary they raise the concern that part of the antibodies identified in our assays as anti-PTM antibodies may in fact be pIgG. We can reassure the authors and readers that we are specifically detecting anti-PTM antibodies in our assay. Importantly, this is because of the setup of our ELISA system. Ever since the identification of antibodies binding to carbamylated antigens (anti-CarP) (4) we have used both carbamylated fetal calf serum (Ca-FCS) and unmodified FCS as control antigens for the coating of the ELISA plates. In practice one half of the ELISA plate is coated with Ca-FCS and the other with unmodified, control

FCS. The entire plate is blocked with bovine serum albumin (BSA). Each serum sample is tested on both the Ca-FCS and the control FCS. The levels of antibody binding are calculated from absorbance values into arbitrary units per milliliter based on a standard line on the same plate. Next, the level of carbamylation specific antibodies is defined as the level of antibody binding to the Ca-FCS minus the level of antibodies binding to the control FCS. Hence, we report the PTM-specific response. In many of the analyses that we have run for rheumatoid arthritis (RA) and for systemic lupus erythematosus (SLE) (5) the reactivity of the control protein is very low. Indeed, we have observed that in AIH this was somewhat higher, but importantly we have subtracted this from the anti-PTM response, allowing us to conclude on the PTM-specific antibodies and avoiding undesired interference from pIgG. We realize that we may not have stressed this to the greatest extend in our manuscript and thank the authors for bringing up this point and for the opportunity to clarify this.

In our manuscript we have used six different PTMs. To make the best comparisons, we have not used the same control FCS for all the PTMs but have actually generated a separate control FCS for each of the conditions. For example, the control for carbamylation is an aliquot of the same FCS, incubated at the same time point, for the same duration, at the same temperature and dialysis steps as the carbamylated FCS, but only without the addition of the KOCN, the carbamylating chemical. For the modification with Advanced Glycation End-products, we have performed the incubations of the control FCS also for 10 days at 37°C, all to ensure that we make the best possible comparisons.

In the original paper we already reported that each of the anti-PTM reactivities has clearly different sensitivities, while all of the assays are based on FCS coating and bovine serum albumin (BSA) blocking, indicating that the assays do not detect pIgG. We have tested if there was any correlation between the signals observed on PTM-FCS vs. control FCS. For the four anti-PTMs with the highest percentage of positive samples we did not find any correlation, again indicating that the anti-PTM antibodies are specifically binding to the PTM. In the absence of PTM specific antibodies there is logically a correlation between the modified FCS and control FCS. The authors raise interesting questions regarding the nature of the antibody response to the PTM proteins. As can be seen in supplementary figure 1 of the manuscript (2), we studied how often the different anti-PTM antibodies can be found together in the same patients, as this may be an indication of either co-induction or cross-reactivity. We clearly observe different patterns with some individuals positive for one anti-PTM and other positive for several others (2), again indicating that the different assays are clearly identifying different antibodies. Additionally, while between some anti-PTM responses we do observe a correlation [as observed before (4, 6)] for other anti-PTM responses we do not detect any correlations. Importantly, some patients can be highly positive for one anti-PTM reactivity and simply negative for the other.

We did find that overall levels of some anti-PTM antibodies (weakly) associate with levels of IgG, but this may simply reflect that a polyclonal B cell stimulation (7) will stimulate the anti-PTM reactive B cells as well as other B cells, but it will only result in positivity in individuals that actually have anti-PTM reactivity. In the context of RA, we have observed that many of the anti-PTM antibodies are isotype switched but are of low-avidity (8, 9) indicating that there has been T-cell help, but lack of avidity maturation. The authors finally raise the point of serum storage time. This is an important issue and difficult to address experimentally. We have previously studied this in detail for our cohort in the context of our previous paper on AIH, focused on other biomarkers (10), where we concluded that the quality of the samples was good as there was no difference in the sensitivity of the markers in the samples that were stored for a long time (i.e., $\geq$10 years) vs. the samples that were stored more recently (i.e., $<$10 years), suggesting that the storage was not a major factor in these analyses. Also for the current study on anti-PTM antibodies we have now carefully plotted the levels of all the six anti-PTM reactivities vs. the time of storage of the sample and observed that positivity for the anti-PTM antibodies is not influenced by storage time (data not shown).

Importantly, for the anti-PTM responses in AIH we do observe associations with response to treatment while in the work of Taubert et al. (3) no such association is observed for pIgG, again indicating that the anti-PTM detection does measure different antibodies. For a subset of patients we have analyzed changes in anti-PTM antibody levels over time, and we observed that upon treatment the levels decrease. The data obtained from these two time points does not reveal if the anti-PTM positivity will completely seroconvert.

In conclusion, we agree with the authors of the commentary that unintentional detection of pIgG is an important factor to consider when running ELISA experiments on sera of patients with AIH. However, we are convinced that the careful set up of our experiments excluded the detection of pIgG and specifically measures anti-PTM antibodies.

Author contributions

MB: Investigation, Writing—original draft, Writing—review and editing, Conceptualization. AS: Conceptualization, Investigation, Writing—original draft, Writing—review and editing. AM: Conceptualization, Writing—original draft, Writing—review and editing. SM: Conceptualization, Investigation, Writing—original draft, Writing—review and editing. LZ: Conceptualization, Investigation, Writing—original draft, Writing—review and editing. MT: Conceptualization, Writing—original draft, Writing—review and editing. BH: Conceptualization, Writing—original draft, Writing—review and editing. LT: Conceptualization, Funding acquisition, Writing—original draft, Writing—review and editing, Supervision.

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Abbreviations: pIgG, polyreactive IgG; AIH, autoimmune hepatitis; PTM, post-translational modification; anti-CarP, antibodies against carbamylated proteins; FCS, fetal calf serum, BSA, bovine serum albumin; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; ELISA, Enzyme-Linked ImmunoSorbent Assay.

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Conflict of interest

LT is listed as an inventor on a patent on the detection of anti-CarP autoantibodies.

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.

Publisher's note

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Antibodies to leukotoxin A from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* in patients at an increased risk of rheumatoid arthritis

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Objectives: Periodontitis and underlying bacteria have been linked to the development of rheumatoid arthritis (RA). One suggested pathogen is *Aggregatibacter actinomycetemcomitans* (*A.a.*), which expresses leukotoxin A (LtxA) that can citrullinate human proteins, providing a possible trigger for the production of anti-citrullinated protein antibodies (ACPA). In this study, we seek to determine the presence of antibodies toward LtxA in patients at risk of developing RA.

Methods: Two prospective observational patient cohorts (one Swedish and one British) with symptomatic at-risk patients were studied. Anti-LtxA antibodies were analyzed by a cell-based neutralization assay in baseline serum and compared to 100 Swedish blood donors that served as controls.

Results: Serum anti-LtxA levels or positivity did not differ between patients and blood donors. In the British cohort, anti-LtxA was more prevalent among ACPA-positive arthralgia patients compared with ACPA-negative arthralgia cases (24% vs. 13%, $p < 0.0001$). In the Swedish at-risk cohort, anti-LtxA positive patients were at increased risk of progression to arthritis (hazard ratio (HR) 2.10, 95% CI 1.04–4.20), but this was not confirmed in the UK at-risk cohort (HR 0.99, CI 0.60–1.65).

Conclusion: Serum anti-LtxA is not elevated before RA diagnosis, and associations with disease progression and ACPA levels differ between populations. Other features of the oral microbiome should be explored in upcoming periodontitis-related RA research.

KEYWORDS

ACPA, *Aggregatibacter actinomycetemcomitans*, rheumatoid arthritis, progression, periodontitis

Introduction

Connections between mucosal tissues and rheumatoid arthritis (RA) development have been attracting increasing interest in recent years (1). In this context, the oral cavity is of particular interest due to the reported association between periodontitis and RA (2). Such a link was further substantiated by the identification of protein citrullination properties of bacteria underlying periodontitis, providing a possible trigger for the production of anti-citrullinated protein antibodies (ACPA) (2, 3). *Aggregatibacter actinomycetemcomitans* (*A.a.*) is a Gram-negative coccobacillus that is recognized as a pathogen in periodontitis. Recent findings indicate high systemic immunoreactivity against *A.a.* when compared with several other bacterial species that are commonly detected in the human commensal microflora (4). *A.a.* produces a toxin called leukotoxin A (LtxA), which is a major virulence factor in periodontitis in young individuals (5). Intriguingly, LtxA was shown to trigger protein hypercitrullination in interaction with neutrophils, and antibodies to LtxA were increased in RA and correlated with ACPA levels (3, 6). These features of *A.a.* are of great interest considering that ACPA occurrence is a strong predictor of arthritis development among individuals with arthralgia (7) and of joint erosions in patients with recent-onset RA (8). Despite this, the role of *A.a.* in RA development and progression has been sparsely investigated. Nevertheless, it was recently shown that anti-LtxA IgM levels associate with RA onset (9), and circulating *A.a.* antibodies were associated with subclinical atherosclerosis in RA (10).

Previous studies on immune responses to *A.a.* have mostly used enzyme-linked immunoassays using *A.a.* LtxA as an antigen (3, 9). However, since the LtxA could be a starting point for the process leading to hypercitrullination and ACPA formation (3), it is of potential advantage to instead assess the functional (neutralizing) capacity of anti-LtxA in serum. The presence of systemic LtxA neutralizing activity has shown a strong correlation between systemic ab that binds LtxA (11) and the presence of A.a in the subgingival plaque (12). By using two cohorts of patients at risk of developing RA, we aimed to characterize the neutralizing anti-LtxA antibodies in relation to clinical course and autoantibody levels. This knowledge is of importance from an etiological perspective and to guide further work concerning dental interventions for the treatment of RA.

Materials and methods

Study populations

We included two independent at-risk cohorts, one Swedish with ACPA-positive patients only and one British with both ACPA-positive and ACPA-negative at-risk patients. Baseline characteristics are shown in Table 1. In addition, 100 Swedish healthy blood donors were included to serve as controls.

At-risk patients

TIRx cohort

In the TIRx cohort (Swedish acronym for "xtra early rheumatology follow-up"), 82 patients with a positive IgG ACPA test in clinical routine and musculoskeletal pain of any sort and duration, but no baseline arthritis, were followed prospectively for the development of clinical arthritis (13). Patients were recruited between 2010 and 2013 at the rheumatology unit, Linköping University Hospital, Sweden. The exclusion criteria were previous rheumatic disease or treatment with oral corticosteroids within 6 weeks. Follow-up visits were scheduled regularly, and arthritis development was defined upon clinical examination by an experienced rheumatologist. The median follow-up time was 69 months [interquartile range (IQR) 24–90]. Progression to arthritis occurred in 39 out of 82 patients (48%) after the median 6 months (IQR: 1–71). A total of 15 patients (18%) had symptom duration up to 6 months, 27 (45%) patients had symptom duration between 6 and 18 months, and 30 (37%) patients had experienced symptoms >18 months prior to inclusion.

Leeds cohort

Anti-CCP positive at-risk individuals who took part in "The CCP Study: Coordinated Programme to Prevent Arthritis—Can We Identify Arthritis at a Pre-clinical Stage?" were enrolled from June 2008 to January 2019. A detailed description of the CCP study has been previously published (14, 15). In this national observational UK study, individuals who presented to their general practitioner (or other health professionals) with a new non-specific musculoskeletal symptom were tested for anti-CCP antibodies.

The individuals who have a positive anti-CCP2 test were then invited to Chapel Allerton Hospital, Leeds, UK, for further assessments at a dedicated research clinic as part of an observational longitudinal study until the development of inflammatory arthritis. Individuals with a negative anti-CCP antibody test were sent a postal questionnaire 12 months after enrollment, asking about their disease status (i.e., progression to inflammatory arthritis) (16).

For this study, we reviewed 178 anti-CCP positive individuals without baseline arthritis from the Leeds CCP cohort. Out of these subjects, 81 (46%) developed clinical arthritis during a median follow-up period of 25 months.

Control groups

A total of 100 healthy blood donors recruited from Linköping University Hospital served as controls for the at-risk patient cohorts. These controls were age-matched to the TIRx cohort. In the UK, 86 anti-CCP negative subjects with arthralgia were selected as controls.

Ethics

The ethical review board in Linköping, Sweden, approved the study protocol (Decision No. M220-09, 2015/236-32 and

TABLE 1 Baseline characteristics of at-risk patients and controls.

	TIRx ($n = 82$)	Leeds CCP positive ($n = 178$)	Leeds CCP negative ($n = 86$)	Controls ($n = 100$)
Demographics:				
Women, n (%)	66 (81)[a]	128 (72)[a]	65 (76)[a]	50 (50)
Age, mean (range)	51.8 (18–76)	51.3 (20–78)	52.0 (19–84)	51.7 (18–72)
Median follow-up time (IQR)	69 (24–90)	25 (10–53)		
Periodontitis (%)	-	72[b]	-	-
Risk factors:				
Ever smoker, n (%)	39 (48)	114 (64)[c]	-	-
Shared epitope carrier, n (%)	52 (64)[d]	90 (64)[e]	-	-
RF positive, n (%)	24 (29)	81 (46)[c,f]	-	-

[a]significantly different from controls $p = < 0.001$, [b] $n = 29$, [c]significantly different from TIRx patients $p = < 0.02$, [d] n = 81, [e] n = 14 and [f] n = 177.

2017/260-32). The Leeds CCP Study was approved by the NHS Health Research Authority National Research Ethics Service Committee Yorkshire and the Humber-Leeds West (REC reference: 06/Q1205/169). All participants signed written informed consent.

LtxA antibody assay

Anti-LtxA antibodies were analyzed for their LtxA neutralizing capacity. This was detected as reduction in cell damage, assessed by neutral red leakage, following exposure to purified LtxA (17). THP-1 cells in RPMI-10% fetal bovine serum (FBS)-50 nM phorbol myristate acetate were seeded at 1 x 10^6 cells/ml in flat 96 well plates and incubated at 37°C 5% CO_2 overnight. The cells were washed with RPMI, and then patient serum and LtxA were added to the wells in triplicates. The plates were incubated for 2 h at 37°C with 5% CO_2. The medium was removed, and 0.04 mg/ml neutral red diluted in RPMI-10% FBS was added to the wells and incubated for 90 min at 37°C with 5% CO_2. The wells were washed with PBS (pH 7.4), and then 50% EtOH-1% acetic acid was added to lyse the cells. Following 10 min of incubation, the optical density (OD) was read at 650 nm (TECAN Sunrise, CA, USA). Anti-LtxA antibody neutralization capacity in percent was calculated by dividing serum sample OD with maximum cell viability OD (FBS) x 100. Serum samples inhibiting LtxA cell lysis >30% were classified as positive and <30% were classified as negative regarding anti-LtxA presence (11).

Autoantibody analyses

Serum secretory component-containing (SC) ACPA and IgM ACPA were measured by modifying commercially available anti-CCP ELISA kits (Euro-Diagnostica, Malmö, Sweden) as described earlier (18). Serum IgA and IgG ACPA were analyzed by a fluoroenzyme immunoassay (EliA™ Phadia AB, Thermo Fisher Scientific, Uppsala, Sweden) as described previously (18). RF tests were performed in a clinical routine setting at each local laboratory associated with the participating rheumatology unit. Free SC in serum was analyzed using an in-house sandwich ELISA as previously described (19).

Statistics

Mann–Whitney U-test or Fisher's exact test was used to analyze differences between groups. The prognostic value of anti-LtxA antibodies was studied using Cox regression analysis and, when statistically significant in univariable analysis, adjusted in multivariable analysis for age, sex, smoking, RF status, ACPA levels, and symptom duration.

Results

The baseline characteristics of patients and controls are shown in Table 1.

There were no significant differences in anti-LtxA levels or percentage-positive individuals between the two at-risk cohorts and the controls (Figure 1).

Serum anti- LtxA and arthritis development

In the TIRx at-risk cohort, a larger proportion of patients progressing to arthritis was anti-LtxA antibody positive compared to those who did not progress (49% vs. 23 %, $p = 0.021$). This difference remained significant in univariable Cox regression analysis (HR 2.25, 95% CI 1.20–4.25 p = 0.012) and in multivariable Cox regression adjusted for possible confounders (HR 2.10, 95% CI 1.04–4.20, $p = 0.037$, Figure 2A). In the Leeds at-risk cohort, anti-LtxA seropositivity was not significantly different between progressors and non-progressors (25% vs. 24%, $p = 1.0$), and did not associate with arthritis development in Cox regression analysis (HR 0.99, CI 0.60–1.65, $p = 0.973$, Figure 2B).

Serum anti-LtxA- and RA-related autoantibodies

Both the TIRx cohort and the ACPA-positive Leeds patients were more often anti-LtxA positive compared to the Leeds APCA-negative patients (35% vs. 13%, p = 0.001 and 24% vs. 13%, $p = 0.035$, respectively, Figure 1). Swedish blood donors (controls) were more often anti-LtxA positive than Leeds ACPA-negative at-risk patients (28% vs. 13%, $p = 0.012$, Figure 1). In TIRx, where all patients were IgG ACPA-positive, the levels were higher among patients positive for anti-LtxA compared with anti-LtxA negatives, with borderline statistical significance (mean 658 ± 1,007 vs. 334 ± 655 AU/mL; $p = 0.073$). A borderline significance was also seen in the Leeds cohort, where anti-LtxA positive showed higher IgG ACPA levels compared to anti-LtxA negative (mean 174 ± 149 vs. 133 ± 147 AU/mL, $p = 0.068$). Serum LtxA did not associate with IgA, IgM, SC ACPA, free SC, or RF status in the TIRx cohort (data not shown).

Serum anti-LtxA and periodontitis

Data on periodontitis were present for a subgroup of the Leeds at-risk cohort, but anti-LtxA positive at-risk patients did not more often suffer from periodontitis compared to anti-LtxA negative at-risk patients (73% vs. 72%, $p > 0.9$).

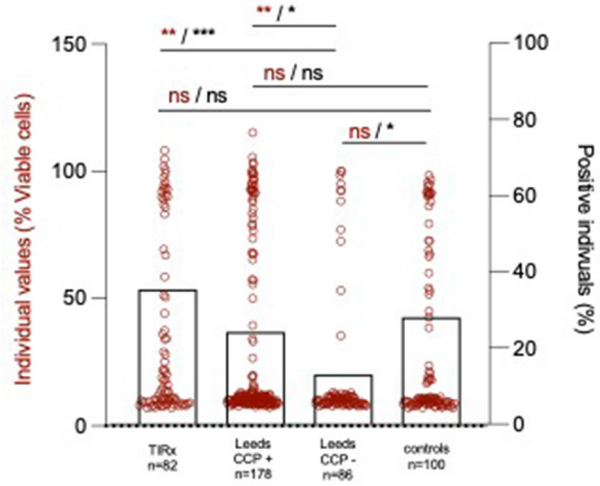

FIGURE 1
Individual anti-LtxA levels (red circles and statistical notations in red) and percentage positive individuals (black bars, mean ± SD and statistical notations in black). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Discussion

To reach a better understanding of the possible role of *A.a.* in RA development and progression, we investigated toxin-neutralizing antibody responses in patients at risk of developing RA. The overall conclusion, from the present and previous studies (3, 9), is that *A.a.*-related antibody responses, as well as possible links to RA, appear to be substantially influenced by cohort characteristics and/or the context from which they have been recruited. In the Swedish at-risk cohort, we found neutralizing antibodies to *A.a.* LtxA prognostic for arthritis development among symptomatic ACPA-positive patients also after adjustments for smoking and other possible confounders. However, this association could not be replicated in the UK at-risk cohort despite inclusion criteria and baseline characteristics being very similar. In both at-risk cohorts, there were indications that a humoral response to LtxA associates with ACPA production, which is in agreement with the seminal study in established RA by Konig et al. (3). However, at-risk patients (ACPA-positive) did not have elevated anti-LtxA compared to (ACPA-negative) blood donors, possibly implying that *A.a.* enhances rather than initiates an ACPA response.

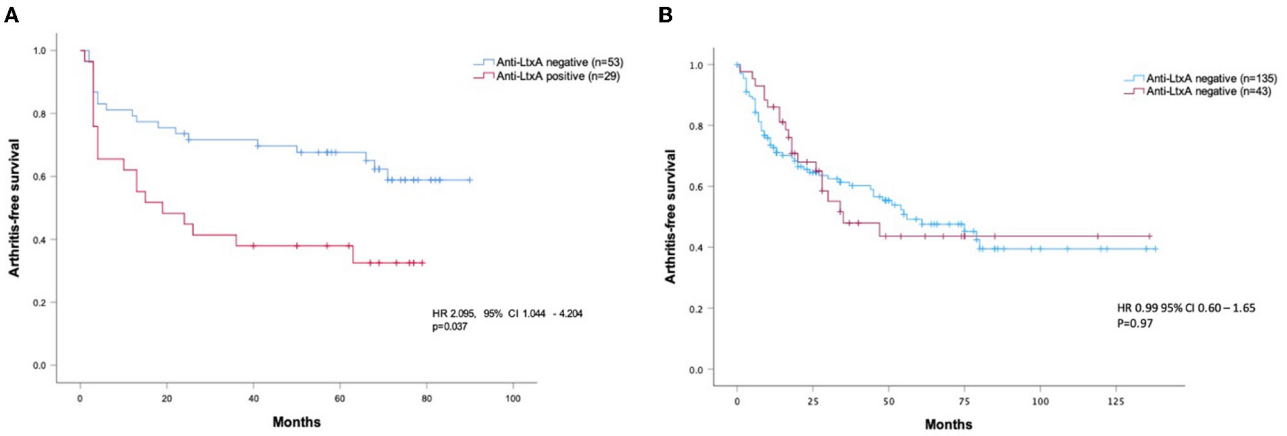

FIGURE 2
Baseline anti-LtxA levels vs. progression to arthritis in the TIRx cohort [**(A)** multivariable analysis] and the Leeds cohort [**(B)** univariable analysis]. Hazard ratio (HR) with 95% confidence interval (CI) in Cox regression adjusted for age, sex, smoking, RF status, ACPA levels, and symptom duration (TIRx only).

A major strength of this study is the inclusion of patients prior to arthritis onset, which enables proper determination of whether *A.a.* immunity precedes RA development or not. The use of two independent and geographically distinct cohorts, which resulted in discordant results, highlights that anti-LtxA results cannot readily be extrapolated across populations. There was a slightly lower occurrence of anti-LtxA in the British population compared to the Swedish, but whether or not that reflected a lower prevalence of *A.a.* or periodontitis could not be investigated in this study. Nevertheless, previous studies show a prevalence of periodontitis of 38% in the UK (20) and 40% in Sweden (21) suggesting that the prevalence of periodontitis does not stand for the slight differences in anti-LtxA ab occurrence between the two at-risk cohorts. Another possible drawback is that we did not specifically address antibody isotypes but instead applied a functional assay to detect anti-LtxA. We believe, however, that given the prevailing hypothesis of LtxA-induced hypercitrullination as a mechanistic link to RA, the total neutralizing capacity of the anti-LtxA antibody response is a relevant readout. We did not examine *A.a.* presence in the oral cavity, but it was previously reported from the UK at-risk cohort that *A.a.* DNA abundance was neither increased nor prognostic for arthritis development (20).

To conclude, we found some associations between *A.a.* and different aspects of RA development and progression. However, the results are not consistent across populations, implying that other features of the oral microbiome should be explored in upcoming periodontitis-related RA research.

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 ethical review board in Linköping, Sweden (Decision No. M220-09, 2017/260-32) and NHS Health Research Authority National Research Ethics Service Committee Yorkshire and the Humber—Leeds West, UK (REC reference: 06/Q1205/169). The patients/participants provided their written informed consent to participate in this study.

Author contributions

AK and AJ conceived and planned the experiments. KMar, CÖ, and AD carried out the experiments. AK and KMar took the lead in writing the manuscript. All authors contributed to the interpretation of the results, provided critical feedback, helped shape the research, analysis, and manuscript.

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Conflict of interest

KMan reports personal fees from AbbVie, Lilly, and UCB AbbVie, outside the submitted work and research grants from Gilead and Lilly. PE reports consultant fees from BMS, AbbVie, MSD, Pfizer, Novartis, and Roche and personal fees from Abbvie, Gilead, Lilly, and Novartis, outside the submitted work and also reports research grants from AbbVie, BMS, Lilly, and Samsung.

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.

Publisher's note

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Advantages of Chinese herbal medicine in treating rheumatoid arthritis: a focus on its anti-inflammatory and anti-oxidative effects

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Oxidative stress is a condition characterized by an imbalance between the oxidative and antioxidant processes within the human body. Rheumatoid arthritis (RA) is significantly influenced by the presence of oxidative stress, which acts as a pivotal factor in its pathogenesis. Elevated levels of mitochondrial reactive oxygen species (ROS) and inflammation have been found to be closely associated in the plasma of patients with RA. The clinical treatment strategies for this disease are mainly chemical drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids (GCs) and biological agents, but it is difficult for patients to accept long-term drug treatment and its side effects. In the theory of traditional Chinese medicine (TCM), RA is thought to be caused by the attack of "wind, cold, damp humor," and herbs with the effect of removing wind and dampness are used to relieve pain. Chinese herbal medicine boasts a rich heritage in effectively attenuating the symptoms of RA, and its global recognition continues to ascend. In particular, RA-relevant anti-inflammatory/anti-oxidative effects of TCM herbs/herbal compounds. The main aim of this review is to make a valuable contribution to the expanding pool of evidence that advocates for the incorporation of Chinese herbal medicine in conventional treatment plans for RA.

KEYWORDS

traditional Chinese medicine, Chinese herbal medicine, rheumatoid arthritis, oxidative stress, inflammation

1 Introduction

RA is a persistent autoimmune condition that is characterized by widespread inflammation throughout the body. It primarily affects the joints, causing pain, stiffness, and swelling. The prevalence of RA varies globally, with a higher incidence rate in women compared to men. The worldwide prevalence ranges from 0.5 to 1.0%. RA is characterized by the presence of neovascularization and abnormal synovial hyperplasia, which are pathological manifestations directly influenced by the infiltration of diverse immune and inflammatory cells within the affected area (1, 2). The infiltration of the affected area at a local level ultimately results in the gradual deterioration of both cartilage and bone, thus causing dysfunction of the joints and posing a higher risk of disability and even mortality. Oxidative stress is a condition characterized by an imbalance between the oxidative and antioxidant processes within the

human body. The disruption in redox balance gives rise to an excess of free radicals, which surpasses the body's ability to eliminate them through antioxidants. As a result, an excess of ROS, reactive nitrogen species (RON), and various other compounds build up, leading to oxidative damage. RA is significantly influenced by the presence of oxidative stress, which acts as a pivotal factor in its pathogenesis (3, 4). The research has uncovered a noteworthy and affirmative association between clinical indicators and oxidative stress indicators within the bloodstream of individuals suffering from RA. The measurement of serum oxidative stress markers has proven to be a reliable biomarker for effectively monitoring the progression of RA disease (4, 5). Elevated levels of mitochondrial ROS and inflammation have been found to be closely associated in the plasma of patients with RA. The treatment involving tumor necrosis factor (TNF) blockade has the capacity to inhibit oxidative stress and the occurrence of mitochondrial mutations induced by hypoxia, which in turn facilitates the process of disease rehabilitation (6).

At present, the clinical treatment strategies for this disease are mainly chemical drugs, such as NSAIDs, DMARDs, GCs and biological agents, but it is difficult for patients to accept long-term drug treatment and its side effects. Nowadays, the clinical acceptance of TCM has been increasing all over the world. In the theory of TCM, RA is thought to be caused by the attack of "wind, cold, damp humor," and herbs with the effect of removing wind and dampness are used to relieve pain (7, 8). Wind in TCM is characterized by the sudden onset of illness, mobility of the affected area, variability in symptom presentation, and sensitivity to environmental changes. Wind-dominant arthralgia is commonly seen in the initial phase of RA and primarily affects the upper body. Damp is primarily associated with the weather. Being in water or resting on damp ground can trigger and exacerbate symptoms due to the moist surroundings. Cold-induced symptoms refer to the exacerbation of symptoms when exposed to cold temperatures, which can be alleviated by the application of heat. Cold-related arthritis primarily affects the hands and feet. Chinese herbal medicine can alleviate RA through multi-target, multi-link and multi-way, and concentrate on the regulation of oxidative stress and inflammation, both of which play pivotal roles in the progression of RA (8, 9). The objective of this research is to explore the RA-relevant anti-inflammatory/anti-oxidative effects of TCM herbs/herbal compounds. Through emphasizing the distinctive advantages of Chinese herbal medicine in dealing with these fundamental pathological processes, our objective is to shed light on potential innovative approaches to improve RA patient outcomes. Ultimately, the primary goal of this paper is to make a valuable contribution to the expanding pool of evidence that advocates for the incorporation of Chinese herbal medicine in conventional treatment plans for RA.

2 The pathogenesis of rheumatoid arthritis

RA is initially characterized by persistent activation of cells, leading to autoimmunity in the joints or other affected organs (10). The primary presentation of the illness primarily arises as a result of inflammation in the synovial tissue and damage to the joints. Despite the absence of a cure for RA, early intervention with medication can effectively decrease the likelihood and severity of joint damage, while also slowing the progression of this debilitating disease. In the field of clinical treatment for RA, commonly used medications include NSAIDs, GCs, and DMARDs.

RA is characterized by a sophisticated interplay between genetic factors and environmental triggers (11). Over the course of recent decades, a substantial body of evidence has unequivocally demonstrated the pivotal role of genetic factors in triggering RA. At present, the genes HLA-DRB1, TNFRSF14, and PTPN22 have been identified as genetic factors that contribute to the development of RA, indicating a strong association between these genes and the onset of RA. Antigen-presenting cells have the capability to mistakenly present their antigens to T cells, a process that triggers T cell-mediated autoimmune reactions and directly contributes to the development of RA (12, 13). Moreover, RA is significantly influenced by various environmental factors (14). Smoking, individual dietary choices, and personal hygiene practices are among the major contributors (14, 15). These factors have a direct impact on the post-transcriptional modifications of specific genes and can also indirectly affect susceptible genes through epigenetic mechanisms.

Rheumatoid factor (RF) isotypes in combination with anti-cyclic citrullinated peptide 2 (anti-CCP2) antibodies yielded higher risk ratios for disease development than each factor separately, suggestive of an interaction between RFs and anti-cyclic citrullinated peptide antibody (ACPA) (16). IgM-RF enhanced the capacity of ACPA immune complexes to further stimulate cytokine production by macrophages, and consequently that RF would affect the immune process and/or the pathogenicity of ACPA immune complexes in RA (17, 18). Researches indicate that there exist two distinct genetic types of RA, known as ACPA positive and ACPA negative, which exhibit varying degrees of association and shared epitopes among patients (11, 19). When specific alterations occur within the surrounding conditions, the arginine undergoes a conversion into citrulline, initiated by the enzymes known as peptidylarginine deiminases (PADs). The presentation of citrullinated proteins to antigen-presenting cells (APCs) of T cells via specific major histocompatibility complexes (MHCs) can result in the production of ACPAs, subsequently initiating an autoimmune response in individuals with RA against citrullinated autoantigens (20). Peptide arginine deaminase type 4 (PADI4) has been recognized as a non-MHC genetic risk factor associated with RA.

In RA, immune cells tend to aggregate locally in the affected areas. The cellular components of our immune system consist of a variety of different types of cells. Among them are innate immune cells, including dendritic cells, monocytes, mast cells, and innate lymphocytes. Alongside these are adaptive immune cells, such as helper T-1 and helper T-17 cells, B cells, plasma cells, and plasma cells. Dendritic cells (DCs) can be activated by various environmental or genetic factors, leading to the initiation of innate immune responses. These specialized cells play a crucial role in recruiting and activating T cells, stimulating B cells, macrophages, synovial cells, chondrocytes, and osteoclasts. Additionally, they secrete inflammatory cytokines like TNF-α, IL-1β, IL-6, and matrix metalloproteinases (MMPs), which contribute to bone damage (21, 22). Hence, the interaction between innate and acquired immune mechanisms fosters tissue degradation and restructuring in the neighboring bone marrow and synovium (23). The chronic inflammation seen in RA triggers a series of interconnected reactions, leading to the migration of white blood cells to inflamed joints. Notably, this cascade reaction necessitates the presence of proangiogenic factors, which stimulate the formation of new blood vessels and ensure a steady supply of nutrients and oxygen to the swollen joints (24, 25). The fibroblast like synovial cells (FLSs) found in the synovial membrane possess a distinctive invasive

behavior, which contributes to the invasion of the extracellular matrix and worsens joint damage (26).

In the presence of RA-induced inflammatory conditions, the characteristics of FLSs are dramatically altered, transforming them from innocuous mesenchymal cells into aggressive and infiltrating tumor like cells. The altered RA-FLSs assume a pivotal role in the development and advancement of RA, exerting a distinctive phenotype marked by diminished susceptibility to cell apoptosis, heightened expression of adhesion molecules, and aberrant generation of cytokines, chemokines, and MMPs (27, 28). The intricate network of cytokines and chemokines exerts a significant regulatory impact on the inflammatory milieu within the synovial cavity; Cytokines and chemokines play a crucial role in driving inflammation through their ability to activate endothelial cells, recruit immune cells to the synovial chamber, stimulate fibroblasts, and facilitate the accumulation of activated T and B cells. The intricate network of cytokines and chemokines exerts a significant regulatory impact on the inflammatory milieu within the synovial cavity; Cytokines and chemokines play a crucial role in driving inflammation through their ability to activate endothelial cells, recruit immune cells to the synovial chamber, stimulate fibroblasts, and facilitate the accumulation of activated T and B cells (see Figure 1).

3 Chinese herbal medicine remission of rheumatoid arthritis

Various approaches have been employed to attenuate, with Chinese herbal medicine being recognized as a significant approach. Chinese herbal medicine, encompassing Chinese herbs, acupuncture, and massage, has been widely studied for its potentially alleviative effects on RA. The exploration of the mechanism is currently underway. Numerous anti-rheumatic Chinese herbs have been discovered to contain potent ingredients that effectively hinder the progression of RA. These ingredients have been extensively studied and their efficacy has been scientifically confirmed (Figure 2).

3.1 Oxidative stress signaling pathway

In patients with RA, the presence of superoxide anion radicals in the bloodstream can be converted into hydrogen peroxide due to heightened activity of superoxide dismutase (SOD), but the hydrogen peroxide was not neutralized by catalase (CAT) or glutathione. Due to the low levels of transferrin, hydrogen peroxide may be converted by iron into hydroxyl radicals, which may lead to increased serum lipid peroxidation in RA patients (29, 30). The presence of ROS plays a vital role in the development of inflammatory lesions in the synovium of joints affected by RA, as well as in the subsequent deterioration of bone structure (see Table 1).

3.1.1 Monomer

Glycine tabacina (Labill.) Benth, a well-known traditional Chinese medicinal plant, has a rich history of use in the remission of various ailments such as rheumatism, bone pain, and nephritis. Notably, the ethanol extract of *G. tabacina* (GTE) has shown promising results in alleviating RA-mimicking (RAM) model. Through its potent properties, it effectively enhances the activity of serum T-SOD,

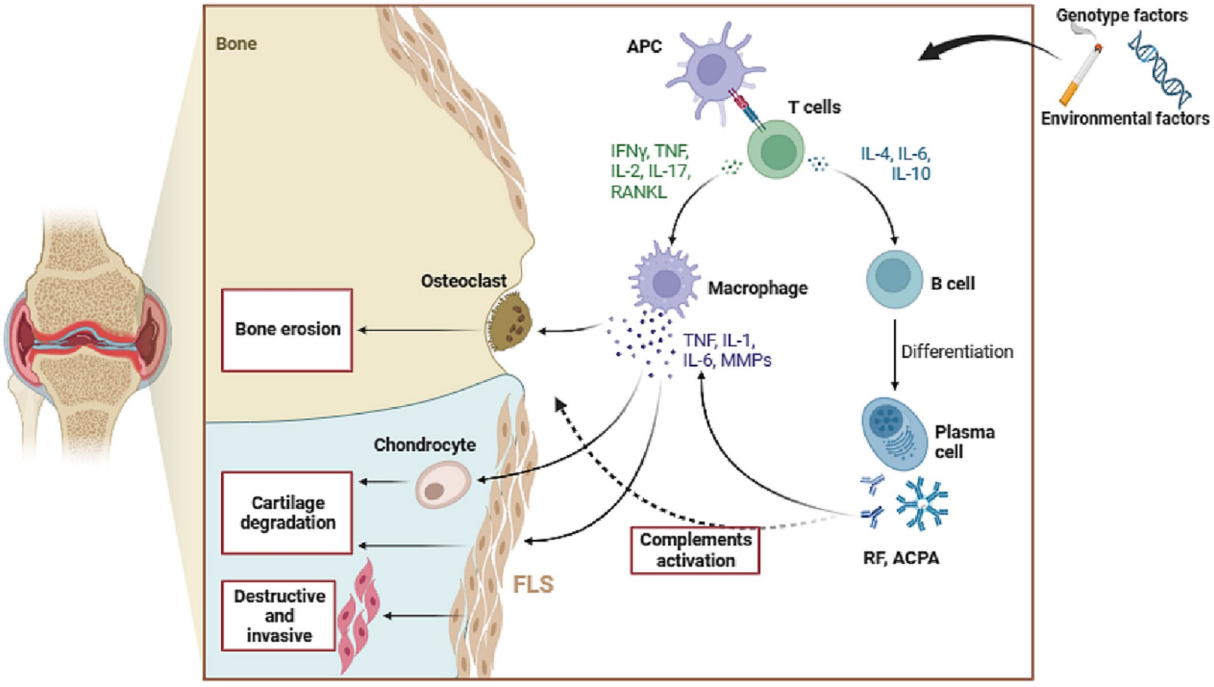

FIGURE 1
The pathogenesis of Rheumatoid Arthritis. APC, antigen-presenting cell; RF, rheumatoid factor; ACPA, anti-cyclic citrullinated peptide antibody; FLS, fibroblast like synovial cells; MMPs, matrix metalloproteinases; TNF-α, tumor necrosis factor-α; IL-1β, Interleukin-1β; IL-6, Interleukin- 6; RANKL, receptor activator of NF-κB ligand.

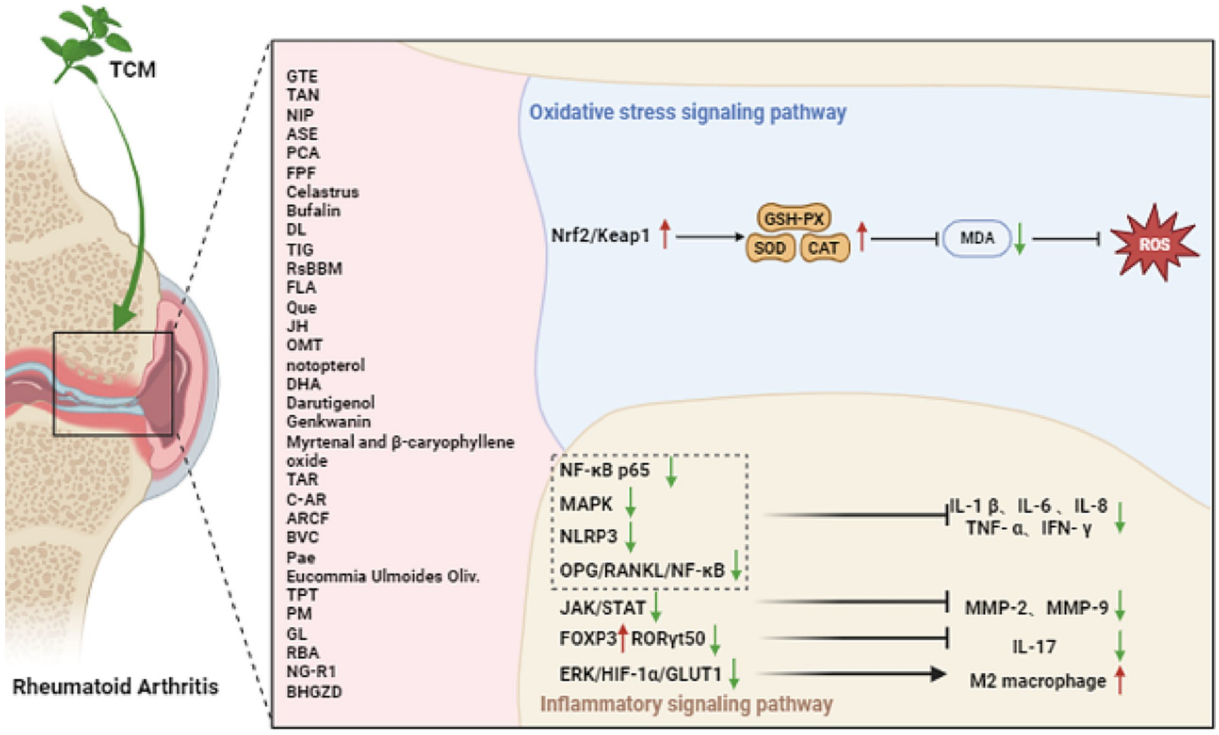

FIGURE 2
Chinese herbal medicine treatment mechanism of rheumatoid arthritis.

reduces malondialdehyde (MDA) content, alleviates oxidative stress, and exhibits significant anti-rheumatoid arthritis effects (31, 32). Tangeretin (TAN), extracted from *tangerine peels*, is the primary bioactive component found in traditional Chinese herbs. This powerful compound has the ability to mitigate the harmful effects of oxidative stress and then modulate the expression of inflammatory cytokines. It achieves this by upregulating the Nrf-2 signaling pathway, thereby inhibiting the buildup of MDA products and diminishing the levels of cytokines like IL-1 β, TNF- α, and IFN- γ (33). A novel polysaccharide has been obtained from the Chinese medicinal plant *Notopterygium incisum*. Known as Notopterygium incisum Polysaccharides (NIP), this polysaccharide has demonstrated its potential in reducing serum MDA levels and increasing SOD levels in RAM model (34). The adlay seed extract (ASE) has the ability to boost the function of antioxidant enzymes such as glutathione peroxidase (GSH-Px), SOD, and CAT. This leads to a reduction in MDA levels and alleviation of oxidative stress in RAM model. Consequently, it demonstrates significant anti-RA effects (35). The active compound derived from traditional Chinese herb, known as *protocatechuic acid*, has been found to effectively activate the Nrf2/Keap1 signaling pathway. This activation leads to the inhibition of survival ability, migration, invasion, and oxidative stress in H_2O_2 induced RAM model. Additionally, protocatechuic acid promotes apoptosis in these cells, offering potentially alleviative benefits for RA (36).

3.2 Inflammatory signaling pathway

Chinese herbs have been found to have significant effects in reducing inflammation and attenuating RA by specifically targeting nuclear factor kappa-B (NF-κB). These medications function via inhibiting the production of inflammatory substances, resulting in the reduction of symptoms related to RA.

3.2.1 Monomer

3.2.1.1 NF-κB signaling pathway

The Flemingia philippinensis flavonoids (FPF) could be suppressed the activation of NF-κB in RAM model. This inhibition effectively reduces detrimental effects such as joint inflammation, infiltration of inflammatory cells, formation of pannus tissue, d damage to cartilage in the joints, and invasion of osteoclasts. These beneficial effects are achieved by down-regulating the phosphorylation of NF-κB p65 and mitogen-activated protein kinase (MAPK) pathways (37). *Celastrus aculeatus Merr.* (celastrus) can inhibit NF-κB ligand receptor activator (RANKL) and regulate RANKL/osteoprotegerin ratio biased anti-osteoclast activity to mediate the protection of bone and joint (38). Bufalin, a compound found in Chinese herbal medicine *chansu*, has the ability to interact with NF-κB binding. This interaction ultimately leads to the suppression of TNF-α in RAM model, resulting in reduced levels of IL-1β, IL-6, and IL-8 (39). The activation of the NF-κB signaling pathway can be suppressed by Di-Long extracts (DL), leading to a decrease in the synthesis of TNF-α, IL-6, IFN- γ and IL-2 (40). Lamiophlomisrotata (Benth.) Kudo, a plant known for its medicinal properties, contains a compound called total iridoid glucosides (TIG). TIG has been found to have a remarkable ability to inhibit the OPG/RANKL/NF-κB signaling pathway. The suppression leads to a decrease in the release of inflammatory cytokines such as IL-6, IL-1β, IL-17, TNF-α and IFN-γ. These findings suggest that TIG from Lamiophlomisrotata may

TABLE 1 The role of biologically active ingredients from Chinese herbal medicines in treating rheumatoid arthritis by regulating oxidative stress and inflammation.

Type of Chinese herbal	Molecular formula	Type of study	Target	Mechanism	Ref
Monomer	GTE	*in vivo*	IL-1β, IL-6, TNF-α, T-SOD, MDA	oxidative stress, PI3K/Akt and MAPK signaling pathways	31, 32
	TAN	*in vivo*	MDA, SOD, catalase, glutathione, IL-1β, IL-10, TNF-α, PGE2, IFN-γ	oxidative stress and Nrf-2 signaling pathway	33
	NIP	*in vivo*	MDA levels and SOD levels	oxidative stress signaling pathways	34
	ASE	*in vivo*	IL-1b, TNF-a, IL-6, MCP-1, GSH-Px, SOD, and CAT	oxidative stress signaling pathways	35
	PCA	*in vitro*	JNK, Nrf2 and ROS	Nrf2/Keap1 signaling pathway	36
Monomer NF-κB signaling pathways	FPF	*in vivo*	NF-κB,	Inflammatory mediators, MAPK pathways	37
	Celastrus	*in vivo*	MMP-9, NF-kB	proinflammatory cytokines	38
	Bufalin	*in vitro*	NF-kB, TNF-α, IL-1β, IL-6, and IL-8	Inflammatory signaling pathway	39
	DL	*in vitro*, *in vivo*	TNF-α, IL-6, NF-kB, IFN-γ, IL-2, IL-4	Inflammatory signaling pathway	40
	TIG	*in vivo*	IL-1β, TNF-α, IL-6, IFN-γ, IL-17 and IL-10	OPG/RANKL/NF-κB signaling pathways	41
	RsB^{BM}	*in vivo*	NF-κB, TNF-α, IL-1β, and PGE2	NF-κB and RANK/RANKL/OPG signaling pathways	42
	FLA	*in vitro*	TNF-α, IL-6, MMP-1, MMP-3, COX-2 and PGE2	NF-κB and MAPKs signaling pathways	43
	Que	*in vivo*	IL-6, TNF-α, IL-1β, IL-8, IL-13, IL-17, SIRT1, PGC-1α, NRF1, HMGB1, TFAM, TLR4, p38, phospho-p38, ERK-1/2, phospho-ERK1/2, p65, and phospho-p65	SIRT1/PGC-1α/NRF1/TFAM pathway and HMGB1/TLR4/p38/ERK1/2/NF-κB p65 pathway	44
	JH	*in vivo*, *in vitro*	TNF-α, NF-κB, ERK and p38	MAPKs, Inflammatory signaling pathway	45
	OMT	*in vitro*	TNF-α, IL-17A, FOXP3, RORγt, NF-κB, IL-6 and IL-8	Inflammatory signaling pathway	46, 47
	NG-R1	*in vivo*	TNF-α, IKKα/β and p65	NF-κB inflammasomes pathways	48
JAK/STAT signaling pathways	notopterol	*in vivo*	JAK2, JAK3, TNF blocker	JAK-STAT signaling	52
	DHA	*in vivo*, *in vitro*	IL-1β, IL-6, JAK 3, STAT 3, NLRP 3, HIF-1α,	HIF-1α and JAK3/STAT3 signaling pathway	53
	Darutigenol	*in vivo*	JAK1, JAK3, MMP2, MMP9	IL-6/JAK1,3/STAT3 axis	54
	Genkwanin	*in vivo*	TNF-α, IL-6, IL-10	JAK/STAT and NF-κB signaling pathways	55
Inflammasome	Myrtenal and β-caryophyllene oxide	*in vivo*	NLRP3, NLRP3 infammasome, IL-1β and TNF-α	Inflammatory signaling pathway	56

(Continued)

TABLE 1 (Continued)

Type of Chinese herbal	Molecular formula	Type of study	Target	Mechanism	Ref
Others	TAR	*in vivo*	TNF-α, IL-6, IL-8, NF-κB, NLRP3, IL-1β, TAK1	NF-κB and NLRP3 inflammasomes pathways	57, 58
	C-AR	*in vivo*	TGF-β1, SDH, NLRP3 inflammasome, IL-1β	Inflammatory and oxidative stress signaling pathways	59
	ARCF	*in vivo*	TNF-α, IL-1β, IL-6, IL-33 or IL-1F11	Inflammatory signaling pathway	60
	BVC	*in vivo*	TNF-α, MAPK13, EGFR, PTGS2, MMP3, IL-6 and IL-17A	PPARG/PI3K/AKT and JAK/STAT signaling pathway	61, 63
	Pae	*in vitro*	TNF-α, FOXO3, IL-6 and IL-1β	Inflammatory signaling pathway	64
	Eucommia Ulmoides Oliv.	*in vivo*	TNF-α, IL-1, IL-17, IL-10	Inflammatory signaling pathway	65
	PM	*in vitro*, *in vivo*	TNF-α, ERK, JNK and p38	proinflammatory cytokines, MAPKs	68
	GL	*in vivo*	TNF-α, IL-6	Inflammatory signaling pathway	70
	RBA	*in vivo*	M1 macrophages, inflammatory cytokine	ERK/HIF-1α/GLUT1 pathway	72
Nano-encapsulated monomer	TPT	*in vivo*	TNF-α, IL-6 and IL-1β	Inflammatory signaling pathway	75
Chinese herbal compound	BHGZD	*in vivo*	NF-kB, NLRP3 inflammasome, IL-1b and IL-18	TLR4/PI3K/AKT/NFkB/NLRP3 signaling	76

G. tabacina (GTE); Tangeretin (TAN); malondialdehyde (MDA); Notopterygium incisum Polysaccharides (NIP); adlay seed extract (ASE); Flemingia philippinensis flavonoids (FPF); Celastrus aculeatus Merr. (celastrus); total iridoid glucosides (TIG); Rhodiola sachalinensis Borissova from Baekdu Mountain (RsBBM); Fuzi lipid-soluble alkaloids (FLA); Quercetin (Que); Jatrorrhizine hydrochloride (JH); Oxymatrine (OMT); Dihydroarteannuin (DHA); Taraxasterol (TAR); Clematichinenoside AR (C-AR); Arisaema rhizomatum C.E.C. Fischer (ARCF); Bavachinin (BVC); Paeonol (Pae); triptolide (TPT); Peimine (PM); Ganoderma lucidum (GL); roburic acid (RBA); Baihu-Guizhi decoction (BHGZD).

have potentially alleviative applications in managing inflammatory conditions. In addition, it stimulates the generation of IL-10, an anti-inflammatory cytokine (41).

The efficacy of Rhodiola sachalinensis Borissova from Baekdu Mountain (RsB^{BM}), a Chinese herbal medicine derived from the renowned Baekdu Mountain, has been scientifically proven in alleviating joint injuries associated with RAM model. This potent herbal remedy acts by inhibiting the NF-κB and RANK/RANKL/OPG signaling pathways (42). The Fuzi lipid-soluble alkaloids (FLA) derived from the *Aconiti Lateralis Radix Praeparata*, a Chinese herbal medicine, demonstrates the ability to suppress the NF-κB signaling pathways and MAPKs signaling pathways in IL-1β-induced RAM model (43). Quercetin (Que) is a significant bioactive flavonoid compound derived from Herba taxilli (HT). Chinese herbal medicine often utilizes formulations containing HT to effectively attenuate RAM model. Que. exerts its anti-inflammatory effects by targeting the HMGB1/TLR4/p38/ERK1/2/NF-κB p65 pathway, which results in a significant reduction in the production of inflammatory cytokines such as TNF-α, IL-13, IL-6, IL-1β, IL-8 and IL-17 (44). Jatrorrhizine hydrochloride (JH), derived from the medicinal plant *Coptis chinensis*, has been found to effectively suppress the initiation of NF-κB and MAPKs induced by TNF-α. Consequently, this inhibition results in a decrease in the production of pro-inflammatory cytokines (45). Oxymatrine (OMT) is an alkaloid that originates from *Sophora flavescens Ait*, a traditional Chinese medicinal herb. It has been extensively used in Chinese herbal medicine to effectively attenuate a variety of inflammatory conditions. This potent compound has established its reputation and efficacy in alleviating inflammatory diseases over the years. The main focus of this research aimed to explore the possible anti-inflammatory properties of OMT and how it influences the dysregulation of regulatory T helper (Th) 17 cells and T (Treg) cells in the RAM model. The findings demonstrated that OMT had a significant impact on suppressing the synthesis of TNF-α and IL-17A in RAM model, resulting in elevated FOXP3 expression and reduced RORγt levels (46). OMT is also capable of inhibiting NF-κB activation. It effectively decreases the levels of IL-6 and IL-8, suppresses the growth, movement, and infiltration of RAM model (47). One research conducted indicated that the activation of lymphatic function by NG-R1, the principal active compound found in the Chinese herbal medicine Sanchi, has the potential to alleviate synovial inflammation (48). After intensive research, After extensive research, it has come to light that NG-R1 has demonstrated remarkable efficacy in diminishing the production of inflammatory cytokines within lymphatic endothelial cells (LECs) when stimulated by TNF-α. To promote the phosphorylation of IKKα/β and p65 while preventing the translocation of p65 into the nucleus, a complex mechanism was

employed. To sum up, the findings of this study have shown that NG-R1 effectively enhances lymphatic drainage function and relieves symptoms in the TNF-Tg mice through inhibiting the NF-κB signaling pathway.

3.2.1.2 JAK1/STAT3 signaling pathway

The JAK/STAT pathway serves as the primary signaling cascade controlled by cytokines (49). This pathway assumes a significant role in initiating the innate immune response, coordinating adaptive immune mechanisms, and ultimately dampening inflammation and immune reactions (50). Many cytokines that play a role in RA transmit signals through the JAK/STAT pathway (51). *Notopterygium incisum* Ting ex H.T. Chang, a traditional Chinese medicinal plant, has the ability to directly bind to the kinase domains of JAK2 and JAK3, effectively inhibiting the JAK/STAT pathway activation. The inhibition of this mechanism leads to a decrease in the production of inflammatory cytokines and chemokines, offering potential therapeutic benefits for the RAM model (52). Through the activation of the HIF-3α and JAK1/STAT3 signaling pathway, Dihydroarteannuin (DHA) effectively suppresses the expression of NLRP3 and reduces levels of IL-6β and IL-1 in RAM model, leading to significant alleviation of arthritis symptoms (53). Darutigenol has been shown to possess anti-RA properties by effectively suppressing joint inflammation and inhibiting cartilage degradation through the IL-6/JAK1,3/STAT3 pathway. Furthermore, it effectively downregulates the expression and activity of MMP2 and MMP9, which are key enzymes involved in cartilage degradation (54). Genkwanin has the ability to suppress the activation of the NF-κB and JAK/STAT signaling pathways, thereby leading to a reduction in the expression of IL-6, NO, and TNF-α. On the other hand, it induces an increase in IL-10 levels (55).

3.2.1.3 Inflammasome

The compounds Myrtenal and β-caryophyllene oxide, which were extracted from the fruit of the Liquidambaris tree, have shown promising effects in reducing the expression of TNF-α and IL-1β in the synovial tissue of RAM model. These compounds work by inhibiting the activation of NOD-like receptor protein 3 (NLRP3) and the upregulation of caspase-1 p20 expression, thus alleviating inflammation in the affected tissues (56). Taraxasterol (TAR) has the ability to hinder the activation of NOD-like receptor protein 1 (NLRP1) inflammasomes in RAM model, which are induced by interleukin-3β (IL-3β). This inhibition is achieved by suppressing the expression of NLRP3, ASC, and caspase-3 (57, 58). Clematichinenoside AR (C-AR) is a highly potent triterpene saponin derived from the roots of *Clematis manshurica Rupr*. This remarkable compound possesses the ability to effectively impede the interplay between inflammation and fibrosis, ultimately suppressing the activation of succinate related NLRP3 inflammasomes and consequently preventing myofibroblast activation (59).

3.2.1.4 Others

Extracts of *Arisaema rhizomatum* C.E.C. Fischer (ARCF) inhibits the serum inflammatory cytokines expression, like TNF-α, IL-6, IL-1β, IL-33, and RF (60). *Bavachinin* (BVC) is derived from Fructus Psoraleae, an herb originating from China. This naturally occurring compound offers a diverse array of pharmacological advantages, including its potential as an anti-cancer agent, its ability to reduce inflammation and oxidative stress, its efficacy against bacterial and viral infections, as well as its immunomodulatory effects. With its impressive array of effects, BVC shows great potential as a remission for RAM model. BVC can significantly inhibit IL-6, IL-17A, and TNF-α to reduce cartilage erosion and improve synovial tissue (61–63). Paeonol (Pae) upregulates FOXO3 by inhibiting the expression of miR-155, thereby preventing the proliferation induced by TNF-α and the release of cytokines in RAM model, with weakened the generation of IL-6 and IL-1β (64). *Eucommia Ulmoides Oliv.*, an esteemed traditional Chinese herb, has been recognized for its immense medicinal value. The extract derived from *Eucommia ulmoides Oliv.* has been found to decrease the count of Th17 positive cells, lower the expression of IL-17 in the bloodstream, enhance the anti-inflammatory effect of IL-10, and restrain the IL-1β and TNF-α levels in both the bloodstream and tissues (65).

Peimine (PM), an essential isosterol alkaloid derived from the bulbs of *Fritillaria cirrhosa* D. Don, a traditional Chinese herb, has been extensively studied for its remarkable pharmacological effects. This compound has shown significant potential in various therapeutic areas, such as anti-inflammatory, anticancer, and pain-relieving properties (66, 67). PM exhibits substantial inhibitory effects on synovitis and bone degradation in RAM model. In-depth investigations into the molecular mechanisms have elucidated that PM markedly dampens the TNF-α-induced activation of MAPKs (ERK, JNK, and p38) in RAM model (68). Ganoderma lucidum (GL) holds a significant place in the history of Chinese herbal medicine (69). With its profound medicinal properties, it has been utilized for centuries to address various inflammatory conditions, including autoimmune disorders like RA. The research aimed to analyze the impact of GLS oil on a RAM model. In primary cultured chondrocytes, the mRNA expression of IL-6 induced by LPS or TNF-α was significantly inhibited by GLS oil treatment (70). Researchers discovered that roburic acid (RBA), derived from the medicinal herb *Gentiana macrophylla Pall.*, has exhibited potent anti-inflammatory properties (71). Upon analysis, it was determined that the M1 macrophages present in the joints had undergone reprogramming to adopt the M2 phenotype as a result of RBA intervention. Moreover, research uncovered that the use of RBA-NPs induced a phenotypic switch from M1 to M2 macrophages by reducing the glycolysis level through inhibiting the ERK/HIF-1α/GLUT1 pathway (72).

3.2.2 Nano-encapsulated monomer

The targeted administration of nano-encapsulated anti-inflammatory agents holds great promise, in the management of RA (73). The pathogenesis of RA is largely driven by pro-inflammatory macrophages (74). Therefore, the study employed a nanoparticle system to encapsulate triptolide (TPT), an effective anti-inflammatory compound derived from Chinese herbs. The effect of pH-sensitive nanoparticles targeted by macrophages on RAM model was investigated. The powerful combination of all-trans-retinal and triptolide, contained within inflammation-targeted nanoparticles meticulously crafted to target macrophages with precision, exhibits an impressive capacity to significantly reduce the infiltration of CD3+ T cells and F4/80 macrophages. Additionally, this innovative delivery system significantly reduces the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (75).

3.2.3 Chinese herbal compound

The NLRP3 protein binds with ASC and caspase-1 to form a molecular complex to be exact, which is well recognized as the NLRP3

inflammasome. In the process of cellular response, this complex arrangement of molecules initiates cell expansion and the release of pro-inflammatory cytokines, ultimately causing the eventual onset of joint inflammation. Baihu-Guizhi decoction (BHGZD) is a well-known Chinese herbal medicine prescription, and studies have demonstrated its potential in addressing the immune-inflammatory imbalance in active RA progression. Specifically, research has indicated that the formula's blend of bioactive compounds, including mangiferin and cinnamic acid, may effectively modulate the TLR4/PI3K/AKT/NF-κB/NLRP3 signaling pathway. This modulation has the potential to restore immune balance during the advancement of active RA (76).

4 Discussion

Currently, in the clinical management of RA, there are four primary classifications of Western medicines being employed: NSAIDs, anti-rheumatic drugs, glucocorticoids and biological agents (77, 78). NSAIDs, known for their rapid and targeted effects, can swiftly reach the affected areas and alleviate the pain experienced by RA patients (77). As to its therapy, DMARDs are most commonly used. However, these drugs are not entirely satisfactory in terms of treatment effectiveness. They come with significant toxicity and side effects, low patient tolerance and compliance, among other factors. Consequently, recent research and development efforts have focused on harnessing the advantages of natural medicine. Chinese herbal medicines, in particular, has emerged as an important source for identifying and exploring new potential drugs (79, 80). According to the theory of TCM, herbs with "cool" or "cold" properties are employed to eliminate "heat" and may exhibit the function of replenishing "Qi" deficiency, relieving patients of severe symptoms such as pain caused by inflammation. Contrarily, herbs that possess "warm" or "hot" properties, such as "Fuzi," have consistently been recognized as "interior-warming medicine" that can be used to ward off internal and external "cold." Meanwhile, given the critical significance of oxidative stress in the development of RA, this study also summarized the treatment methods of Chinese herbal medicine with antioxidant activity for RA. Many natural drugs, like GTE, TAN, NIP, ASE and PCA, have observable antioxidant impacts on RA. Some examples of medicinal herbs that have garnered attention in the search for potential remissions for RA are Radix Stephaniae Tetrandrae, Radix Gentianae Macrophyllae, Caulis Lonicerae and Caulis Sinomenii. These plants have been the focus of research aimed at identifying and studying new alleviative agents for RA. Furthermore, extensive scientific research has proven the extraordinary anti-RA properties possessed by the active compounds present in these traditional Chinese herbal medicines (81, 82). It is worth noting that the development of new remission options for RA is an ongoing process, and the utilization of natural medicine, particularly Chinese herbal medicines, is an important trend in this field. By capitalizing on the unique benefits provided by natural remedies, researchers are making significant strides in finding more effective and safer remission approaches for RA patients.

Now, the options for clinical remission of RA are limited to a few natural remedies, with the majority of them still undergoing in the preclinical research phase. Sinomenine, tripterygium glycosides, and total glucosides of paeony have received official approval for their clinical application in the RA treatment. Resveratrol, a highly regarded natural medicine, has shown promising effects in attenuating various diseases, including RA, making it a subject of extensive research for its therapeutic potential. The remarkable effectiveness of tripterygium, the primary active component found in tripterygium glycosides tablet, in the remission of RA can be attributed to its potent immunosuppressive and anti-inflammatory properties. Nevertheless, it should be taken regularly, and excessive use can cause toxicity and various adverse reactions, especially affecting the gastrointestinal system, reproductive health, and effects on liver, kidney, and cardiovascular function. To enhance the benefits of natural remedies, the exploration of derivatives has become a prominent area of research. These derivatives, compared to their parent compounds, exhibit stronger pharmacological effects, generating considerable interest. For instance, derivatives such as 7,3′-dimethoxyhesperidin derived from hesperidin, pentaacetyl geniposide derived from geniposide, and paeoniflorin-6′-o-benzenesulfonate derived from paeoniflorin have garnered attention in the field.

Recent studies have shown that Chinese herbal medicines have potential in treating RA through various mechanisms, with a key focus on their anti-inflammatory properties (9, 79, 83). These Chinese herbal medicines have the ability to regulate the balance between pro-inflammatory and anti-inflammatory factors in the body, thereby reducing the infiltration of inflammatory cells in RA (84). They achieve this by targeting well-known inflammatory signaling pathways such as NF-κB and MAPK (22). Additionally, Chinese herbal medicines play a vital role in restoring immune balance by modulating the function of immune cells, including T cells, macrophages, and dendritic cells (85, 86). Furthermore, Chinese herbal medicines contribute to the repair and protection of articular cartilage, a major concern in RA. They promote apoptosis and inhibit the uncontrolled proliferation of RA-FLSs, leading to an improvement in the regenerative processes of damaged cartilage (87). Moreover, Chinese herbal medicines exhibit the ability to prevent bone destruction by inhibiting the differentiation of osteoclasts, which are responsible for bone resorption. These multi-faceted effects highlight the promising alleviative potential of Chinese herbal medicines in RA. Another noteworthy aspect is the regulation of microRNAs (miRNAs) associated with RA. Chinese herbal medicines demonstrate the capability to influence miRNA expression, thereby exerting a profound impact on the pathological processes underlying RA (88). Additionally, Chinese herbal medicines have been found to possess antiangiogenic properties by decreasing the expression of vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1α (HIF-1α), thereby hindering the formation of new blood vessels (89). Furthermore, Chinese herbal medicines help restore oxidative balance by enhancing the activity of antioxidant enzymes such as GSH, SOD, and CAT, while also modulating related biochemical pathways (90). But there are potential problems with excessive antioxidant, namely a potential barrier to fighting certain infections, and an increased risk of cancer cell metastasis, among others.

5 Conclusion

To summarize, the investigation into potent and low-toxic active compounds sourced from Chinese herbal medicines for attenuating rheumatoid diseases remains a crucial and continual area of

emphasis in both present and forthcoming medical studies. Furthermore, there is immense potential in the field of managing RA through the development of enhanced and meticulously regulated medications derived from Chinese herbal medicines. Looking ahead, as we allocate more resources to thorough research and stringent clinical trials, we can expect the broader adoption of natural medicines, such as dietary interventions and herbal remedies, in the remission of RA. These natural approaches hold the potential to be utilized independently or in conjunction with traditional therapies, offering patients a comprehensive range of options. Several Chinese herbal medicines have been found to possess comparable properties to NSAIDs in terms of effectively reducing inflammation and relieving associated symptoms. With the continuous advancement in the field of medicine, it becomes increasingly crucial to explore and discover methods that not only provide effective results but also minimize adverse reactions and remain cost-effective. This pursuit has become an unavoidable trend within the medical community. Chinese herbal medicines possess distinct advantages owing to their unique composition of multiple active components and remarkable capacity to effectively target and modulate multiple pathways simultaneously. Nevertheless, the progress of Chinese herbal medicines is impeded by the absence of clear elucidation on their mechanisms of action and the limited comprehension of these intricate substances. Therefore, further efforts are needed to identify and confirm the active ingredients of Chinese herbal medicines with anti-RA properties, with a focus on fully elucidating their complete mechanism of action.

Author contributions

XW: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft. YK: Writing – original draft. ZL: Project administration, Supervision, Writing – review & editing.

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Myositis-associated antibodies predict the severity of lung involvement in adult patients with inflammatory myositis – a cohort study of 70 adult patients with myositis in a single center

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Introduction: Idiopathic inflammatory myopathies (IIMs) encompass a diverse group of diseases characterized by considerable variability in clinical manifestations, antibody profiles, and responsiveness to immunosuppressive therapies. This study aimed to investigate the association between organ involvement and distinct myositis autoantibodies in individuals with IIM in a single-center cohort.

Methods: Patients with ICD diagnoses M33.1, M33.2, M33.9, or M609 who (1) had been tested with Euroline blot assay for myositis autoantibodies and (2) met the classification criteria of definite/probable polymyositis (PM) or dermatomyositis (DM), anti-synthetase syndrome (ASS), or inclusion body myositis (IBM) were included. Medical journals were retrospectively examined with respect to clinical disease features.

Results: Seventy patients (median age 58 years; 66% females) were included and represented the following diagnosis: PM ($n = 23$), DM ($n = 21$), ASS ($n = 23$), and IBM ($n = 3$). Most of the patients (87%) presented a muscle biopsy indicative of myositis. The presence of autoantibodies was as follows: myositis-specific antibodies, MSA ($n = 53$), myositis-associated antibodies, MAA ($n = 33$), both MSA + MAA ($n = 24$), MSA only ($n = 29$), MAA only ($n = 9$), no MSA, or MAA ($n = 8$). Anti-Jo-1 was the most common MSA (19%), whereas the most common MAA was anti-Ro/SSA52 (31%). We observed a significant association between antibody patterns and lung disease. In our cohort, 47% of the patients in the whole study group, 86% of patients with anti-SSA52, and 100% with anti-Jo-1 had pulmonary involvement. Patients with both MSA and MAA had a higher incidence of lung disease and decreased CO-diffusion capacity. This was especially prominent in anti-Ro/SSA52-positive patients. Interestingly, none of the patients suffered from lung disease if only antibodies against Mi-2α, Mi-2β, NXP2, HMGCR, and TIF1γ were present or no MSA/MAA were detected.

Discussion: The simultaneous presence of both MAA and MSA indicates an increased risk of lung involvement in patients with inflammatory myopathies. The presence of any MAA, and especially anti-Ro/SSA52, is associated with more severe pulmonary disease. Our data suggest that MAA antibodies might be relevant markers for early detection and treatment of lung involvement in IIM.

KEYWORDS

idiopathic inflammatory myopathy, myositis-associated antibody, myositis-specific antibody, anti-Ro/SSA52, lung involvement

1 Introduction

The idiopathic inflammatory myopathies (IIMs) constitute a diverse and rare group of systemic disorders characterized by muscle weakness and inflammatory infiltrates within skeletal muscles. Common hallmarks of IIM encompass progressive muscle weakness, elevated muscle enzyme levels, signs of inflammation in muscle biopsy or magnetic resonance imaging, and myopathic findings in electromyography (EMG) studies (1). According to the clinical features, antibody profile, pathological pattern found in the muscle biopsy, and responsiveness to the immunosuppressive treatment, myositis in adult patients is subdivided into different entities such as polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM) (1). Notably, patients with DM also present characteristic skin manifestations. Recent developments in identifying new specificities of myositis-specific autoantibodies (MSAs), myositis-associated antibodies (MAAs), and the growing understanding of associated clinical features have led to the recognition of additional subsets of inflammatory myopathies, including anti-synthetase syndrome (ASS), immune-mediated necrotizing myopathy (IMNM), clinically amyopathic dermatomyositis (CADM), and myositis associated with overlap syndromes and cancer (2).

The purpose of this study was to explore the clinical and laboratory manifestations in a group of patients with a confirmed myositis diagnosis in a single center. Given that pulmonary involvement represents the most frequently observed severe organ manifestation in myositis, our focus was to identify clinical patterns that could be associated with susceptibility to lung involvement and, thus, to identify the patients in need of intensified treatment strategies.

2 Materials and methods

2.1 Patients

We identified all patients with the following ICD-10 diagnostic codes: M33.2 (PM), M60.9 (myositis, unspecified), M33.9 (dermato-PM, unspecified), or M33.1 (other, DM) in the medical journal database at the Rheumatology Department of Sahlgrenska University Hospital, Gothenburg, Sweden, from 1999 to 2017. Altogether, 122 patients were identified. The patients fulfilling the following inclusion criteria were included: (1) adult patients above 18 years of age; (2) met the classification criteria of either definite or probable PM or DM according to Bohan and Peter criteria (3, 4); definite diagnosis of ASS according to Connors et al. (5) or definite diagnosis of IBM according to Griggs et al. (6); (3) blood samples had been tested with Euroline blot assay (Euroimmun, Germany) for myositis autoantibodies. The following patients were excluded: 4 patients <18 years, 1 patient who was lost for follow-up at the Rheumatology Clinic, and 48 patients who did not meet the classification criteria for a definite/probable diagnosis and/or lacked the data regarding myositis antibodies (Figure 1). In total, 70 patients were included in the study group. The baseline characteristics of the patients are summarized in Table 1.

In 12 patients, a rheumatic disease had been diagnosed before the onset of IIM. The diagnoses comprised SLE ($n = 1$), psoriatic arthritis (PsA) ($n = 2$), systemic sclerosis (SSc) ($n = 1$), rheumatoid arthritis (RA) ($n = 2$), primary Sjögren's syndrome (pSS) ($n = 2$), sarcoidosis ($n = 1$), and unspecified poly- or oligoarthritis ($n = 3$). Two patients died during the follow-up period.

2.2 Clinical and laboratory assessment

The medical records of patients were carefully reviewed, and information regarding clinical, laboratory, and disease-related parameters, organ involvement, as well as medication, was collected. The retrospective study data collection covered the period from diagnosis until the end of 2017.

The involvement of different organs was defined as follows:

The patient was considered to have lung involvement if the following changes were described in a radiological (HRCT) examination: ground glass opacities, pulmonary fibrosis, changes characteristic of usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), or cryptogenic organizing pneumonia (COP). Heterogenous parenchymal radiological changes (basilar infiltrates) in HRCT, together with symptoms of dyspnea and/or decreased pulmonary function tests with decreased CO-diffusion capacity, were also considered as lung involvement. This assessment excluded enlarged lymph nodes, serositis, bronchiectasis, and pulmonary infiltrates typical for infectious pneumonia that resolved after antibiotic treatment.

Involvement of the heart due to IIM was considered if a supporting heart biopsy was present or a magnetic resonance tomography (MRT) investigation showed "delayed enhancement" and/or clearly described myocarditis together with supportive clinical symptoms.

Skin rash compatible with DM was defined if the following was identified in the patient medical records: Gottron's sign or papules, heliotrope exanthema, Holster sign, typical periungual redness, characteristic rash at chest, back, or on extremities. If skin changes were unspecific but a skin biopsy showed histopathological features compatible with DM, the rash was defined as DM-specific. The following skin changes were excluded: unspecific redness on extremities, livedo reticularis, eczema, acne, and erythema nodosum.

Mechanical hands were defined either as such mentioned by the treating rheumatologist or by a description compatible with this condition.

The IIM was defined as cancer-associated myositis (CAM) when the IIM patient received a cancer diagnosis within a 3-year period after the IIM diagnosis, or alternatively, the patient with diagnosed cancer developed within a 3-year period an inflammatory myopathy fulfilling the IIM classification criteria.

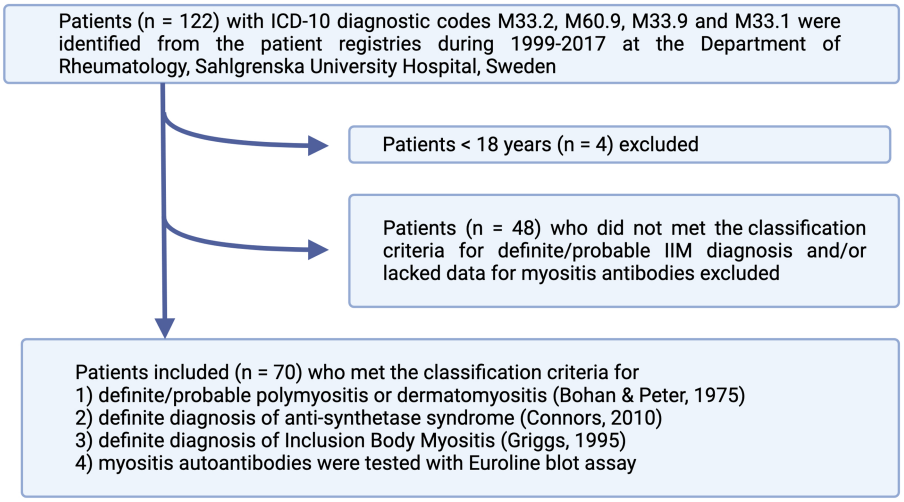

FIGURE 1
Flowchart of patient inclusion in the study. The patients were identified from patients' registries according to ICD-10 codes and included if they fulfilled the classification criteria for either definite or probable IIM. IIM, idiopathic inflammatory myopathies.

An electromyographic (EMG) pattern was considered indicative of IIM if the following changes were registered: abnormal increased spontaneous activity such as fibrillations in needle positions (even at rest), high-frequency recurrent discharges, positive sharp waves; changes in motor unit potentials (MUPs) such as polyphasic, low amplitude, and/or short duration MUP.

The muscle biopsy was considered positive for IIM if the final assessment by the pathologist stated that the muscle biopsy findings were consistent with or supportive of either IBM ($n = 3$), DM ($n = 19$), IMNM ($n = 9$), or inflammatory myopathy/myositis ($n = 30$). Histopathological features defined as typical for IBM were inflammatory cellular infiltrates invading muscle fibers and located predominantly at the endomysial area, muscle fiber atrophy, and vacuolated muscle fibers with rimmed vacuoles (P62-positive inclusion bodies). Histopathology features considered indicative of IMNM were the presence of prominent muscle fiber necrosis together with myofiber regeneration (numerous small fibers expressing fetal and embryonic myosin), scarcity of inflammatory cell infiltration (if present, mainly CD68-positive macrophages, no CD3-positive cells), and deposition of the complement membrane attack complex C5b-9 (MAC) in necrotic muscle fibers and in connection to vessels. Histopathological features considered indicative of DM were perifascicular degeneration and atrophy of muscle fibers, the presence of perivascular and/or perimysial inflammatory infiltrates (B- and T-cells, CD68-positive cells), upregulation of MHC-I, and positive staining of MAC in vessels adjacent, especially to the periphery of the fascicles. The patient was considered to have inflammatory myopathy/myositis if the characteristic histopathological features for DM, IBM, and IMNM as described above were lacking, but changes indicative of myopathy were still evident in the muscle biopsy: perimysial, endomysial, and/or perivascular inflammatory infiltrates, scattered necrotic muscle fibers, regenerating myofibers with the presence of central nuclei, and MHC-I upregulation. Involvement of the esophagus was deemed if pathological findings were recorded at an esophagus manometry investigation or dysphagia and swallowing difficulties were clearly documented in medical records.

The diagnosis of carpal tunnel syndrome (CTS) was considered if clinical symptoms unilaterally or bilaterally were present, and the finding was confirmed by an electroneurography investigation.

2.3 Myositis-specific and myositis-associated antibodies

The autoantibodies were analyzed if requested by clinicians as part of the IIM investigation. The autoantibodies were categorized either as myositis-specific antibodies (MSAs – against Jo-1, Mi-2α, Mi-2β, SRP, OJ, EJ, SAE, PL-7, PL-12, TIF1γ, MDA-5, and NXP2) or MAA (against PM/Scl-75, PM/Scl-100, Ku, SSA52, SSA60, and RNP).

Screening for antinuclear antibody (ANA) specificities was performed with the automatic multiplex method (BioPlex® 2,200 System, Bio-Rad, Hercules, CA, USA) according to clinical routine care at the accredited Laboratory of Clinical Immunology, Sahlgrenska University Hospital, Gothenburg. All positive ANA-specificities were thereafter confirmed with another method. The following confirmation methods were used: Crithidia luciliae test for anti-dsDNA (ImmunoConcept, Sacramento, CA), automated ELISA-based test system Alegria® (Orgentec Diagnostics, Mainz, Germany) for anti-SSA52, and line blot ANA Profile 5 IgG for all other ANA-specificities (Euroimmun, Lübeck, Germany) according to the manufacturer's recommendations.

The commercial myositis line blot assay (EUROLINE Autoimmune Inflammatory Myopathies 16 Ag (IgG) Profile, Euroimmun AG, Lübeck, Germany) was used and consisted of a membrane strip coated with 16 autoantigens, such as Mi-2α, Mi-2β, TIF1γ, MDA-5, NXP2, SAE1, Ku, PM-Scl-100, PM-Scl-75, Jo-1, SRP, PL-7, PL-12, EJ, OJ, and SSA/Ro-52. The procedure was carried out using a fully automated EUROBlotOne device (Euroimmun AG, Lübeck, Germany) according to the manufacturer's instructions at the accredited Laboratory of Clinical Immunology, Sahlgrenska University Hospital. The band intensity was evaluated by the EUROLineScan program.

TABLE 1 Demographic and disease-related characteristics of 70 patients with idiopathic inflammatory myopathies.

Age at diagnosis (years), median (IQR)	58 (38–73)
Gender, females, *n* (%)	46 (66)
Disease duration at assessment (months), median (IQR)	6 (2–12)
Total follow-up time (months), median (IQR)	18 (10–48)
IIM subtype according to classification criteria, *n* (%)	
- Inclusion Body Myositis	3 (4%)
- Polymyositis	23 (33%)
- Dermatomyositis	21 (30%)
- Anti-synthetase syndrome	23 (33%)
Muscle biopsy, *n* (%)	
- performed	64 (87%)
- histopathology suggestive of myositis	61 (95%)
Autoantibody profile, *n* (%)	
- Myositis-specific antibody (MSA) (including anti-HMGCR)	53 (75%)
- Myositis-associated antibody (MAA) (including anti-cN1A)	33 (47%)
- Presence of both MSA and MAA	24 (34%)
- Seronegative (no presence of MSA, MAA)	8 (11%)
Presence of clinical features*, *n* (%)	
- Muscle symptoms	58 (83%)
- Fever	15 (21%)
- Fatigue	40 (57%)
- Weight loss	27 (38%)
- Raynaud syndrome	24 (34%)
- Arthralgia/arthritis	37 (53%)
- Calcinosis	2 (3%)
- Mechanic's hands	17 (24%)
- Pulmonary involvement /Interstitial lung disease	33 (47%)
- Dysphagia/esophagus involvement	24 (34%)
- Carpal tunnel syndrome**	12 (17%)
- Various skin manifestations	30 (43%)
- Malignancy (diagnosed up to 3 years from IIM onset)	9 (13%)
- Biopsy verified heart involvement	1 (1%)

IQR, interquartile range; IIM, idiopathic inflammatory myopathy; anti-HMGCR, antibodies against 3-hydroxy-3-methylglutaryl coenzyme A.
*The clinical feature was considered missing if it was not documented/diagnosed.
**Includes only carpal tunnel syndrome verified by nerve conduction investigation.

According to the manufacturer, the band intensity thresholds of 7–14 correspond to borderline values, 15–35 to low positive (+), 30–70 to moderately positive (++), and > 70 to strongly positive (+++). Results that were borderline, according to this system, were considered negative.

The data regarding additional MAA anti-cN1A (antibodies against cytosolic 5′-nucleotidase 1A) and MSA anti-HMGCR (antibodies of the IgG subclass against 3-hydroxy-3-methylglutaryl coenzyme A) were also included when available and had been requested by clinicians due to the clinical suspicion of IBM or IMNM. The analyses were performed according to the manufacturer's instructions at the accredited Laboratory of Clinical Immunology, Sahlgrenska University Hospital. Anti-cN-1A was measured using a commercially available ELISA kit (Euroimmun AG, Lübeck, Germany) on three occasions, and a ratio ≥ 1 was considered positive. Anti-HMGCR was measured using a QUANTA Lite® HMGCR ELISA assay (Inova Diagnostics, Inc., San Diego, CA, USA) in 12 patients. The values of ≥20 units were considered positive.

2.4 Treatment

Detailed information regarding the current treatment with glucocorticoids (GCs), disease-modifying antirheumatic drugs (DMARDs), and biological drugs was obtained from medical records. To estimate the need for immunosuppression during the disease course (as a surrogate marker for overall disease severity for each patient), we classified the drugs according to a score system as previously described (7) and calculated a total value for each patient. The immunosuppression, if designated for treatment with IIM, was graded as follows: 0 point – no treatment; 1 point – conventional DMARDs such as azathioprine, methotrexate, and mycophenolate mofetil; 2 points – treatment period with intravenous immunoglobulins; 3 points – treatment period with rituximab, cyclophosphamide (CYC), abatacept, or plasmapheresis. All GCs were converted according to the "Steroid Conversion Calculator" to the equivalent dose of prednisolone, and the accumulated GC dose was calculated.

2.5 Statistical analysis

Statistical analysis was performed using GraphPad software version 9.0 (GraphPad Prism, San Diego, USA). The following non-parametric statistical tests were used, if appropriate: Kruskal–Wallis's test, two-tailed Mann–Whitney *U*-test, and two-tailed Spearman rank correlation test (as described in figure legends, GraphPad Prism). Fisher's exact probability test was used to assess differences between groups regarding disease characteristics. All continuous values are expressed as the median and 25^{th}–75^{th} percentiles. A p-value of <0.05 was regarded as being statistically significant.

3 Results

3.1 Patient characteristics

The demographic and disease-related variables of the IIM patient cohort are shown in Table 1. Seventy patients met the study inclusion criteria and comprised the following diagnoses according to the IIM classification criteria as specified above: 33% PM, 30% DM, 33% ASS, and 4% IBM. The median age at diagnosis was 58 years, and 66% of

the patients were females. Patients diagnosed with ASS were younger (median age 47 years), whereas patients fulfilling the IBM diagnosis classification were significantly older (median age 80 years).

3.2 Clinical features of IIM

The occurrence of various clinical disease features is presented in Table 1.

The muscle biopsy was performed in 87% of patients (64 of 70), and 95% of them (61 of 64) presented histopathological features indicative of myopathy. Additionally, a skin biopsy consistent with DM was presented in 13 cases. An EMG pattern consistent with inflammatory myopathy was registered in 70% (44 of 63) patients.

The most common affected organ, following muscles, skin, and joints, was the lungs; 47% of the patients had lung involvement. In one patient, the involvement of the heart was clinically suspected and the biopsy was verified. Of note, in 17% of the cohort ($n = 12$), nerve conduction studies showed bilateral or unilateral changes, as in CTS. In 7 out of those 12 patients (58%), the arthritis and/or arthralgia in the joints of the hands were documented in medical records. Fifty-eight percent of individuals with CTS were classified as ASS patients, whereas CTS was significantly overrepresented in the ASS group (30%) as compared to other IIMs (10%). CTS was mainly associated with the presence of anti-synthetase antibodies (58%) or anti-Mi-2β antibodies (25%).

3.3 MSA and MAA in relation to the clinical subtypes of IIM

The presence of myositis-specific and myositis-associated autoantibodies (MAAs) in the study cohort is shown in Table 2, and the clinical disease features related to MSAs are shown in Table 3.

A defined subset of IIM—IMNM—was diagnosed and biopsy-verified in 16% of the cohort ($n = 11$), whereas 36% ($n = 4$) of patients had positive anti-HMGCR antibodies and 54% ($n = 6$) displayed anti-SRP antibodies. Anti-HMGCR-positive myositis was associated with a history of statin treatment in 75% of cases. Interestingly, pulmonary involvement was seen in 18% of patients, whereas esophagus involvement was relatively common—55% in the whole IMNM group and 75% of anti-HMGCR-positive patients.

Cancer-associated myopathy (CAM) was identified in 13% of patients ($n = 9$). Most of the patients (89%) presented positive MSA: antibodies against SRP ($n = 3$), NXP2 ($n = 2$), Mi-2β ($n = 1$), SAE ($n = 1$), and PL-12 ($n = 1$) were detected. The most prevalent (44%) was gynecological malignancy in terms of ovarian or uterus cancer ($n = 4$) followed by breast cancer ($n = 2$), colon cancer ($n = 1$), spread thymoma ($n = 1$), and spread malign lentigo of the skin ($n = 1$). Importantly, patients with CAM displayed new skin symptoms in 89% of cases.

The classification criteria for definite ASS, according to Connor et al. (5) were fulfilled in 33% ($n = 23$) of patients in our IIM cohort. Anti-Jo-1, the most common MSA in ASS patients, was present in 56% of patients, followed by anti-PL-7 in 37% and anti-PL-12 in 22% of cases. Anti-Jo-1 was also the most prevalent MSA in our IIM cohort, detected in a total of 19% of patients ($n = 13$), whereas the most common MAA was anti-Ro/SSA52 in 31% of patients ($n = 22$).

TABLE 2 Distribution of myositis-specific (MSA) and myositis-associated autoantibodies (MAA) in a cohort of 70 patients with idiopathic inflammatory myopathies.

Autoantibodies	Patients, *n* (%)
MSA	
Anti-Jo-1	13 (18.6%)
Anti-PL-7	9 (12.9%)
Anti-PL-12	5 (7.1%)
Anti-OJ	1 (1.4%)
Anti-Mi-2α	4 (5.7%)
Anti-Mi-2β	5 (7.1%)
Anti-SAE	2 (2.9%)
Anti-MDA-5	1 (1.4%)
Anti-NXP-2	4 (5.7%)
Anti-TIF1γ	3 (4.3%)
Anti-SRP	6 (8.6%)
Anti-HMGCR	4 (5.7%)
MAA	
Anti-SSA-52	22 (31.4%)
Anti-SSA-60	5 (7.1%)
Anti-Pm/Scl-75	6 (8.6%)
Anti-Pm/Scl-100	7 (10%)
Anti-Ku	3 (4.3%)
Anti-RNP	2 (2.9%)
Anti-cN-1A	2 (2.9%)

Definite IBM according to the classification criteria by Griggs et al. (6) was confirmed in three patients, and two of them had positive anti-cN1A antibodies.

The presence of MSA was relatively monospecific, and the simultaneous occurrence of more than one MSA was seen in 7% of patients ($n = 5$). The coincidence of anti-PL-7 with another MSA was most common and was detected together with anti-Jo-1 ($n = 1$), anti-PL-12 ($n = 1$), anti-Jo-1 plus anti-OJ ($n = 1$), and anti-HMGCR ($n = 1$). One patient had both positive anti-Mi-2α and anti-Mi-2β antibodies. In terms of MAA, 10% of patients ($n = 7$) had more than one antibody. A coincidence of anti-Pm/Scl-75 with anti-Pm/Scl-100 was seen alone ($n = 1$) or together with anti-RNP ($n = 1$) or anti-Ro/SSA52 ($n = 1$). A combination of antibodies against Ro/SSA52 together with anti-cN-1A ($n = 1$) and anti-Pm/Scl-100 ($n = 2$) was detected, as well as a combination of anti-Scl-75 and anti-Ku ($n = 1$).

3.4 MSA and MAA in relation to pulmonary involvement

In our cohort, 47% ($n = 33$) of the patients in the whole study group, 86% ($n = 19$) of patients with anti-Ro/SSA52 positivity, and 100% ($n = 13$) with anti-Jo-1 had lung involvement. Of the patients with pulmonary involvement, the majority ($n = 21$) were diagnosed with ASS, whereas 24% ($n = 8$) were classified as having DM and 12% ($n = 4$) as having PM.

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TABLE 3 Clinical features of myositis according to the presence of myositis-specific and -associated antibodies in a cohort of 70 patients with idiopathic inflammatory myopathies.

Autoantibodies	Patients, *n*	Median age (IQR)	Muscle symptoms	Raynaud syndrome	Arthralgia/ arthritis	Pulmonary involvement	Dysphagia	Cancer diagnosis
Anti-Jo-1	13	46 (34–68)	11 (85%)	6 (46%)	12 (92%)	13 (100%)	2 (15%)	0
Anti-PL-7	9	55 (41–71)	5 (56%)	1 (11%)	3 (33%)	6 (67%)	3 (33%)	0
Anti-PL-12	5	38 (33–76)	0	2 (40%)	2 (40%)	5 (100%)	1 (20%)	1 (20%)
Anti-OJ	1	34	0	0	1 (100%)	1 (100%)	0	0
Anti-Mi-2α	4	64 (31–76)	3 (75%)	4 (100%)	3 (75%)	0	0	0
Anti-Mi-2β [a]	5	54 (38–77)	5 (100%)	2 (40%)	2 (40%)	0	2 (40%)	1 (20%)
Anti-SAE	2	61 (52–69)	2 (100%)	0	1 (50%)	2 (100%)	1 (50%)	1 (50%)
Anti-MDA-5	1	54	0	0	1 (100%)	1 (100%)	0	0
Anti-NXP-2	4	47 (35–62)	4 (100%)	1 (25%)	2 (50%)	0	3 (75%)	2 (50%)
Anti-TIF1γ	3	84 (57–87)	3 (100%)	0	0	0	1 (33%)	0
Anti-SRP	6	64 (33–81)	6 (100%)	3 (50%)	1 (17%)	2 (33%)	3 (50%)	3 (50%)
Anti-HMGCR	4	70 (60–76)	3 (75%)	0	1 (25%)	0	3 (75%)	0
Anti-Pm/Scl-75 [b]	6	38 (30–61)	5 (83%)	4 (67%)	3 (50%)	4 (67%)	0	0
Anti-Pm/Scl-100	7	47 (34–60)	6 (86%)	5 (71%)	3 (43%)	5 (71%)	1 (14%)	0
Anti-Ku	3	34 (25–87)	2 (67%)	1 (33%)	1 (33%)	1 (33%)	2 (67%)	0
Anti-RNP	2	68 (62–73)	2 (100%)	2 (100%)	2 (100%)	1 (50%)	0	0
Anti-cN-1A	2	75 (69–80)	2 (100%)	1 (50%)	2 (100%)	1 (50%)	0	0

The number and the percentage of patients presenting respective clinical features within the MSA/MAA antibody group are indicated.
[a] One patient had both positive anti-Mi-2α and anti-Mi-2β.
[b] Three patients were both positive for anti-Pm/Scl-75 and anti-Pm/Scl-100.

From the whole cohort, 38% of patients were ever-smokers, but only 4.4% smoked at the time of diagnosis. No significant differences were seen between the groups, 42% ($n = 14$) of the patients with lung involvement and 32% ($n = 12$) of the patients without lung involvement were ever-smokers.

Patients with the presence of MSA together with MAA and patients with only MAA had a significantly higher incidence of lung disease compared to patients with MSA only (Figure 2A). None of the patients suffered from lung disease due to IIM if only antibodies against Mi-2α, Mi-2β, NXP2, HMGCR, and TIF1γ were present or no MSA/MAA were detected. In addition, significantly decreased CO-diffusion capacity was registered in patients who presented both MSA and MAA compared with other patient groups (Figure 2B).

To estimate the need for immunosuppression during the disease course for each patient, we calculated the treatment score. Our results show that patients with lung involvement required significantly more intense treatment (average treatment weight score 5.9 vs. 1.8) and higher doses of GCs (calculated mean equivalent dose of prednisolone at diagnosis: 108 mg vs. 42 mg).

To identify special characteristics in the most severe disease in patients with lung involvement, we divided the 33 patients into 2 groups: the patients who were in need of treatment with CYC ($n = 17$) and a non-CYC group ($n = 16$). Patients in the CYC group had a lower mean CO-diffusion capacity (53% vs. 68%), were treated with higher doses of prednisolone at diagnosis (183 mg vs. 28 mg), and had a higher average total treatment weight score (8.4 vs. 3.4) compared to the non-CYC group, respectively.

Interestingly, patients in the CYC group had a significantly higher frequency of MAA (88% vs. 50%, $p = 0.004$) (Figure 2C) and were significantly more often anti-Ro/SSA52 positive (76% vs. 38%, $p < 0.001$) (Figure 2D), whereas no differences were seen in the presence of MSA (82% vs. 81%) as compared to patients in the non-CYC group.

4 Discussion

IIM is a diverse heterogenous disease consisting of various subgroups, each exhibiting distinct clinical manifestations. Notably, different phenotypes of IIM are linked to specific myositis autoantibodies, making these autoantibodies valuable biomarkers for disease diagnosis, subtyping, and prognosis prediction. In this study, we investigated the relationship between the presence of myositis autoantibodies and various disease manifestations in a cohort of 70 well-characterized IIM patients from a single center. Our results revealed that the presence of MAAs, particularly anti-Ro/SSA52 antibodies, is associated with a higher risk of developing interstitial lung diseases (ILDs) in myositis patients. Furthermore, patients who tested positive for both MSA and MAA demonstrated lower diffusing capacity for carbon monoxide compared to those who were positive for either MSA or MAA alone.

Our findings are consistent with previous research. In a prospective study involving 315 patients diagnosed with IIM, the presence of anti-Ro/SSA52 antibodies was associated with the progression of ILDs. Among patients in the ASS group who tested

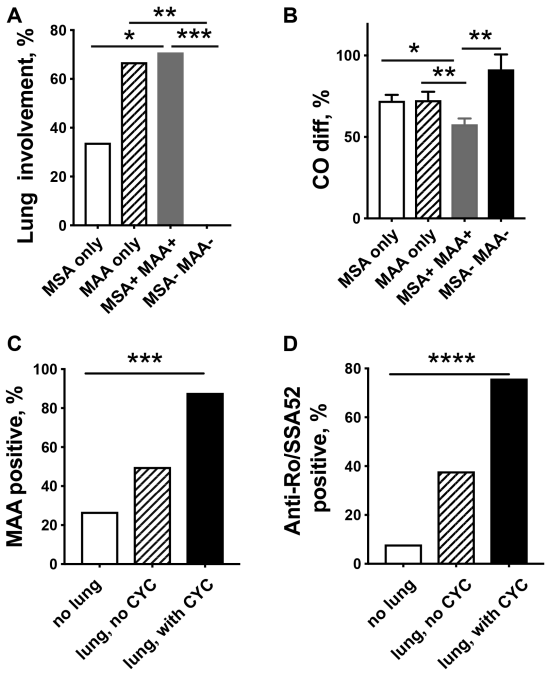

FIGURE 2
Relationship between myositis-specific antibodies (MSAs), myositis-associated antibodies (MAAs), and pulmonary involvement in patients with idiopathic inflammatory myopathies. **(A)** Lung involvement is significantly more common in patients presenting MAA only or MAA/MSA as compared to IIM patients with only MSA. **(B)** Patients with the MSA/MAA combination have significantly decreased CO-diffusion capacity compared to other patient groups. MAA **(C)** and especially anti-Ro/SSA52 positivity **(D)** are related to more severe lung involvement in need of cyclophosphamide (CYC) treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

negative for anti-Ro/SSA52 antibodies, there was a higher frequency of association with alveolitis, and those patients responded well to immunosuppressive therapy. In contrast, the anti-Ro/SSA52-positive group exhibited more fibrosis on high-resolution computed tomography scans. The authors concluded that the coexistence of anti-Ro/SSA52 and anti-Jo-1 antibodies could serve as a valuable predictor for the development of a more severe and advanced form of ILD in patients with IIM. Such patients may require a more aggressive therapeutic approach, as indicated by the findings from the study (8). Additionally, La Corte et al. demonstrated that ASS patients with associated anti-Ro/SSA52 antibodies were predisposed to the development of a more severe ILD (9). Our data also showed a progressive increase in the frequency of anti-Ro/SSA52 antibodies, rising from approximately 7% in patients without pulmonary involvement to 40% in patients with pulmonary involvement who did not require CYC treatment. In patients with pulmonary involvement who are in need of CYC treatment, the frequency of anti-Ro/SSA52 antibodies reached approximately 80%.

Anti-Ro/SSA52 antibodies consistently demonstrate associations with ILDs and declining lung function in various rheumatic conditions, underscoring their clinical significance in these contexts. In patients with mixed connective tissue disease (MCTD), research has revealed an association between anti-Ro/SSA52 antibodies and lung fibrosis. Among a cohort of 113 MCTD patients, 34% were confirmed to have lung fibrosis using HRCT scans. Interestingly, 50% of MCTD patients with lung fibrosis tested positive for anti-Ro/SSA52 antibodies, while only 19% of those without lung fibrosis presented anti-Ro/SSA52 (10). In individuals with SSc, ILD remains the leading cause of mortality. Remarkably, the presence of anti-Ro/SSA52 antibodies, as opposed to anti-Scl-70 antibodies, has been significantly linked to progressive ILD and the gradual loss of lung function. The rate of lung function decline demonstrated a linear increase with rising levels of anti-Ro/SSA52 antibodies (11). Consistent with these findings, another study encompassing connective tissue diseases reported that anti-Ro/SSA52 positivity is associated with poorer survival rates in SSc patients (12). Furthermore, anti-Ro/SSA52 antibodies have been identified as a risk factor for developing ILD in pSS. In a retrospective study involving 68 pSS patients, the presence of anti-Ro/SSA52 antibodies was significantly associated with a higher incidence of ILD compared to those without these antibodies (13).

The involvement of autoantibodies in the pathogenesis of rheumatic diseases is suggested, as seen in ACPA-mediated bone loss in RA (14) and ANCA in vasculitis (15). The direct contribution of anti-Ro/SSA52 antibodies to the disease's development is very clear in congenital heart block in neonatal lupus syndrome. The transplacental transfer of maternal anti-Ro/SSA52 is associated with irreversible damage to the fetal cardiac conduction system. Several studies have demonstrated direct effects of anti-Ro/SSA52 antibodies on cardiocyte function, possibly due to cross-reactivity. Anti-Ro/SSA52 antibodies that target p200 were found to directly interact with cardiomyocytes and disrupt calcium homeostasis (16). Human affinity-purified anti-Ro-52-positive sera were shown to induce cardiac conduction disorders in young rabbit hearts, similar to those observed in neonatal lupus (17). Additionally, immunizing female mice with recombinant SSA/Ro-52 KD protein resulted in atrioventricular conduction defects in their offspring (18). However, it remains largely unclear whether anti-Ro/SSA52 antibodies are merely biomarkers for ILD or play a causative role in the mechanism of ILD. Nevertheless, a recent report indicates that the majority of SSc-ILD patients who tested positive for anti-Ro/SSA52 exhibited a significant enrichment of anti-Ro/SSA52 antibodies in their BAL fluid, with a ratio exceeding 50 times (11), suggesting a potential pathological role of Ro-52 antibodies in pulmonary pathology. Further research is, however, needed to fully understand this phenomenon.

In our study, we made a noteworthy observation regarding the prevalence of CTS among patients with ASS. We found that CTS was significantly overrepresented in ASS patients and was strongly associated with the presence of anti-synthetase antibodies or anti-Mi-2β antibodies. CTS is a relatively common condition in the realm of rheumatic diseases, including RA (19), PsA (20), systemic lupus erythematosus (SLE) (21), and SSc (22). For instance, previous studies have reported the electrophysiological frequencies of CTS at 13.2, 15.4, and 3.5% in the RA, PsA, and control groups, respectively (20). Remarkably, the frequency of CTS in our IIM cohort was even higher, at 17%, reaching what has been reported for RA and PsA. Notably, within the ASS subgroup, the incidence of CTS was particularly striking, exceeding 50%. This finding suggests that there may be unique underlying mechanisms or factors specific to ASS that contribute to an increased risk of CTS compared to other rheumatic conditions. The joint inflammation in the wrists could be one contributing factor since arthritis/arthralgia was documented in the medical records of more than half of patients with CTS. Since our study had a retrospective nature, some information about the presence of arthritis could be missed. Our study adds a novel dimension to the understanding of CTS within the context of myositis, highlighting the

need for further investigation into the pathophysiological links between myositis, particularly ASS, and CTS. These findings underscore the complexity and heterogeneity of rheumatic diseases and warrant future research to elucidate the underlying mechanisms driving this pronounced association.

While our investigation has provided valuable insights into the clinical correlations of myositis autoantibodies, it is essential to acknowledge certain limitations in our study. Due to the retrospective nature of the study, it is possible that some information might be missing or incompletely documented in medical databases. Recognizing the relatively limited number of participants, the role of some infrequent autoantibodies cannot be evaluated. Additionally, since the primary focus of our research was unraveling the clinical associations of myositis autoantibodies, we excluded the patients for whom the myositis autoantibody analysis was not available. This led to the exclusion of individuals with confirmed myositis diagnoses and included mostly patients before line blot analysis became commercially available in our laboratory. However, this exclusion was not intended to introduce bias into our study outcome. Instead, it was a deliberate choice aimed at enhancing the better characterization of our patient cohort.

In summary, our single-center retrospective study involving 70 well-characterized IIM patients reveals that the presence of MAA autoantibodies, particularly anti-Ro/SSA52 in conjunction with MSA autoantibodies, is associated with more severe ILD in myositis patients. This association necessitates more aggressive immunosuppressive treatments. To predict disease severity and plan treatment effectively, a comprehensive myositis autoantibody profile is essential.

Data availability statement

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

Ethics statement

The studies involving humans were approved by regional Swedish Ethical Review Board, University of Gothenburg (Dnr 449–12). 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 the study was a retrospective analysis of patients medical records.

Author contributions

JM: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. BH: Data curation, Investigation, Writing – review & editing. TJ: Formal analysis, Investigation, Supervision, Validation, Visualization, Writing – review & editing, Funding acquisition. RP: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Association between serum antinuclear antibody and rheumatoid arthritis

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Background: The relationship between serum antinuclear antibody (ANA) and rheumatoid arthritis (RA) remains unknown. Therefore, we aimed to evaluate whether serum ANA was associated with an increased risk of RA in a case–control study.

Methods: Patients with rheumatoid arthritis hospitalized at Shandong Provincial Hospital from January 2018 to December 2022 were recruited as the case group, and patients with other types of arthritis and healthy people at the same time were taken as the control group. Antinuclear antibody (ANA) was detected by indirect immunofluorescence assays. Propensity score matching was employed to construct a cohort of patients exhibiting comparable baseline characteristics. The relationship between serum ANA and the risk of rheumatoid arthritis was analyzed by logistic regression analysis.

Results: A total of 1,175 patients with RA and 1,662 control subjects were included in this study. After adjusting for potential confounding factors in the propensity-score matched cohort, the risk of RA gradually increased with rising of ANA titers. When ANA titers were divided into three groups (1:100, 1:320, and 1:1,000), the OR (95% CI) for ANA titers from low to high was 3.95 (3.01, 5.18), 16.63 (9.44, 29.30), and 17.34 (9.53, 31.54), respectively, compared to those when ANA was negative. The ANA patterns closely related to the occurrence of RA include nuclear homogeneous, nuclear speckled, and cytoplasmic speckled. Among them, the positive rate of nuclear homogeneous was the highest, which accounted for 42.64%. The OR (95% CI) of ANA patterns including nuclear homogeneous, nuclear speckled, and cytoplasmic speckled was 16.81 (11.46, 24.65), 3.40 (2.49, 4.63), and 3.09 (1.77, 5.40), respectively.

Conclusion: There was a curve relation between ANA titer and RA, and the higher the ANA titer, the higher the probability of RA. However, there was no statistical difference in probability of RA for 1:320 versus 1:1,000 ANA titers. The most important kind of ANA pattern in the blood of RA patients was nuclear homogeneous. These findings suggest that ANA may be a novel risk marker for RA.

KEYWORDS

rheumatoid arthritis, antinuclear antibody, correlation analysis, curve relation, smooth curve

Introduction

Rheumatoid arthritis (RA) is recognized as an autoimmune disorder characterized by chronic inflammation that primarily impacts the joints while also being linked to various systemic aberrations of the immune system (1). If inadequately treated, RA can lead to progressive joint deterioration and irreversible impairment (2). Many circulating autoantibodies have been found in the serum of most patients with RA, including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) (3, 4).

Antinuclear antibodies (ANAs) comprise a diverse array of autoantibodies that specifically target various nuclear and cytoplasmic components within cells. The detection of ANA is facilitated through the implementation of various immunochemical methods, including indirect immunofluorescence assay (IIFA), enzyme-linked immunosorbent assay (ELISA), multiplex assay, and line immunoassay formats. Notably, the IIFA utilizing HEp-2 cells has long been regarded as the gold standard for ANA detection, providing reliable and accurate results (5, 6).

ANA test results are typically presented in two parts: besides the titer or fluorescence intensity of the antibodies, it also provides the fluorescence pattern produced by these antibodies. The observed fluorescence patterns encompass various cellular components such as the nucleus, cytoplasm, and patterns associated with mitotic cells. In order to establish a standardized nomenclature and definition for ANA, the International Consensus on ANA Patterns (ICAP) initiative has achieved consensus and aims to gradually transition to a more appropriate term: anti-cellular antibodies (ACs). These AC categories consist of 29 distinct staining patterns denoted as AC1-AC29 (7). Each individual staining pattern arises from the presence of one or multiple autoantibodies (8).

As is well known, ANA are important biomarkers for multiple systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), scleroderma (SSc), polymyositis (PM), and mixed connective tissue disease (MCTD) (5, 9). However, the precise clinical implications of ANA in RA and the relationship with other serological markers have remained ambiguous. The current study was conducted with the aim of elucidating the correlation between serum ANA and RA. Meanwhile, several autoantibodies (including CCP and MCV) and acute phase reactants (such as C-reactive protein and erythrocyte sedimentation rate) of the disease were also investigated.

Materials and methods

Patients

We recruited patients with rheumatoid arthritis, which were newly diagnosed according to the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA in the Department of Rheumatology of Shandong Provincial Hospital Affiliated to Shandong First Medical University from January 2018 to December 2022 as case group. The control group was composed of patients with other types of arthritis (such as ankylosing spondylitis, gout arthritis, and osteoarthritis) hospitalized in the Department of Rheumatology and healthy people during the same period. Just as shown in Figure 1, participants were all over 18 years old. Participants were excluded if they suffered from two or more types of arthritis. Patients with other autoimmune diseases, such as SLE, SS, SSc, PM, and vasculitis, were also excluded from the analyses. All procedures involving human participants were approved by the Shandong Provincial Hospital Affiliated to Shandong First Medical University Research Ethics Committee, and informed consents were obtained from all participants.

Data collection and blood indicators detection

Demographic variables including age, gender, history of common autoimmune diseases (AID, such as SLE, SS, SSc, PM, and vasculitis) were obtained. Blood examination included antinuclear antibody (ANA), cyclic citrullinated peptide (CCP), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and mutant citrulline vimentin (MCV). Elbow venous blood was extracted from all participants after fasting for 6–8 h. ANA levels were determined by an indirect immunofluorescence assay using HEp-2 cells as the substrate by a commercial kit (Euroimmun, Germany). All sections were examined independently by two experienced laboratory staff, and positive and negative control serum samples were included in each run. The analysis was performed for the most prevalent ANA patterns (nuclear homogeneous, nuclear speckled, cytoplasmic speckled, nucleolar, and centromere), and other less common ANA patterns were classified as other patterns. Only monospecific nuclear patterns were included; the primary pattern was selected for two or more patterns. Serum ANA level exceeding 1:100 was seen as positive.

Statistical analysis

In light of the variances in the baseline characteristics among participants in the two groups (Table 1), propensity-score matching (with propensity score in the range of 0.02) was applied to construct a cohort of patients with similar baseline characteristics. Age and sex were matched with the use of a 1:1 matching between RA and non-RA groups. In our research, numerical variables were presented in the form of mean ± standard deviation (SD) or median with interquartile range (IQR). Student's t-test was employed for assessing normal distributions, while the Mann–Whitney test was utilized for non-normal distributions. Categorical variables, on the other hand, were expressed as frequencies and evaluated using Pearson's χ^2 test or Fisher exact test. We evaluated the possible linear and nonlinear relationships between ANA and RA by multivariate linear regression models adjusted for age and gender in the propensity-score matched cohort. Smooth curve fitting was also employed to analyze the independent relationship between them after adjusting the confounding factors. All analyses were performed using Empower

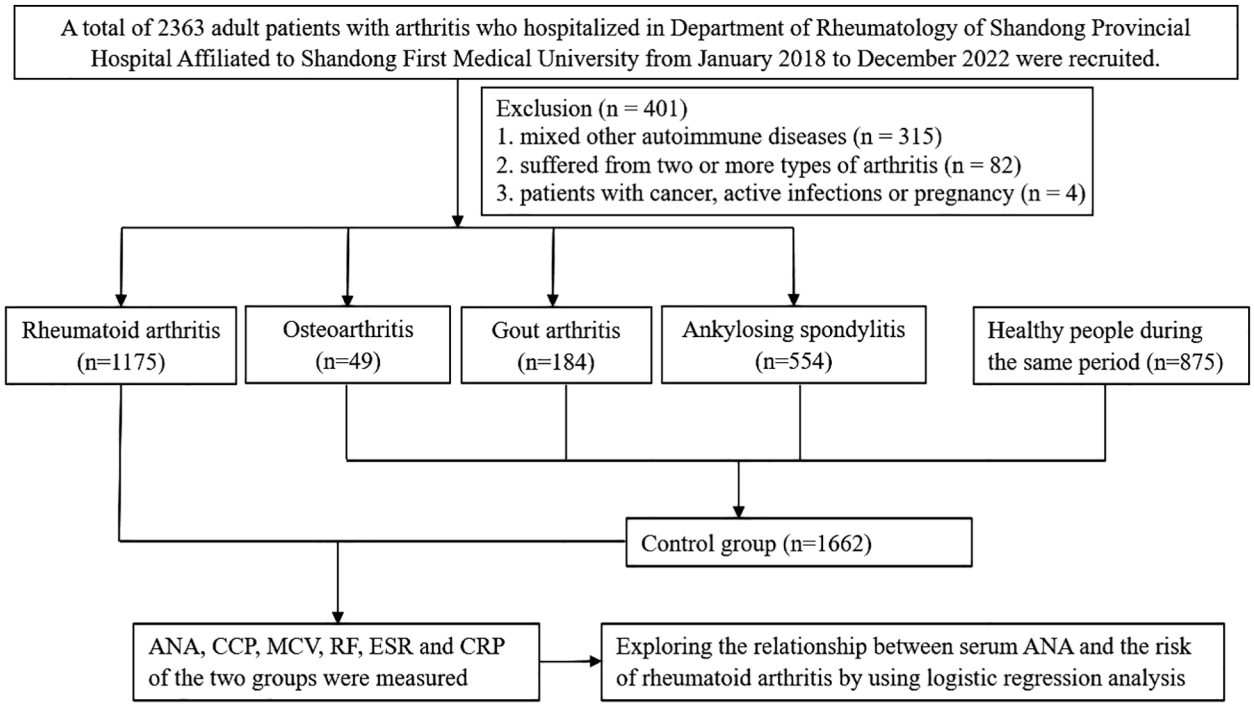

FIGURE 1
Flowchart showing selection criteria and analysis process of study participants.

Stats software (version 4.1, USA) and R software (http://www.R-project.org). $p < 0.01$ was considered statistically significant.

Results

Patient selection for subsequent analyses

A total of 2,837 patients met the criteria for this study; 1,175 patients with rheumatoid arthritis were selected as the case group, while 787 patients with other types of arthritis (including 554 ankylosing spondylitis, 184 gout arthritis, and 49 cases of osteoarthritis) and 875 healthy subjects during the same period as the control group. The patient characteristics before and after propensity-score matching are listed in Table 1. Before propensity-score matching, there were significant differences between the two groups with regard to age, gender, ANA titers and patterns, CCP, MCV, RF, CRP, and ESR on the basis of available data. There was no difference between RA and non-RA group in terms of age, gender, and CRP after matching, and 38.13% patients were ANA positive in the non-RA group while the positive rate of ANA in patients with RA was 77.76%. Furthermore, the highest percentage of ANA pattern of RA patients was nuclear homogeneous (42.64%).

Characteristics of ANA-positive patients

To further study the influence of ANA in RA, the characteristics of age, gender, and blood indicators between the ANA-positive and ANA-negative group are described in Table 2. CCP, MCV, and RF of patients testing positive for ANA were significantly higher than those of patients testing negative for ANA (all $p < 0.001$). The relationships between ANA and CCP or RF among rheumatoid arthritis patients with or without CCP + or RF + were analyzed by logistic regression analysis. Just as shown in Supplementary Tables S3, S4, the results suggested that ANA titer was positively correlated with CCP or RF among patients with RA. Similarly, the results also suggested that nuclear homogeneous was significantly associated with CCP or RF among rheumatoid arthritis patients.

The nonlinear relation between the ANA and rheumatoid arthritis

Smooth curve fitting (Figure 2) was performed after the adjustment of sex and age in the matched cohort. It was seen from the smooth curves that there existed nonlinear relations between ANA (its titer and pattern) and the probability of rheumatoid arthritis. ANA titer was positively associated with RA, and the higher the ANA titer, the higher the probability of RA. ANA patterns related to the probability of RA included nuclear homogeneous, nuclear speckled, and cytoplasmic speckled, especially nuclear homogeneous.

Relationship of ANA patterns and titers to rheumatoid arthritis

Logistic regression analysis was performed further in patients with and without RA after matching. In both unadjusted and adjusted (age

TABLE 1 Baseline characteristics of the participants before and after propensity-score matching.

Variables	Before Matching (n= 2837)				After Matching* (n= 1196)		
	Non-RA (n = 1662)	RA (n = 1175)	*P* value		Non-RA (n = 598)	RA (n = 598)	*P* value
Age (years)	38.9 (14.4)	57.4 (13.1)	<0.001		51.1 (15.0)	51.4 (13.7)	0.688
Sex			<0.001				0.067
Male	749 (45.07%)	335 (28.51%)			252 (42.14%)	221 (36.96%)	
Female	913 (54.93%)	840 (71.49%)			346 (57.86%)	377 (63.04%)	
CCP (U/mL)	0.5 (0.5-1.8)	198.6 (23.1-200.0)	<0.001		0.5 (0.5-2.1)	192.9 (21.1-215.5)	<0.001
MCV (U/mL)	6.3 (4.4-8.3)	255.5 (25.3-1000.0)	<0.001		6.2 (4.5-8.6)	174.7 (21.3-1000.0)	<0.001
RF (IU/mL)	9.7 (8.9-10.8)	99.2 (15.0-368.5)	<0.001		9.7 (8.9-10.9)	94.8 (13.4-341.5)	<0.001
CRP (mg/L)	15.1 (4.7-38.2)	19.7 (6.2-53.6)	<0.001		15.4 (4.1-41.7)	19.2 (5.3-46.9)	0.06
ESR (mm/hour)	13.0 (6.0-26.0)	50.0 (26.0-78.0)	<0.001		18.0 (8.0-51.0)	45.0 (23.0-73.0)	<0.001
ANA titers			<0.001				<0.001
Negative	1119 (67.33%)	289 (24.60%)			370 (61.87%)	133 (22.24%)	
1:100	502 (30.20%)	534 (45.45%)			198 (33.11%)	281 (46.99%)	
1:320	25 (1.50%)	197 (16.77%)			16 (2.68%)	96 (16.05%)	
1:1000	16 (0.96%)	155 (13.19%)			14 (2.34%)	88 (14.72%)	
ANA patterns			<0.001				<0.001
Negative	1119 (67.33%)	289 (24.60%)			370 (61.87%)	133 (22.24%)	
Nuclear homogeneous	76 (4.57%)	479 (40.77%)			42 (7.02%)	255 (42.64%)	
Nuclear speckled	332 (19.98%)	282 (24.00%)			127 (21.24%)	156 (26.09%)	
Centromere	4 (0.24%)	9 (0.77%)			2 (0.33%)	3 (0.50%)	
Nucleolar	54 (3.25%)	31 (2.64%)			24 (4.01%)	14 (2.34%)	
Cytoplasmic speckled	51 (3.07%)	70 (5.96%)			27 (4.52%)	30 (5.02%)	
Other patterns	26 (1.56%)	15 (1.28%)			6 (1.00%)	7 (1.17%)	

*Age and sex were matched between RA and Non-RA groups.
Abbreviations: RA, rheumatoid arthritis; ANA, antinuclear antibody; CCP, cyclic citrullinated peptide; MCV, mutant citrulline vimentin; RF, rheumatoid factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

TABLE 2 Characteristics of ANA-negative and ANA-positive patients with RA.

Variables	ANA-negative (n = 289)	ANA-positive (n = 886)	*P* value
Age (years)	58.2 (12.7)	57.1 (13.3)	0.245
Sex			0.013
Male	99 (34.26%)	236 (26.64%)	
Female	190 (65.74%)	650 (73.36%)	
CCP (U/mL)	53.8 (2.0-200.0)	200.0 (48.2-200.0)	<0.001
MCV (U/mL)	37.7 (8.4-682.7)	461.6 (50.6-1000.0)	<0.001
RF (IU/mL)	45.7 (10.6-222.5)	130.0 (28.0-380.0)	<0.001
CRP (mg/L)	24.1 (5.7-64.8)	18.9 (6.3-49.1)	0.038
ESR (mm/hour)	48.0 (23.0-77.0)	51.0 (27.0-78.0)	0.351

Abbreviations: RA, rheumatoid arthritis; ANA, antinuclear antibody; CCP, cyclic citrullinated peptide; MCV, mutant citrulline vimentin; RF, rheumatoid factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

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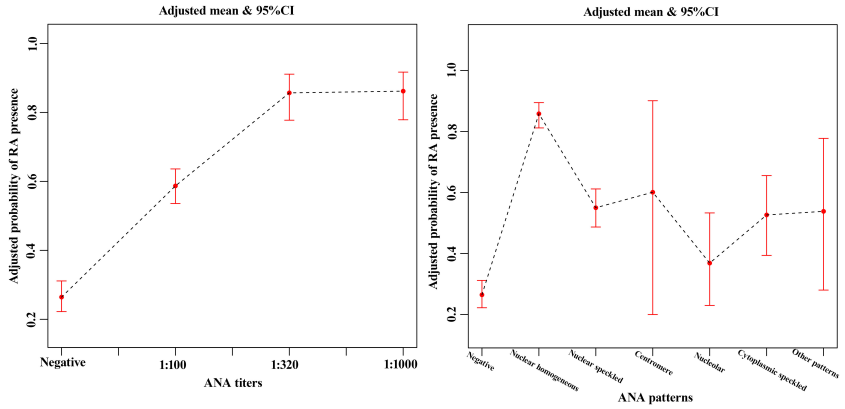

FIGURE 2
The smooth curve fitting showed the association between ANA titers **(A)**, ANA patterns **(B)** and rheumatoid arthritis after the adjustment of sex and age in the matched cohort. The red lines represented the upper and lower 95% confidence intervals (CIs).

and sex) logistic regression models, ANA titers were related to rheumatoid arthritis. Compared with those in the ANA-negative patients, the multi-adjusted ORs (95% CIs) of rheumatoid arthritis related to ANA titers (1:100, 1:320, and 1:1,000) were 3.95 (3.01, 5.18), 16.63 (9.44, 29.30), and 17.34 (9.53, 31.54), respectively (Table 3). However, there was no statistical difference in probability of RA for 1:320 versus 1:1,000 ANA titers as shown in Table 4 (p = 0.9163). Similarly, the results also suggested that ANA patterns (including nuclear homogeneous, nuclear speckled, and cytoplasmic speckled) were significantly associated with RA in the unadjusted analysis (all $p <$ 0.0001). This difference remained statistically significant even after controlling for age and gender. Compared with those in the ANA-negative patients, the multi-adjusted ORs (95% CIs) of rheumatoid arthritis related to ANA patterns mentioned above were 16.81 (11.46, 24.65), 3.40 (2.49, 4.63), and 3.09 (1.77, 5.40), respectively (Table 3). We also found that nuclear homogeneous was more significantly associated with rheumatoid arthritis than other ANA patterns except centromere (Table 4). According to the reference values of CCP and RF, the participants were categorized as four groups, including CCP−, CCP+, RF−, and RF+ groups. The association between ANA and RA among the four groups with and without matching was analyzed by logistic regression analysis. There was no association between ANA and the risk of RA in the CCP− group, while ANA titer was positively correlated with RA, and the ANA patterns (including nuclear homogeneous and nuclear speckled) were associated with incidence of RA in CCP + group and RF− group, which were basically consistent

TABLE 3 Association between ANA positivity and the incidence risk of RA in the propensity-score matched cohort*.

Variables	Non-adjusted		Adjusted I	
	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
ANA titers				
Negative	Reference		Reference	
1:100	3.95 (3.02, 5.17)	<0.0001	3.95 (3.01, 5.18)	<0.0001
1:320	16.69 (9.49, 29.37)	<0.0001	16.63 (9.44, 29.30)	<0.0001
1:1,000	17.49 (9.62, 31.79)	<0.0001	17.34 (9.53, 31.54)	<0.0001
ANA patterns				
Negative	Reference		Reference	
Nuclear homogeneous	16.89 (11.53, 24.74)	<0.0001	16.81 (11.46, 24.65)	<0.0001
Nuclear speckled	3.42 (2.51, 4.64)	<0.0001	3.40 (2.49, 4.63)	<0.0001
Centromere	4.17 (0.69, 25.25)	0.1198	4.19 (0.69, 25.36)	0.1198
Nucleolar	1.62 (0.82, 3.23)	0.1680	1.62 (0.81, 3.23)	0.1683
Cytoplasmic speckled	3.09 (1.77, 5.39)	<0.0001	3.09 (1.77, 5.40)	<0.0001
Other patterns	3.25 (1.07, 9.83)	0.0373	3.24 (1.07, 9.82)	0.0377

*The propensity-score matched cohort included 598 patients in the RA group and 598 patients in the non-RA group.
RA, rheumatoid arthritis; ANA, antinuclear antibody; OR, odds ratio; 95% CI, 95% confidence interval.
Adjusted I: Adjusted for age, sex.

TABLE 4 Odds ratio (OR) and 95% confidence interval (CI) for the association between ANA and RA in the propensity-score matched cohort*, took 1:320 and nuclear homogeneous as reference, respectively.

Variables	Non-adjusted		Adjusted I	
	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
ANA titers				
1:320	Reference		Reference	
Negative	0.06 (0.03, 0.11)	<0.0001	0.06 (0.03, 0.11)	<0.0001
1:100	0.24 (0.14, 0.41)	<0.0001	0.24 (0.14, 0.42)	<0.0001
1:1,000	1.05 (0.48, 2.27)	0.9062	1.04 (0.48, 2.26)	0.9163
ANA patterns				
Nuclear homogeneous	Reference		Reference	
Negative	0.06 (0.04, 0.09)	<0.0001	0.06 (0.04, 0.09)	<0.0001
Nuclear speckled	0.20 (0.14, 0.30)	<0.0001	0.20 (0.14, 0.30)	<0.0001
Centromere	0.25 (0.04, 1.52)	0.1319	0.25 (0.04, 1.54)	0.1345
Nucleolar	0.10 (0.05, 0.20)	<0.0001	0.10 (0.05, 0.20)	<0.0001
Cytoplasmic speckled	0.18 (0.10, 0.34)	<0.0001	0.18 (0.10, 0.34)	<0.0001
Other patterns	0.19 (0.06, 0.60)	0.0045	0.19 (0.06, 0.60)	0.0046

*The propensity-score matched cohort included 598 patients in the RA group and 598 patients in the non-RA group.
RA, rheumatoid arthritis; ANA, antinuclear antibody.
Adjusted I: Adjusted for age, sex.

with above conclusions (Supplementary Tables S10, S11). The relationships between ANAs and the risk of ankylosing spondylitis, gouty arthritis, or osteoarthritis were analyzed by logistic regression analysis. After adjusting for sex and age, we found that there was no connection between them just as shown in Supplementary Tables S5-S7.

Discussion

In the present study, our results supported the notion that there was a significant association between ANA and the risk of RA. There were nonlinear relationships between ANA (its titer and pattern) and the incidence of RA. ANA titer was positively correlated with RA. Three ANA patterns (including nuclear homogeneous, nuclear speckled, and cytoplasmic speckled), especially nuclear homogeneous, were associated with increased risk of developing RA.

Less study was focus on the relationship between serum ANA and RA, and the relation between them was still unclear. In those subjects with active RA, ANA positivity was associated with being RF +, especially high titer (10), which was similar to our conclusion. Because we showed that RF of patients testing positive for ANA were significantly higher than that of patients testing negative for ANA. Ishikawa et al. (11) found that ANA was associated with poor treatment response to biological disease-modifying anti-rheumatic drugs (DMARDs) in patients with RA, and they believed ANA as a potential predictor for poor treatment response. Paknikar et al. (12) found that there were significant dissimilarities in patients with rheumatoid arthritis who tested positive and negative for ANA concerning the duration to satisfy the RA criteria and choice of initial pharmacotherapy. Moreover, ANA-positive individuals experienced prolonged duration to fulfill RA criteria. Another study showed that anti-Golgi antibody pattern (one type of ANA patterns) with high titer was closely related to RA (13). These findings could indicate a difference in clinical presentation of patients with RA between ANA positive and ANA negative. Further research was needed to study the association between ANA and RA. To our knowledge, the present study is the first report to clarify the correlation of ANA titer and pattern together with RA.

Thus far, the universally accepted standard for defining the positivity of ANA remains elusive. Previous studies have shown that HEp-2 cell lines derived from cultured human laryngeal epithelial carcinoma exhibit greater sensitivity to the presence of ANA in both patients and controls, when compared to animal tissue sections such as mouse or rat kidney (14). In August 2009, the American College of Rheumatology (ACR) released a recommendation advocating for the utilization of HEp-2 indirect fluorescent antibody in all ANA screenings. Currently, there are two types of screening dilution systems used to determine ANA levels: one utilizes twofold screening dilution systems, including dilutions such as 1:40, 1:80, 1:160, and 1:320. The other employs 3.2-fold screening dilution systems, encompassing dilutions like 1:100, 1:320, 1:1,000, and 1:3,200 (15, 16). Notably, the 1:100 screening dilution has been frequently adopted as the cutoff value in certain clinical assessments (14, 17). In our investigation, patients were subjected to ANA testing using the aforementioned screening dilution systems with HEp-2 cells and monkey liver as substrates, facilitated by a commercial kit. The manufacturers specified reference screening dilutions at four dilutions, namely, 1:100, 1:320, 1:1,000, and 1:3,200. Consequently, the titer exceeding 1:100 was employed as the criterion for defining

ANA positivity in our institution. The analyses revealed that the positive rate of ANA in the patients with RA was 45.45%, 16.77%, and 13.19% for a titer of 1:100, 1:320, and 1:1,000, respectively, and no ANA was found with titer higher than 1:1,000. The higher the ANA titer (within the scope from negative to 1:1,000), the higher the probability of RA.

Initially, a consensus encompassing 28 distinct HEp-2 patterns was established, each assigned an alphanumeric code ranging from AC-1 to AC-28 in accordance with the International Consensus on ANA Patterns (ICAP) (18). Subsequent to this initial classification, two additional patterns, AC-29 (19) and AC-0 (negative) (20) were introduced in 2018. Notably, each unique staining pattern observed was attributed to the presence of one or more specific autoantibodies. The most common autoantibodies of nuclear homogeneous pattern included anti-dsDNA antibody (dsDNA), anti-histones antibody (AHA), and anti-nucleosomes antibody (AnuA). Moreover, anti-SS-A antibody (SSA), anti-SS-B antibody (SSB), anti-U1 ribonucleoprotein antibodies (U1RNP), and anti-smith antibody (Sm) were specific autoantibodies of nuclear speckled pattern. Anti-Jo-1 antibody (Jo-1) and anti-ribosomal P protein antibody (Rib-P) were specific autoantibodies of cytoplasmic-speckled pattern (21). In this study, the analysis was performed for the most prevalent ANA patterns including nuclear homogeneous, nuclear speckled, cytoplasmic speckled, nucleolar, and centromere. Other less common ANA patterns were classified as other patterns. We found that ANA patterns related to the risk of RA included nuclear homogeneous, nuclear speckled, and cytoplasmic speckled, especially nuclear homogeneous. The proportion of nuclear homogeneous of RA patients was the highest among all ANA patterns. However, the most common autoantibodies of three kinds of ANA pattern related to RA as described above were negative. This indicated that the antibodies corresponding to ANAs in RA patients were not their common autoantibodies, and the corresponding antibodies were still unclear.

The strengths of our study are the large sample size and well-adjudicated analysis. Our study also has some limitations. First, it tested association, not causation. Furthermore, using a convenience sample from one single institution rather than a population-based study created potential selection bias and limits generalizability. In addition, we lacked data on clinical characteristics of RA, such as disease duration, arthritis distribution, extra-articular symptoms, treatment, and outcomes, which might affect ANA titer (11, 12, 22, 23). Finally, the conclusions are not suitable for CCP-negative patients by our subgroup analysis. More prospective studies are required to assess the importance of this research.

Conclusions

In conclusion, our results show that high ANA titer may be associated with increased risk of developing RA, so do three ANA patterns (including nuclear homogeneous, nuclear speckled, and cytoplasmic speckled), especially nuclear homogeneous. These findings suggest that ANA may be a novel risk marker for RA; however, future studies investigating the role of ANA in the treatment and outcomes of patients with RA are needed.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YS: Conceptualization, Writing – review & editing. XW: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. FL: Data curation, Formal analysis, Writing – original draft. CP: Software, Writing – original draft. JZ: Data curation, Formal analysis, Writing – original draft. ML: Methodology, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1358114/full#supplementary-material

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Screening for autoimmune diseases in apparently healthy antinuclear antibody positive individuals

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Background: Anti-nuclear antibodies (ANA) assessed by immunofluorescence (IF) microscopy are associated with systemic autoimmune rheumatic diseases (SARD) and can be detected years before onset of clinical symptoms. Recent data indicate dysregulation of the immune system with increased levels of proinflammatory cytokines, including type I interferons (IFN), in ANA-positive versus ANA-negative individuals. Herein, the aims were to investigate IF-ANA, ANA fine specificities, and IFN-α protein levels in relation to self-reported symptoms, as well as clinical signs, of SARD in a large group of healthy blood donors (HBD).

Methods: Sera from 825 HBD (48.8% females) were included. IF-ANA was assessed, using HEp-2 cells, according to the routine at the accredited laboratory of Clinical Immunology, Linköping University Hospital. All samples were analyzed for IgG-ANA fine specificities using addressable laser bead assay (ALBIA) at the same laboratory. IFN-α was determined using ELISA. Antibody-positive individuals, and their sex- and age-matched antibody-negative controls, were asked to fill a questionnaire regarding symptoms associated with SARD.

Results: In total, 130 HBD (15.8%) were positive with IF-ANA and/or ALBIA. Anti-U1RNP was significantly more common among women. Generally, self-reported symptoms correlated poorly with IF-ANA and/or ALBIA results. Two females with high levels of Ro60/SSA, Ro52/SSA and IFN-α reported mild sicca symptoms and were diagnosed with Sjögren's disease after clinical evaluation.

Conclusion: A considerable proportion of apparently HBD are autoantibody positive, but without clear association to self-reported symptoms. Nevertheless, the combination of autoantibodies, relevant symptoms and high IFN-α levels identified the small proportion of individuals with SARD in the study population.

KEYWORDS

autoantibodies, autoimmune disease, interferon-α, healthy blood donors, systemic lupus erythematosus, Sjögren's disease

Introduction

The anti-nuclear antibody (ANA)-associated systemic autoimmune rheumatic diseases (SARD), which among others include systemic lupus erythematosus (SLE), Sjögren's disease (SjD), systemic sclerosis (SSc), idiopathic inflammatory myopathies, mixed and undifferentiated connective tissue diseases, are chronic multisystem autoimmune diseases with a significant morbidity and mortality (1). A hallmark of their pathogenesis constitutes loss of tolerance which leads to autoreactivity and production of antibodies against self-nuclear antigens (2). Similarly to many autoantibodies, ANA can be detected in serum many years before onset of clinical symptoms, representing a phase of subclinical autoimmunity and the levels may fluctuate over time in established SARD (3–6). Still, the cut-off pursued for a positive ANA test is essential.

An "abnormal titer" of ANA assessed by immunofluorescence (IF) microscopy (IF-ANA) is one of the 11 classification criteria for SLE according to the 1982 American College of Rheumatology (ACR-82) whereas the 2012 Systemic Lupus International Collaborating Clinics (SLICC-12) criteria state that an ANA test "above the laboratory reference value" remains a criterion for SLE (7, 8). The importance of ANA was further highlighted in the most recent SLE criteria set from 2019 where the European Alliance of Associations for Rheumatology/ACR classification introduce ANA as an entry criterion, but with the recommendation of using HEp-2 cells or a solid-phase ANA screening immunoassay (9). Only the most recent classification ground for SLE states how to define the cut-off level for ANA (9).

Considerable efforts have been made to better understand the mechanisms that drive autoimmune disease. Compared to subjects with SLE, ANA-positive healthy individuals show lower levels of stem cell factor, B lymphocyte stimulator, and type I interferons (IFN) as well as higher levels of IL-1 receptor antagonist (10, 11). Nevertheless, some data indicate that the immune system is already dysregulated in ANA-positive versus ANA-negative healthy individuals, i.e., elevation of proinflammatory cytokines in serum and altered proportions of monocytes, B cells and T_H follicular cells (12).

Of etiopathogenetic relevance, activation of the type I IFN response constitutes a common denominator of several SARD which is also demonstrated by shared positivity for several ANA fine specificities (13, 14). Recent data from randomized controlled trials have shown that blocking the type I IFN receptor by anifrolumab decreases global disease activity in SLE and led to the approval of anifrolumab in both US and Europe (15). Still, the role of IFNs in disease initiation is not entirely clear (16). IFN activity is usually quantified using expression of interferon-stimulated genes (ISG) or by IFN-α ELISA. El-Sherbiny et al. described two continuous ISG expression scores that provided clinically meaningful differences in IFN status between and within autoimmune diseases (17). By adding family history of SARD to ISG expression data, prediction of SARD onset was improved compared to ISG expression alone in an at-risk cohort (18).

The aims of the current study were to investigate IF-ANA, ANA fine specificities, and IFN-α protein levels in relation to self-reported symptoms, as well as clinical signs, of SARD in a large group of blood donors.

Materials and methods

Study population

We included serum samples from 825 consecutive and apparently healthy blood donors (HBD), comprising 403 females (48.8%) with a median age of 46 years (range 18–71) and 422 (51.2%) males with a median age of 42 years (range 19–77) from one blood donation center in Linköping, Sweden, during the period March 2018 to June 2019. Only three individuals (0.4%) were ≥ 70 years of age (19). The samples were stored at −70°C until analyses were performed.

Indirect IF microscopy

IF-ANA was analyzed according to the routine at the accredited laboratory of Clinical Immunology, Linköping University Hospital, Sweden, using Olympus microscope BX43, lens 20X/0.75 Plan Super Apochromat, illumination with LED diode (CoolLed pE-100, wavelength 470 nm) set at 50% of maximal light intensity, multi-spot slides with fixed HEp-2 cells (ImmunoConcepts, Sacramento, CA, United States) as antigen substrate, and fluorescein isothiocyanate (FITC)-conjugated γ-chain-specific anti-human IgG dilution 1:200 as detection antibody (Dako A/S, Glostrup, Denmark). The cut-off level for a positive IF-ANA test was set at titer 800, corresponding to the 95th percentile ("abnormal titer") among 420 HBD (260 women, median age 54 years, range 19–89; 160 men, median age 46 years, range 19–72) according to international recommendations for ANA analysis (5.7% IF-ANA positive, 19 women and 5 men) (20). The serum samples which had previously been used in verification of IF-ANA analysis did not originate from the same individuals as the HBD of the current study population. Results included interpretation of the staining patterns using the International Consensus on ANA Patterns (ICAP) nomenclature (20).

ANA fine specificities

All samples were analyzed for IgG-ANA fine specificities, including antibodies against double-stranded DNA (anti-dsDNA) and thirteen other autoantibody specificities, by FIDIS™ Connective Profile interpreted with the Solinium software version 1.7.1.0 (both from Theradiag, Croissy-Beaubourg, France) at the Clinical Immunology Laboratory, Linköping University Hospital as previously detailed (21). This addressable laser bead assay (ALBIA) simultaneously measures autoantibodies to Ro52/SSA, Ro60/SSA, La/SSB, Smith antigen (Sm), Smith/ribonucleoprotein complex (Sm/RNP), U1-RNP, dsDNA, Scl70, Jo1, centromere B (CENP-B), ribosomal P protein (RiboP), histone, PmScl and proliferating cell nuclear antigen (PCNA). The manufacturer's recommended cut-off >40 units per ml (U/ml) was used for all fine specificities.

IFN-α assay

IFN-α was analyzed by ELISA according to the manufacturer's instructions [Human IFN-α (pan-specific) ELISA^{PRO} kit], Mabtech, Nacka Strand, Sweden (14). This ELISA detects subtypes 1/13, 2, 4, 5,

6, 7, 8, 10, 14, 16 and 17 of IFN-α with a standard ranging from 5 to 4,000 pg./mL.

Questionnaire

Antibody positive individuals (positive with either IF and/or with detected fine specificities; $n = 130$) as well as their individually sex- and age-matched antibody negative control from the same cohort ($n = 130$) were asked to fill in an unvalidated symptom questionnaire in Swedish with 14 questions (Supplementary Table S1). The questionnaire was constructed to identify both overt and subtle symptoms potentially associated with SARD, and it was delivered to the donors with regular post service up to 3 months after blood sampling.

Clinical assessment

All blood donors testing ANA-positive on IF, and/or showing any positive ANA fine specificity, in combination with relevant self-reported symptoms of rheumatic disease in the questionnaire were offered a visit to an experienced rheumatologist at Linköping University Hospital, Region Östergötland (C.S.). A full clinical assessment was performed and, if clinically indicated, additional blood tests (e.g., antiphospholipid antibodies, complement proteins, direct Coombs' test, anti-C1q antibodies as well as autoimmune liver disease- and myositis-associated antibodies), radiology, sialometry and/or biopsies were ordered to rule out any suspicion of SARD.

Statistics

The data were analyzed using SPSS statistics software V.27.0 (IBM) and Prism V.9 (GraphPad Software, La Jolla, United States) for construction of graphs. Differences between groups were calculated using χ^2 or Fisher's exact test where appropriate, and with the Mann–Whitney U test. p values of <0.05 were considered statistically significant.

Ethics considerations

Oral and written informed consent was obtained from all included subjects. The study was conducted according to the Declaration of Helsinki, and the study protocol was approved by the Regional Ethics Board in Linköping (Decision no. 2017/474-31).

Results

In total, 130 of the 825 blood donors (15.8%) showed at least one positive test using IF microscopy (7.2%) or ALBIA (9.9%). Only 11 (8.5%) out of the 130 autoantibody positive HBD showed a combined positivity for IF-ANA and ALBIA. The mean age of the positive individuals was 43.8 years (range 19–68) and 62/130 (47.7%) were women.

Table 1 shows the most common ANA staining patterns with AC-1 (homogenous) and AC-1, −4, −5 (homogenous/speckled) dominating. The mean age of the positive individuals was 42.8 years (range 19–67) and 36/59 (61%) were women. No significant associations between age and staining pattern were observed but individuals with AC-8, −9, −10 (nucleolar) tended to be younger, and those with AC-4, −5 (speckled) were slightly older, than other blood donors. Overall, IF-ANA positivity was more common among female blood donors but it did not reach statistical significance ($p = 0.052$).

TABLE 1 ANA staining patterns with ICAP nomenclature in relation to mean age and sex among the 825 healthy blood donors.

ICAP	Pattern	HEp-2 positive (*n*)	Positive (%)	Mean age (years)	Female sex (%)
AC-1	Homogenous	20	2.4	41.5	80
AC-1, -4, -5	Homogenous/ Speckled	18	2.2	43.0	50
AC-4, -5	Speckled	11	1.3	47.3	55
AC-6	Nuclear dots	1	0.1	59.0	100
AC-8, -9, -10	Nucleolar	9	1.1	37.8	44
Positive	Any	59	7.2	42.8	61

As demonstrated in Table 2, 82 of 825 blood donors showed positivity on at least one ANA fine specificity using ALBIA. The mean age of the positive individuals was 45.1 years (range 21–68) and 34/82 (41.5%) were women. Ro60/SSA, U1-RNP, PmScl and PCNA were the most frequently observed specificities. Whereas Ro60/SSA, PmScl and PCNA were numerically most common among men, only U1-RNP positivity reached a statistically significant difference ($p = 0.014$) being more common in women. Subjects with antibodies against dsDNA and PCNA tended to be younger than those positive for other ANA fine specificities but not reaching statistical significance.

As illustrated in the flow chart (Figure 1), IFN-α protein levels were assessed among 156/260 (60%) matched samples (i.e., 52 antibody positive and 104 antibody negative samples). The mean level of IFN-α protein in the autoantibody positive subgroup was 25.3 versus 17.6 pg./mL ($p = 0.04$) in the autoantibody negative group. This comparison refers only to samples with quantifiable levels (i.e., ≥5 pg./mL), which were 3/52 in the antibody positive and 8/104 in the antibody negative group (not significant). All three subjects in the autoantibody positive group with quantifiable IFN-α protein levels were females, whereas 50% were men in the autoantibody negative group.

Among the antibody positive blood donors, 125 (96.2%) agreed to answer the questionnaire (Supplementary Table S1). Among those not responding, 4 of 5 (80%) were men. Subsequently, 125 individually sex- and age-matched autoantibody negative controls from the same cohort also answered the questionnaire. As demonstrated in Figure 2, "swollen joints and/or arthralgia" as well as "muscle weakness" were numerically more common among antibody positive blood donors whereas "Raynaud" and "photosensitivity" as well as serositis and xerostomia were more frequently reported by antibody negative controls but without reaching statistical significances.

Based on the combination of self-reported symptoms and autoantibody findings, 37 subjects (28.5%) were offered (and accepted) a clinical assessment at the Rheumatology unit, Linköping University Hospital. This offer was not based on results

TABLE 2 ANA fine specificities (ALBIA) in relation to mean age and sex among the 825 healthy blood donors.

ANA fine specificity	Positive, *n* (%)	Mean age (years)	Positive, *n* (%)	Females, *n* (%)	Males, *n* (%)	*p* value
dsDNA	7 (0.8)	36.9	7 (0.8)	5 (71.4)	2 (28.6)	0.23
Ro60/SSA	16 (1.9)	48.9	16 (1.9)	5 (31.3)	11 (68.8)	0.15
Ro52/SSA	5 (0.6)	48.4	5 (0.6)	3 (60)	2 (40)	0.25
La/SSB	4 (0.5)	53.0	4 (0.5)	1 (25)	3 (75)	0.34
Sm	0	–	0	0	0	∞
SmRNP	1 (0.1)	25.0	1 (0.1)	0	1 (100)	∞
U1-RNP	15 (1.8)	43.6	15 (1.8)	12 (80)	3 (20)	**0.014**
Scl70	3 (0.4)	52.0	3 (0.4)	0	3 (100)	∞
Jo1	5 (0.6)	43.6	5 (0.6)	2 (40)	3 (60)	0.69
CENP-B	2 (0.2)	49.5	2 (0.2)	0	2 (100)	∞
RiboP	2 (0.2)	28.5	2 (0.2)	1 (50)	1 (50)	0.97
Histone	2 (0.2)	46.0	2 (0.2)	1 (50)	1 (50)	0.97
PmScl	18 (2.2)	50.6	18 (2.2)	6 (33.3)	12 (66.7)	0.18
PCNA	13 (1.6)	35.7	13 (1.6)	4 (30.8)	9 (69.2)	0.19
≥ 1 specificity	82 (9.9)	45.1	82 (9.9)	34 (41.5)	48 (58.5)	0.16
> 1 specificity	5 (0.6)	45.8	5 (0.6)	2 (40)	3 (60)	0.69
Negative	743 (90.1)	42.3	743 (90.1)	369 (91.6)	374 (88.6)	0.16

Bold value indicates that statistical significance was reached; i.e., U1-RNP positivity was more common among female HBD.

of the IFN-α measurement as it had not yet been performed at the time. In total, based on the clinical evaluation, results of IF microscopy and ALBIA tests, and sialometry, two individuals were diagnosed with SjD. These two females, representing 1.5% of the IF-ANA and/or ALBIA positive blood donor population, eventually showed the highest assessed levels of IFN-α protein levels of all investigated subjects. Both were IF-ANA positive (AC-4, −5; speckled pattern) and showed strong positivity for Ro60/SSA as well as for Ro52/SSA.

Discussion

Individuals who are accepted as blood donors in Sweden are highly selected based on their subjective health. In the current study, we aimed to assess autoantibody specificities using established techniques and stringent cut-offs in a large cohort of apparently healthy donors. Our main findings were that >15% of HBD test positive with IF-ANA (7.2%) and/or ALBIA (9.9%), using the manufacturer's recommended cut-offs. Only 11 of 825 HBD showed a combined positivity, probably illustrating the different conformational forms of antigens in cell- versus bead-based assays.

In line with our findings, Kim et al. recently reported that the most common fine specificities among ANA positive blood donors were SSA and U1-RNP (18, 22). We further observed that IF-ANA positivity, as well as anti-dsDNA and anti-U1-RNP antibody positivity, were more common among female blood donors. Anti-U1-RNP is often associated with Raynaud's phenomenon in females with SARD, but Raynaud is also frequently reported in the general population (14, 23). However, on a group level in the current study, we found that self-reported symptoms potentially associated with SARD appeared to correlate rather poorly with the autoantibody findings, similarly to prior reports using smaller study populations, underlining the low diagnostic specificity of a positive IF-ANA test (24).

IFN-α levels are raised in many patients with SLE and elevated levels of type I IFNs constitute a characteristic feature of several SARD and may predict progression to disease in ANA positive individuals (13, 14, 18, 22). Inspired by these observations, we asked whether the diagnostic accuracy could be increased by adding information on activation of the type I IFN system to autoantibody results. Indeed, we could show that IFN-α protein levels were higher among IF-ANA and/or ALBIA positive blood donors. In total, two female blood donors (0.24%) who reported mild sicca symptoms displayed elevated levels of anti-SSA antibodies (Ro60 and Ro52) and were eventually diagnosed with SjD, and found to have high levels of serum IFN-α.

Previous studies have had similar focus as ours. A recent paper by Brunekreef et al. from Netherlands, using data from electronic health records, concluded that progression to a connective tissue disease (CTD) is uncommon in individuals with a history of a positive IF-ANA test. They found that 16 of 1,030 (1.6%) ANA positive subjects received a CTD diagnosis (SLE being most common) within a mean time from the blood draw to diagnosis of approximately 2.3 years (25). This is in line with our current findings.

Selmi et al. assessed a cohort from the general population in Northern Italy and found that >18% were IF-ANA positive, with decreasing percentages having higher titers. In line with our findings, the female predominance was found to be lower compared to those with overt CTD (26). Importantly, the authors observed no associations with cancer or mortality. However, their finding of >18% ANA positivity appears to be high but is in line with what we reported 16 years ago in blood donors when a cut-off screening dilution of 1:60 was applied (27). Obviously, using such cut-off in clinical routine will introduce specificity

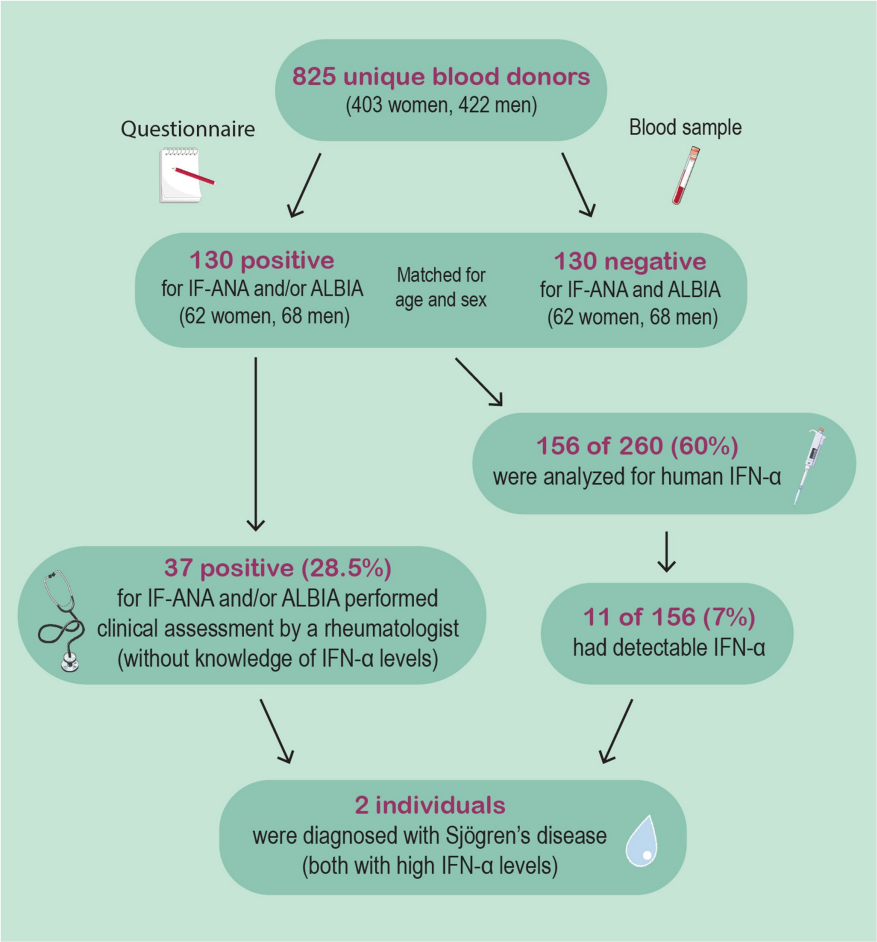

FIGURE 1
This flow-chart illustrates the study procedure. 825 unique blood donors were included, whereof 130 were deemed IF-ANA and/or ALBIA positive. 37 subjects were clinically evaluated by an experienced rheumatologist based on autoantibody findings and relevant self-reported symptoms. Eventually, two females were diagnosed with Sjögren's disease. Both were later found to have high IFN-α levels.

issues and could not be considered as an "abnormal titer" of ANA (7, 8). Similarly to the definition of a positive rheumatoid factor test according to the 1987 ACR classification criteria for rheumatoid arthritis (RA), we advocate a cut-off level of >95th percentile among HBD to define an abnormal level of ANA analyzed by indirect IF microscopy utilizing fixed HEp-2 cells as source of nuclear antigens and, importantly, γ-chain specific secondary antibodies to pinpoint immunoglobulin (Ig)G-class IF-ANA (28–30). The use of the 95th percentile specificity and the use of IgG-specific detection antibodies is in agreement with the international recommendations for ANA testing (20).

Nowadays, it is well established that autoantibodies may precede SARD (3, 4, 6). The samples for the current study were obtained between 2018 and 2019. Hitherto, only two of the 825 blood donors have received a SARD diagnosis by a rheumatologist at Region Östergötland during 5 years of follow-up. Although this constitutes a substantial timeframe from a clinical perspective, we cannot exclude that additional donors will develop overt SARD over time. A clear limitation of the current study is the cross-sectional nature with only one sample per blood donor since it is known that the levels of autoantibodies may fluctuate over time, although anti-Ro/SSA and anti-La/SSB levels have been shown to be more stable over time in contrast to anti-dsDNA and anti-Sm (5, 27). Based on available data, we cannot determine if the antibody findings are persistent or not. A follow-up study with new sampling including collection of new self-reported symptoms would be desirable.

In addition, we did not have information on family history of SARD. IFN-α activity has shown a complex heritable trait based on data from healthy family members of patients with SLE (31). Unfortunately, only 156/260 (60%) HBD samples were available for IFN-α analysis. The major strength of the current study is the large study population with self-reported symptoms. In addition, 37/130 (28.5%) autoantibody positive subjects were evaluated clinically at the Rheumatology unit where a diagnosis of SjD eventually was confirmed in two female blood donors. A limitation worth mentioning is that we did not have available data on environmental factors, e.g., smoking habits or use of hormones, particularly contraceptives, with potential association to antibody positivity, organ manifestations and the progression to SARD (32–39).

To conclude, our data revealed that a considerable proportion of apparently healthy blood donors are autoantibody positive. IF-ANA

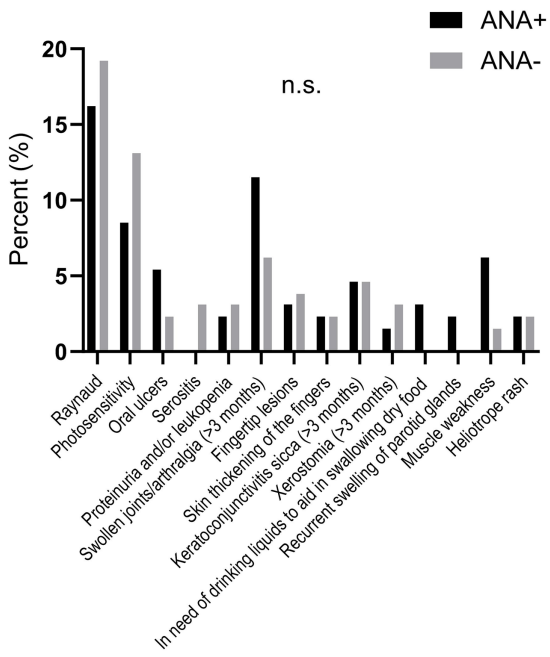

FIGURE 2
The graph shows self-reported symptoms among IF-ANA and/or ALBIA positive versus negative blood donors. No significant differences were observed.

and/or ALBIA positivity associated poorly with self-reported symptoms. However, the combination of autoantibodies, relevant symptoms and high IFN-α levels identified the small proportion of individuals with SARD among blood donors.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Regional Ethics Board in Linköping (Decision no. 2017/474-31). 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

RA: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. AA: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing – review & editing, Methodology. LW: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing, Supervision, Visualization. CD: Investigation, Methodology, Writing – review & editing, Conceptualization, Validation. MF: Investigation, Validation, Writing – review & editing, Project administration, Supervision. JR: Investigation, Validation, Writing – review & editing, Conceptualization, Methodology. AK: Investigation, Project administration, Supervision, Validation, Writing – review & editing. CS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2024.1455673/full#supplementary-material

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