# CHRONIC AUTOIMMUNE ARTHRITIS, INFECTIONS AND VACCINES

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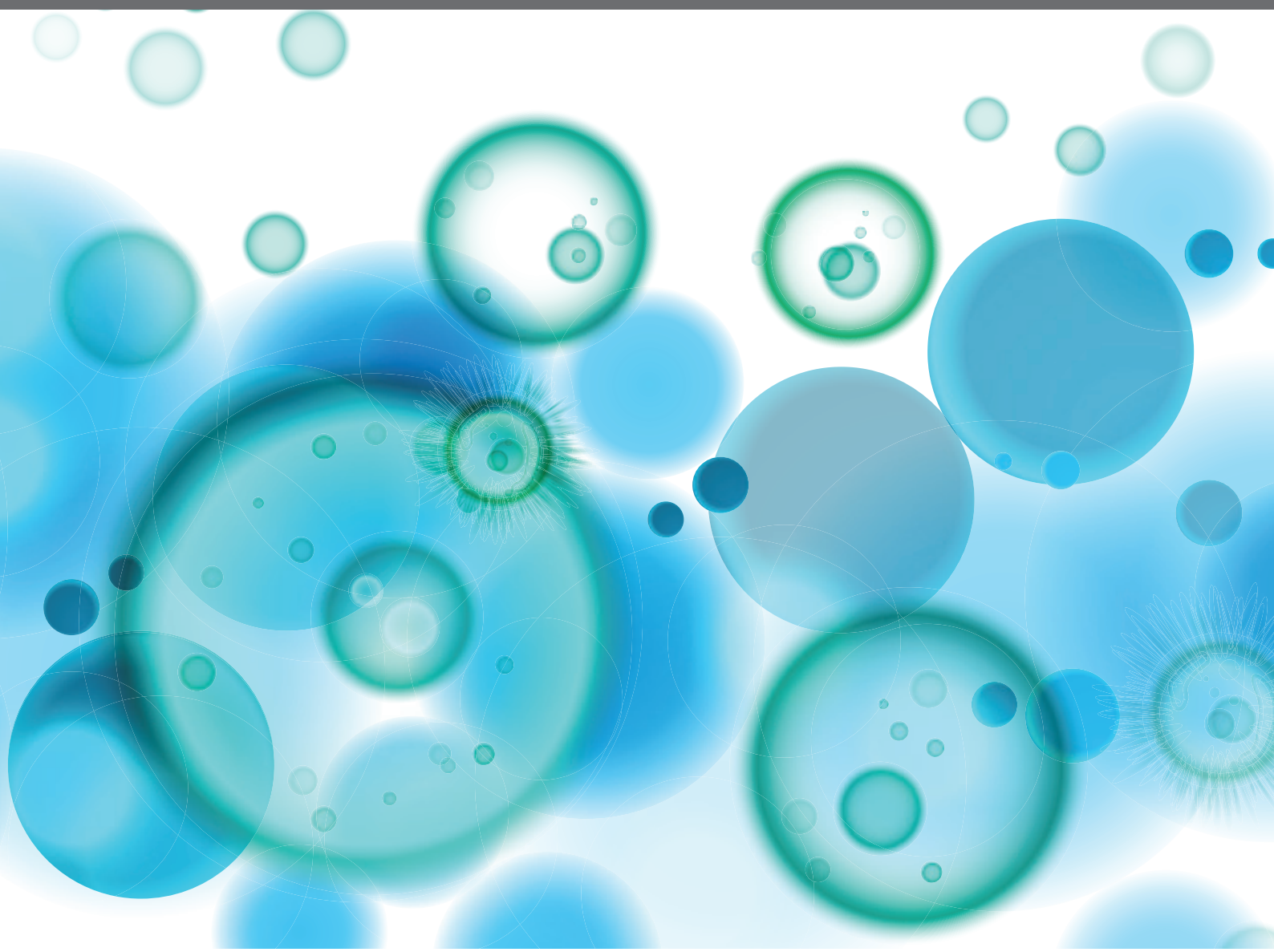

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# CHRONIC AUTOIMMUNE ARTHRITIS, INFECTIONS AND VACCINES

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# Editorial: Chronic autoimmune arthritis, infections and vaccines

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KEYWORDS

**SARS-CoV-2 infection, COVID19, autoimmune disease, arthritis, vaccines, immunosuppressive therapy**

Editorial on the Research Topic
Chronic autoimmune arthritis, infections and vaccines

The link between autoimmunity and infection continues to represent an intriguing immunologic conundrum for scientist and a frequent clinical challenge for patients and physicians.

Patients with chronic autoimmune arthritis indeed have an increased risk of infections, mainly due to the dysregulation of their immune system and the use of immunosuppressive therapy. Infections in these patients are more frequent, have a more severe clinical course, eventually with prolonged viral persistence, compared to the general population and represent a frequent cause of death.

Besides, infections can trigger autoimmune diseases *via* different immunologic mechanisms such as molecular mimicry, epitope spreading, by-stander activation and can also induce disease relapses.

SARS-CoV-2 infection represents a dramatic example of this complex connection.

It is known, indeed, that different autoimmune manifestations can complicate SARS-CoV-2 infection such as uncontrolled host-immune response leading to life-threatening condition known as cytokine release syndrome, or autoimmune hemolytic anemia, immune thrombocytopenic purpura, Guillain-Barre syndrome, and the detection of different autoantibodies.

This Research Topic includes seventeen contributions, fifteen original articles and two review articles, providing several new insights into the efficacy and safety of SARS-CoV-2 vaccine in autoimmune patients, immunologic biomarkers for diagnosis and therapeutic outcome of autoimmune arthritis.

The work of D'Abramo et al underlines the need of a prompt, combined multi-target tailored therapy for immunosuppressed patients with severe COVID-19. They described a case series of 21 COVID-19 patients under B cell depletion therapy, effectively treated

with a combined therapy based on intravenous remdesevir and steroid associated with SARS-CoV-2 monoclonal antibodies against Spike glycoprotein and/or hyper-immune convalescent plasma

Sjowall et al compared SARS-CoV-2 antibodies in longitudinal samples collected prior to vaccination with Systemic Lupus Erythematosus (SLE) progression and antinuclear antibody levels. Their data from early 2021 indicate that a large proportion of Swedish SLE patients had serological signs of exposure to SARS-CoV-2 but apparently with a minor impact on the SLE course.

The development of several SARS-CoV-2 vaccines represented a major step-forward, but it raises special concerns for patients with autoimmune arthritis such as the choice of the best vaccine formulation and the management of ongoing immunosuppressive treatment in infected patients or during the scheduled vaccination.

Picchianti Diamanti et al provided an updated review of the main controversial issues in terms of safety, efficacy and effectiveness of SARS-CoV-2 vaccines in autoimmune patients and the role of immunosuppressive therapy. Immunomodulating agents such as dexamethasone and other biological agents (ie, tocilizumab, sarilumab, anakinra and baricitinib) demonstrated clinical and virological efficacy for the management of COVID-19 patients, however, they have to be tailored to COVID-19 severity and clinical setting, considering the strict correct window of opportunity to optimize patient's outcome. On the other hand, autoimmune patients are a priority group for vaccination, but special measures should be adopted to improve vaccine safety and effectiveness, such as temporary suspension of some immunosuppressive drugs and the preferential use of mRNA-based vaccine

In their research article Picchianti Diamanti et al, showed for the first time that antibody-specific and whole-blood spike-specific T-cell responses induced by the COVID-19 mRNA-vaccine are present in the majority of Rheumatoid Arthritis (RA) patients, who underwent a strategy of temporary suspension of immunosuppressive treatment during vaccine administration, in the absence of disease relapses and major adverse events. However, the levels of specific responses were lower than Health Care Workers (HCWs) and reduced by some ongoing immunosuppressive therapy, in particular by the CTLA-4Ig abatacept.

In a following work on the same patient population Farroni et al, deeply characterized the kinetic of both humoral and cellular immune responses to BNT162b2 vaccine. The authors showed a significant reduction of the humoral response after 6 months from the first dose in both HCWs and RA patients regardless of the immunosuppressive therapy, whereas the T-cell response remained mostly stable. Looking at the T and B cell response overall, they stratified patients in full responders (both humoral and cellular, 40%), partially responders (only humoral or only T-cell response, 51%) and not responders (9%). The last two groups are likely to be at higher risk for COVID-19 despite the complete vaccination, underlining the need for a tailored strategy such as longer suspension of some immunosuppressive drugs and earlier adoption of a booster dose.

Through millions of people being vaccinated with COVID-19 mRNA vaccine is not surprising that rare reports of adverse events are emerging. In their Research article, Luchetti et al, suggests that the anti-Spike antibodies may play a key role in the induction of an abnormal and deregulated immune response. They evaluated the onset of clinical and laboratory immune manifestations related to COVID-19 or SARS-CoV-2 vaccination in a large cohort of hospitalized patients, with recently SARS-CoV-2 infection, or with autoimmune rheumatic diseases (ARDs) in remission and flared after SARS-CoV-2 infection, or finally outpatients with symptoms of probable immune-mediated origin after SARS-CoV-2 vaccination. They observed different clinical manifestations of ARD such as arthralgia/myalgia, pericarditis, thrombocytopenia as well as some cases of newly diagnosed ARD after the recovery from COVID-19 as well as after SARS-CoV-2 vaccination.

In the clinical case described by Hakroush and Tampe, there was a temporal association between the second dose of BNT162b2 vaccination and the development of anti-neutrophil cytoplasmic antibody associated vasculitis (AAV), rhabdomyolysis and pauci-immune crescentic glomerulonephritis (GN) suggesting a strong/uncontrolled neutrophilic immune response to mRNA vaccine as a potential trigger. Prompted treatment with intravenous cyclophosphamide followed by oral prednisone rescued kidney function, proteinuria dropped down and serological testing revealed that also ANCA IF turned negative.

Watanabe et al described a case of new-onset RA following COVID-19 vaccination. Flares or new-onset of autoimmune disorders have been reported soon after the COVID-19 vaccination, however in such a case, serum cytokine levels, after vaccination, resembled a typical genuine RA. Indeed, interleukin-6, tumor necrosis factor-alpha, type I interferon, were elevated at the active phase, whereas remission induced by methotrexate and tocilizumab was accompanied by a marked reduction of these cytokines.

Different interesting manuscripts focused on the pathogenesis, early diagnosis and therapy of RA were also submitted.

An important role in the pathogenesis of RA is played by the family of peptidylarginine deiminases (PADs) that have been linked to the anticitrullinated protein antibodies (ACPA) production. In their case-control study, Guzman-Guzman et al. found that the TTT haplotype of SNPs on the PADI2 gene confers genetic susceptibility to RA and radiographic joint damage in women from southern Mexico.

The experimental study of Brevet et al, gives a very interesting insight into the different roles that autoantibodies could have in RA. In fact, anti-carbamylated fibrinogen IgG

antibodies (ACa-Fib IgG) were associated with a more inflammatory and erosive disease at baseline, and maybe correlated with systemic inflammation, but not with rapid radiological progression, which remains strongly related to ACPA antibodies.

Microparticles, also called extracellular vesicles (EVs), are small membrane-coated vesicles that are released from various cells during cell activation and apoptosis thus can be a source of autoantigens. They can also have important procoagulant properties based on the availability of phosphatidylserine (PS) exposed on the surface after stimulation. Stojanovic et al, found elevated levels of circulating EVs in patients with established RA, in relation to the inflammatory burden and coagulation activation in the disease.

Early diagnosis of autoimmune diseases is critical to preventing disease progression.

In their meaningful clinical research study, Ahn et al investigated whether plasma tumor M2-PK is elevated in patients with RA and whether its levels correlate with disease activity. They demonstrated that the level of plasma M2-PK in RA patients is increased, and it is positively correlated with the disease activity. After treatment with immunosuppressive agents the level of M2-PK decreases concomitantly to the reduction of inflammation; however, M2-PK was hardly detected in the synovial tissue.

The differential diagnosis of seronegative RA can be a challenge for clinicians considering the absence of serological biomarkers. In their work, the Italian Group of Bason et al, through the use of random peptide library identified autoantibodies that could be used as serum biomarkers for the diagnosis of this patient population. In particular they found a peptide in the sera of seronegative RA patients and not in healthy controls, that shares homology with some self-antigens, such as Protein-tyrosine kinase 2 beta, B cell scaffold protein, Liprin-alfa1 and Cytotoxic T lymphocyte protein 4.

In the present era, of microarray and high-throughput sequencing analysis, is important to join efforts in comparing and converging the information on gene expression profiles, present in various platforms, to identify potential novel genes and biomarkers.

By selecting five microarray datasets and a high throughput sequencing dataset Zhou et al identified differently expressed genes in immune cell infiltrating the synovial tissues in RA patients as compared to the ones of healthy donors. Authors found that CD8 T cells expressing the alpha chain of the CD8, the chemokine receptor CCL5, the chemokine CXCR4 and Granzyme A are a good diagnostic biomarker candidate for RA. And that CXCR4 activated memory CD4/Tfh cells expressing Granzyme A participate in early pathogenesis of RA. Moreover, RA synovia contained M1 type macrophages, generated by the inflammatory synovial microenvironment, which in turn sustain inflammation and recruitment of monocyte/neutrophils.

Yu et al examined RA's diagnostic signatures and the effect of immune cell infiltration through integrated bioinformatic analysis and machine-learning strategies, further verified by qRT-PCR.

They found that lymphocyte-specific protein 1 (LSP1), Granulysin (GNLY), and Mesenchymal homobox 2 (MEOX2) regarded as RA's diagnostic markers and showed their statistically significant difference by qRT-PCR.

Finally, two manuscripts were more focused on RA therapy and predictive biomarkers of therapeutic response.

Hu et al, performed a systematic review on the efficacy of denosumab in patients with RA, focusing on the percent changes in bone mineral density (BMD), and the changes in modified total Sharp score (mTSS), modified Sharp erosion score and joint space narrowing score. Pooled analyses showed that denosumab treatment was superior to bisphosphonates for the improvement of lumbar spine and femoral neck BMD, as well as for the reduction of joint destruction evaluated through mTSS and modified Sharp erosion score.

In their study, Cai et al, have carried out a correlation analysis and single-sample gene set enrichment analysis (ssGSEA) finding 46 common genes between RA patients' infliximab (IFX)-responders or non-responders and a specific 25-gene signature in datasets of the non-responder patients and Derlin-1 (DERL1) was identified as the hub gene. Interestingly, DERL1 demonstrated to be involved in the immune response and autophagy regulation, since DERL1-siRNA partially inhibited autophagosome formation in RA-fibroblast-like synoviocytes and may have potential predictive value for the therapeutic effect of IFX.

In conclusion, we are pleased that this Research Topic attracted several innovative and valuable scientific articles that faced this issue from different point of view, by using single case reports, real life clinical studies and a plethora of technologies such as bioinformatics and molecular biology.

We hope that this Research Topic will help to share the advances in the knowledge of SARS-CoV-2 infection and vaccination, as well as new potential biomarkers for prompt diagnosis and optimal management of autoimmune rheumatic diseases.

## Author contributions

APD, EN, MML, BL, MMR conceived the Research Topic, revised submitted manuscripts and wrote/revised the Editorial. All authors contributed to the article and approved the submitted version.

## Conflict of interest

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

## Publisher's note

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# Increased Expression of Extracellular Vesicles Is Associated With the Procoagulant State in Patients With Established Rheumatoid Arthritis

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This study sought to identify different subpopulations of extracellular vesicles (EVs) in plasma from female patients with established rheumatoid arthritis (RA) in relation to the activation of coagulation and fibrin formation in these patients. Forty women were included in the study, 20 patients and 20 age-matched healthy controls. The mean disease duration in patients was 13.0 (5.0–25.0) years, with medium to high disease activity despite ongoing treatment with low-dose prednisolone and methotrexate. There were no differences between the investigated groups regarding the presence of traditional cardiovascular risk factors. The concentration of phosphatidylserine-positive ($PS^+$) EVs; platelet ($CD42a^+$), leucocyte ($CD45^+$), monocyte ($CD14^+$), and endothelial ($CD144^+$)-derived EVs; and EVs-expressing tissue factor ($CD142^+$), P-selectin ($CD62P^+$), and E-selectin ($CD62E^+$) were determined by flow cytometry analysis. Overall hemostasis potential (OHP) was assessed to follow the hemostatic disturbances, including the parameters for overall coagulation potential (OCP) and overall fibrinolytic potential (OFP). Fibrin clot turbidity was measured together with clot lysis time, and scanning electron microscopy was performed. Increased concentrations of $PS^+$, $CD42a^+$, $CD142^+$, $CD45^+$, $CD14^+$, and $CD62P^+$ EVs were found in plasma from patients with RA compared to healthy controls, and the concentrations of $PS^+$, $CD42a^+$, $CD14^+$, and $CD62P^+$ EVs were positively correlated with the inflammatory parameters in RA patients. Positive correlations were also found between the levels of $PS^+$ and $CD42a^+$ EVs and OCP as well as between the levels of $PS^+$, $CD42a^+$, and $CD62P^+$EVs and OHP. The levels of $PS^+$, $CD42a^+$, $CD14^+$, $CD62P^+$, and $CD62E^+$ EVs were negatively correlated with OFP. Elevated levels of circulating EVs of different cell origins were found in patients with established RA, in relation to the inflammatory burden and coagulation activation in the disease.

**Keywords: rheumatoid arthritis, extracellular vesicles, hemostasis, inflammation, fibrin structure**

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disease causing synovitis and destructive arthritis and is accompanied by out-of-joint disease manifestations, a strong systemic inflammatory response with accelerated development of atherosclerosis and shortened life expectancy and as such represents a significant burden for both individuals and society (1–5). The most common onset of illness is in the forties (6), with a significantly higher disease activity in the female population (7). Patients with RA have high prevalence of cardiovascular disease (CVD) as well as an increased risk of developing fatal cardiovascular events such as acute myocardial infarction, stroke, and heart failure (8, 9).

The pathogenesis of RA is complex and comprises interactions between genetics, epigenetic modifications, and environmental factors such as smoking, all of which contribute to the production of specific autoantibodies against citrullinated proteins (ACPAs), systemic inflammatory response, and joint destruction.

Recently, the role of microparticles was implicated in the pathogenesis of RA. Microparticles, also called extracellular vesicles (EVs), are small membrane-coated vesicles 0.1–1.0 μm in diameter that are released from various cells during cell activation and apoptosis. Depending on the cell of origin, EVs incorporate nuclear, cytoplasmic, and membrane molecules as they detach from the cells, and they express cellular antigens on their surface (10). EVs can be a source of autoantigens and can induce the formation of immune complexes or may be involved in the transfer of miRNA and inflammatory cytokines (11, 12). EVs also have important procoagulant properties based on the availability of phosphatidylserine (PS) exposed on the surface after stimulation (13).

A pivotal study revealed increased levels of platelet-derived EVs (PEVs) in plasma from RA patients, and these levels correlated with disease activity (14), while Boilard et al. demonstrated the presence of a large number of PEVs in the synovial fluid of RA patients (15). This might be a surprising finding because the origin of PEVs in the joint spaces is not known, thus suggesting platelet activation in the joint or the transit of PEVs. Stimulation of the collagen receptor glycoprotein VI on platelets seems to be the key trigger for the collagen-induced generation of PEVs in arthritis pathophysiology (15). However, some data suggest that EVs in the joint can originate from granulocytes or monocytes (16).

Higher proportions of leucocyte-derived EVs (LEVs) have been found in the plasma of RA patients compared to the plasma of patients with osteoarthritis (OA) and healthy controls (HCs), while increased levels of LEVs and monocyte and T-cell–derived EVs were also present in the synovial fluid of patients with RA compared to OA patients (12). Biro E et al. considered the role of complement in the pathogenesis of RA and detected LEVs expressing the complement components C1q, C4, and/or C3 in the synovial fluid of RA patients, but plasma levels of LEVs expressing complement were much lower in both patients with RA and in controls (17). Elevated levels of EVs expressing C1q were found in patients with early RA and remained increased even during the combination therapy with prednisolone, methotrexate, and sulfasalazine and despite improved disease activity as measured by DAS28 scores (18). Additionally, increased EV amounts correlated with the presence of traditional cardiovascular risk factors (diabetes mellitus, hypertension, dyslipidemia and obesity) in a cohort of 114 RA patients, and EVs isolated from RA patients were able to promote endothelial activation *in vitro* (19).

Still, the procoagulant effect of EVs has not previously been explored in patients with RA. This is of particular interest in patients with long-lasting disease and accumulated inflammatory burden leading to the activation of coagulation, diminished fibrinolysis, and accelerated prothrombotic condition. Considering the above observations, we aimed to identify different subpopulations of EVs in the plasma of female patients with established RA in relation to the hemostatic disturbances associated with chronic systemic inflammation in this disease.

## MATERIAL AND METHODS

### Study Participants

Twenty women with established RA referred to the outpatient clinic of the Department of Rheumatology, Clinical Centre Kragujevac, Serbia, were included in the study (mean age 51.85 ± 9.43 years). These patients were previously included as part of a larger study investigating hemostatic disturbances in women with RA (20). RA was diagnosed according to the classification criteria of the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 (21). The exclusion criteria comprised a history of diabetes mellitus, malignancy, or inflammatory bowel disease, liver or renal insufficiency, previous cerebrovascular or cardiovascular disorders (including inherited thrombophilia, antiphospholipid syndrome, and hyperhomocysteinemia), or venous thromboembolism.

RA assessments included a detailed medical history, the presence of extra-articular disease, current disease activity assessed by the 28-point disease activity score (DAS28) (22), and physical function using the Health Assessment Questionnaire (HAQ) (23). All current medications were recorded. Additionally, at the time of blood sampling the patients did not receive any anticoagulants, antithrombotic agents (acetylsalicylic acid), or non-steroidal anti-inflammatory drugs.

Twenty age and sex-matched healthy women (mean age 52.55 ± 7.27 years) were included in the study as healthy controls (HC). None of the women included in the study were taking oral contraceptives or receiving hormone replacement treatment.

Written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee of the Clinical Center Kragujevac prior to the onset of the study. The investigation was conducted in accordance with the principles outlined in the Declaration of Helsinki and principles of Good Clinical Practice.

### Blood Sampling

Blood was collected in tubes containing 0.129 M sodium citrate (BD Vacutainer Blood Collection System) using 21-gauge needles (BD Vacutainer needles). Platelet-poor plasma was obtained within 60 min of sampling by centrifugation at 2,000 × *g* for 15 min at room temperature, then divided into aliquots and stored frozen at –70°C until further analysis.

## Isolation of EVs

Platelet-poor plasma was thawed in a water bath for approximately 5 min (37°C) and thereafter centrifuged at 2,000 × *g* for 20 min at room temperature. The supernatant was then re-centrifuged at 13,000 g for 2 min at room temperature. The obtained supernatant was used for further analysis.

## Flow Cytometry Analyses of EVs

Subsequently, 20 µl of the supernatant was incubated for 20 min in the dark with lactadherin-FITC (BD Biosciences, USA) together with CD42a-PE (PEVs; BD Biosciences, USA), CD14-PE [Monocyte EVs (MoEVs); BD Biosciences, USA], CD45-APC [Leucocyte EVs (LEVs); BD Biosciences, USA), CD62P-PE (P-selectin, BD Biosciences, USA), CD62E-PE (Endothelial EVs – E-selectine; BD Biosciences, USA); CD142-PE (Tissue factor (TF), BD Biosciences, USA), CD144-PE (Endothelial EVs; BD Biosciences, USA).

EVs were measured by flow cytometry on a BD FACSCanto™ instrument. The MV-gate was determined using Megamix plus beads FSC (BioCytex, Marseille, France), which is a mix of beads with diameters of 0.1, 0.3, 0.5, and 0.1 µm. EVs were defined as vesicles <1.0 µm in size and positive for lactadherin. Conjugate isotype-matched immunoglobulins (IgG1-FITC, IgG1-PE, and IgG1-APC) with no reactivity against human antigens were used as negative controls to define the positive and negative gates. The absolute number of EVs was calculated by means of the following formula: Concentration (µl) = (Events reading × 550)/(44 × 20), where 550 µl is the total volume of sample in the tube analyzed, 44 µl is the volume of the analyzed sample over 90 s, and 20 µl is the volume of the plasma sample added in the tube (24). The concentration of EVs is expressed as $10^6$ EVs/l, and the intra and interassay coefficients of variation for EV measurements were less than 9%.

## Global Hemostatic Assays

### Determination of Overall Hemostatic Potential

We employed a modification of the assay described by He et al. (25) in order to assess overall hemostatic potential (OHP) in plasma. Absorbance (Abs) at 405 nm was measured every 12 seconds for 60 minutes, and the area under the curve was calculated by summation of the Abs values (Abs-sum) and expressed as the OHP value. Two additional parameters were also analyzed – the overall coagulation potential (OCP), determined as the area under the fibrin aggregation curve obtained without the addition of tissue plasminogen activator (t-PA), and the overall fibrinolysis potential (OFP), calculated as the difference between the two areas as OFP (%) = ((OCP – OHP)/OCP) × 100. The intra- and inter-assay coefficients of variation for OHP were 1.6% and 6.8% and for OCP were 1.2% and 5.7%, respectively. The detailed protocol was previously described (20).

## Determination of Clot Lysis Time

CLT was determined based on the fibrin aggregation curve for the determination of OHP and was defined as the time from the midpoint of the clear-to-maximum-turbid transition (which corresponds to the "clotting time") to the midpoint of the maximum turbid-to-clear transition.

## Analysis of Fibrin Clot Formation

Fibrin clot density was assessed with the turbidimetric clotting assay according to the method described by Carter et al. (26). The turbidimetric curve for determination of OCP was used to assess the maximum absorbance (Max Abs) as a measure of the clot density and defined as the increase in Abs from baseline to the maximum value (20).

## Scanning Electron Microscopy

The clots formed during fibrin generation for the determination of OCP were washed with PBS (phosphate-buffered saline) solution and fixed in 2.5% glutaraldehyde in Hepes-buffered saline for 60 min at room temperature and stored at 4°C. Samples were analyzed under an Ultra 55 field emission scanning electron microscope (Carl Zeiss, Oberkochen, Germany) at 3 kV. The detailed protocol was previously described (20).

## Routine Laboratory Analysis

Laboratory analyses of C-reactive protein (CRP) (turbidimetric method, Beckman Coulter AU680 analyser), erythrocyte sedimentation rate (ESR) (Westergren method, Vacuette ESR analyser), fibrinogen concentration (Clauss method, ACL TOP analyser by Instrumentation Laboratory), lipid profile (cholesterol, triglycerides, HDL, and LDL, all by Beckman Coulter AU680 analyser), rheumatoid factor (turbidimetric method, Beckman Coulter AU680 analyser), and ACPA (Roche electrochemiluminescence immunoassay, Cobas e411 analyser), were performed at the Central Laboratory of the Clinical Center Kragujevac.

## Statistical Analysis

All data were analyzed using SPSS 20.0 (IBM Corp. Released 2011), GraphPad Prism 5 (Version for Windows, GraphPad Software, La Jolla California, USA), and FlowJo software 8.7.1 (Treestar, Ashland, OR). The results are expressed as means (SD) or median (IQR) depending on the data type and distribution. Distribution of the data was checked by the Shapiro–Wilk test. Independent samples t-tests (parametric) and Mann–Whitney U-tests (non-parametric) were used to assess the differences in estimated variables between groups. A p-value <0.05 was regarded as statistically significant. Correlation between variables was examined using Spearman correlation analysis.

# RESULTS

## Characteristics of the Study Population

The mean disease duration in patients was 13.0 ± 6.6 years, and disease activity was medium to high (DAS28 was 4.1 ± 1.2) at the moment of blood sampling. The mean HAQ value was 1.2 ± 0.2, and the majority of patients were rheumatoid factor positive (n = 18; 90%) and ACPA positive (n = 20; 100%). All patients were treated with methotrexate (15–25 mg per week) and prednisolone (≤10 mg per day), and only two patients (10%) were in remission (DAS28<2.5). Patients were not previously treated with biologic

agents. Three patients had extra-articular manifestations in the form of rheumatoid nodules on the elbows and the small joints of the hands.

There were no differences between the HC and RA patients regarding the presence of traditional CVD risk factors or regarding lipid profile.

Patients with RA had higher CRP, ESR and fibrinogen levels compared to HC. Demographic and clinical characteristics of the participants as well as the presence of traditional CVD risk factors and laboratory parameters are presented in **Table 1**.

**TABLE 1** | Clinical characteristics and laboratory parameters of the study population.

| Subjects characteristics | Healthy controls | RA patients | p value |
|---|---|---|---|
| Number of patients | 20 | 20 | |
| Average age (years) | 52.5 ± 7.2 | 51.8 ± 9.4 | NS |
| BMI (kg/m$^2$) | 25.3 ± 3.5 | 25.6 ± 5.2 | NS |
| Disease duration (years) | NA | 13.0 ± 6.6 | / |
| DAS28 | NA | 4.1± 1.2 | / |
| HAQ | NA | 1.2 ± 0.2 | / |
| RF positive, n (%) | NA | 18 (90) | / |
| ACPA positive, n (%) | NA | 20 (100) | / |
| Ongoing treatment | | | |
| Prednisolone 10mg, n(%) | NA | 12 (60) | |
| Prednisolone 5mg, n(%) | NA | 8 (40) | |
| Methotrexate 15 mg, n(%) | NA | 7 (35) | |
| Methotrexate 20 mg, n(%) | NA | 11 (55) | |
| Methotrexate 25 mg, n(%) | NA | 2 (10) | |
| Smokers (n,%) | | | |
| Current smokers | 10 (50) | 7 (35) | NS |
| Non-smokers | 8 (40) | 12 (60) | NS |
| Past smokers | 2 (10) | 1 (5) | NS |
| Cholesterol (mmol/L) | 6.2 ± 1.1 | 5.8 ± 1.1 | NS |
| Triglycerides (mmol/L) | 1.4 ± 0.7 | 1.3 ± 0.5 | NS |
| HDL (mmol/L) | 1.56 ± 0.28 | 1.44 ± 0.24 | NS |
| LDL (mmol/L) | 4.0 ± 1.0 | 3.8 ± 1.1 | NS |
| ESR (mm/h) | 10.7 ± 8.5 | 29.0 (7.0-56.0) | p < 0.001 |
| CRP (mg/L) | 1.9 ± 1.0 | 6.8 (0.8-56.2) | p < 0.001 |
| Fibrinogen (g/L) | 3.1 ± 0.6 | 3.9 ± 0.5 | p < 0.001 |

*The values are expressed as means ± SD or median (IQR). BMI, body mass index; DAS28, Disease Activity Score 28; HAQ, Health Assessment Questionnaire; RF, rheumatoid factor; ACPA, citrullinated protein antigen; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein; NA, not applicable; NS, not significant.*

## Global Hemostatic Parameters

As presented in **Table 2**, OCP (Abs-sum) and OHP (Abs-sum) were significantly higher, while OFP (%) was significantly lower in RA patients compared to HC. Parameters of fibrin clotting showed higher Max Abs values and longer CLT in RA patients indicating increased clot turbidity and diminished fibrinolysis in these patients.

**TABLE 2** | Parameters of global hemostatic assays in the study population.

| Parameters | Healthy controls | RA patients | p value |
|---|---|---|---|
| OHP (Abs-sum) | 345.0 ± 42.8 | 390.4 ± 60.2 | p = 0.003 |
| OCP (Abs-sum) | 126.5 ± 28.1 | 166.3 ± 49.6 | p = 0.009 |
| OFP (%) | 63.4 ± 6.6 | 58.0 ± 8.6 | p = 0.032 |
| Max Abs | 1.6 ± 0.3 | 1.8 ± 0.2 | p = 0.037 |
| CLT (sec) | 21.5 ± 3.7 | 25.1 ± 4.4 | p = 0.008 |

*The values are expressed as means ± SD. OHP, Overall hemostasis potential; OCP, Overall coagulation potential; OFP, Overall fibrinolytic potential; Max Abs, increase in absorbance from baseline to maximum value; CLT, clot lysis time.*

## EVs

As presented in **Table 3** and **Figure 1**, the concentration of PS$^+$ EVs was significantly higher in RA patients compared to HC. Furthermore, PEVs, LEVs, MoEVs and EVs expressing TF and P-selectin were significantly higher in RA patients. EVs of endothelial origin (CD144$^+$ or CD62E$^+$) were higher in RA patients, but the difference was not statistically significant. The stratified analyses according to the daily corticosteroid dosage have not shown significant differences in the levels of investigated EVs.

## Representative Samples

Representative dot-plots for PS$^+$EVs (Lactadherin+ EVs) regardless of cellular origin, together with PEVs (CD42a+) as the most common EVs, are presented on **Figure 2**.

## Correlations

The correlations between the levels EVs and inflammatory parameters ESR and CRP as well as the parameters of the global hemostatic assays are presented in **Table 4**.

## Analysis of Clot Structure

Scanning electron microscopy showed dense fibrin structures in the plasma from an RA patient compared to the control sample. The clot from the RA patient was made of thinner fibers that were tightly packed into the network with smaller intrinsic pores and thus less susceptible to fibrinolysis (**Figure 3**), and this was similar to our previous findings of fibrin clots in women with RA in regard to the menopausal status (20).

Measurement of the fiber thickness of the representative samples was performed in five different areas, and the mean value of 10 fibers/area was used as the final measure of fiber thickness in these samples. Thinner fibers were found in the patient compared with control (136 nm ± 49 nm vs 157.0 ± 56.0 nm).

# DISCUSSION

We determined the presence of circulating EVs in relation to hemostatic disturbances in women with established RA, indicating the presence of a hypercoagulable state in these patients. Despite treatment, the patients with RA included in our study presented with ongoing inflammatory activity contributing to the extensive production of circulating EVs of platelet, leucocyte, and monocyte origin as well as increased expression of PS, TF, and P-selectin on the surface of EVs. In addition, amplified fibrin formation together with increased clot turbidity and diminished fibrinolysis was confirmed in these patients as measured by the global hemostatic parameters OCP, OHP, Max Abs, and OFP, respectively (20). We postulate that EVs play an important role in the delicate interaction between inflammatory processes and the activation of coagulation in the course of RA. The major advantage of our study is the investigation of circulating EVs of different origin in relation to hemostatic and inflammatory parameters in a

**TABLE 3** | The concentration of different subtypes of EVs in healthy controls and rheumatoid arthritis patients.

| EV concentration ($10^6$ EVs/l) | Healthy controls (n = 20) | Rheumatoid arthritis patients (n = 20) | p-value |
|---|---|---|---|
| $PS^+$ EVs | 934.4 (695.6–1479.0) | 1918.0 (950.0.5–3822.0) | p = 0.01 |
| PEVs ($CD42a^+$) | 218.1 (141.3–335.0) | 620.9 (202.2–1111.0) | p = 0.02 |
| $TF^+$ EVs ($CD142^+$) | 37.5 (26.2–48.7) | 50.3 (35.0–117.8) | p = 0.03 |
| LEVs ($CD45^+$) | 53.4 (43.4–62.2) | 83.1 (62.2–145.3) | p = 0.008 |
| MoEVs ($CD14^+$) | 74.7 (66.2–94.7) | 320.8 (235.0–527.5) | p < 0.001 |
| P-selectin ($CD62P^+$) | 12.0 (10.0–19.4) | 20.0 (14.3–28.7) | p = 0.008 |
| EEVs ($CD144^+$) | 35.0 (25.0–47.5) | 36.2 (26.8–72.2) | NS |
| E-selectin ($CD62E^+$) | 33.1 (22.5–52.5) | 60.3 (18.7–106.6) | NS |

*The results are expressed as median (IQR). NS, non-significant difference between the groups; EVs, extracellular vesicles; PS, phosphatidylserine; PEVs, platelet-derived EVs; LEVs, leucocyte-derived EVs; MoEVs, monocyte-derived EVs; EEVs, endothelial EVs.*

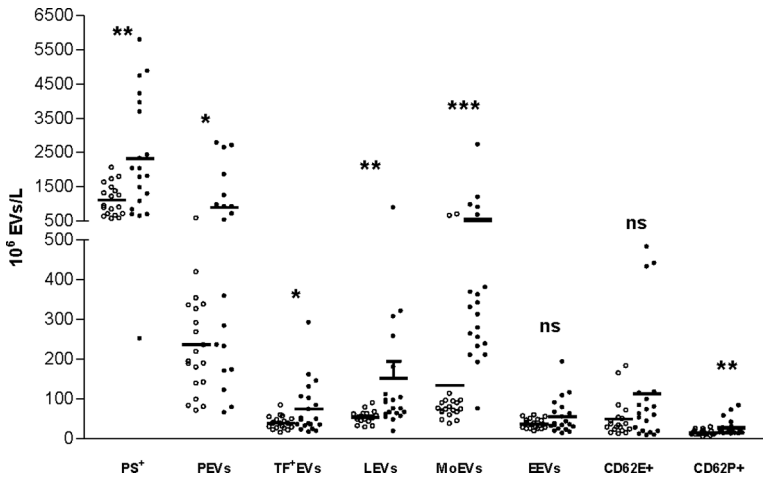


**FIGURE 1** | Distribution of different subtypes of EVs in HCs and patients with RA. EVs, extracellular vesicles; Ps, phosphatidylserine; TF, tissue factor; PEVs, platelet-derived EVs; LEVs, leucocyte-derived EVs; MoEVs, monocyte-derived EVs; EEVs, endothelial EVs. *p < 0.05; **p < 0.01; ***p < 0.001; ns, non-significant; white: healthy controls; black: rheumatoid arthritis.

homogenous group of RA patients in comparison to strictly matched control subjects.

While the formation of EVs is a physiological response to cell activation or cell death, a dramatic increase in circulating EVs has been detected in conditions associated with inflammatory states of different severity and different prothrombotic conditions (27–29). Identification of EV origin is vital because it indicates the dominant cell types involved in the pathogenesis of these disorders (14, 18, 30, 31). Several studies have tried to elucidate the role of circulating EVs as well as EVs detected in the synovial fluid in the pathogenesis of RA, as reviewed in Boilard et al. (32).

We have shown elevated levels of EVs expressing PS irrespective of the cell origin in plasma from patients with RA. The phospholipid membranes of EVs containing PS represent an adequate platform for the activation of coagulation processes leading to increased thrombin and fibrin formation, as measured by OCP and OHP (28, 29). Further, we present the association between the intensity of inflammation in RA and the concentration of released $PS^+$EVs. Thus, the negatively charged phospholipid membrane of EVs might play an important role in the interactions between the inflammatory state and procoagulant condition in the circulation of RA patients.

In line with previous studies, our results verify that the majority of circulating EVs in RA patients are of platelet origin (14, 29, 33), reflecting platelet activation in RA as an essential part of the inflammatory reaction. PEVs are involved in the interactions between platelets and inflammatory cells and in production of pro-inflammatory cytokines (31). Previously, a direct correlation was found between the levels of circulating PEVs and disease activity in RA patients (14, 29, 33). We were not able to confirm this association, probably due to the small number of investigated patients. Still, there was clear association of circulating PEVs with the inflammatory parameters, as well as the parameters of activation of coagulation and impaired fibrinolysis in our patients. As an additional marker of platelet activation, we noticed increased expression of P-selectin on circulating EVs in plasma from RA patients. P-selectin mediates the interaction between platelets and neutrophils (11) and may be a sign of ongoing platelet activation in RA, even if the disease is clinically silent (34). Amplified expression of P-selectin was also found in the synovial fluid of RA patients, though not as high as in the circulation (33).

Moreover, circulating PEVs have the ability to incorporate into the fibrin clots (35). We have recently confirmed the close interaction between PEVs and fibrin fibers, particularly at

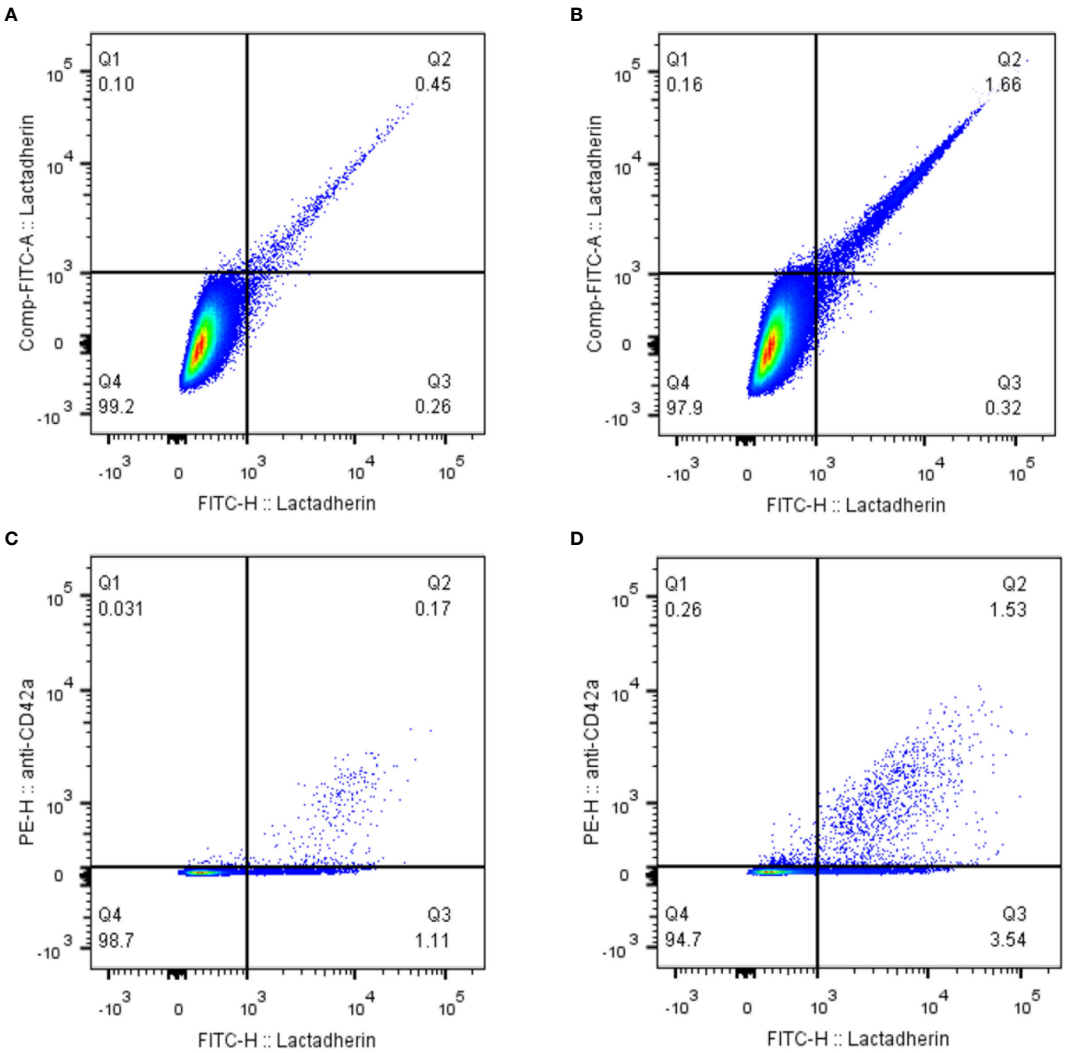


**FIGURE 2** | Representative samples of PS+EVs and PEVs. EVs positive for lactadherin ($PS^+$EVs) in a control sample **(A)** and RA patient **(B)**. Platelet-derived EVs in a control sample **(C)** and RA patient **(D)**. EVs, extracellular vesicles.

**TABLE 4** | Correlations between the levels of EVs and inflammatory parameters and the parameters of the global haemostatic assays.

| Parameters | CRP | ESR | OCP | OHP | OFP | Max Abs | CLT |
|---|---|---|---|---|---|---|---|
| $PS^+$EVs | p = 0.048 | p = 0.009 | p = 0.013 | p = 0.001 | p = 0.006 | p = 0.001 | p = 0.002 |
| | *r = 0.32* | *r = 0.41* | *r = 0.39* | *r = 0.51* | *r = –0.43* | *r = 0.511* | *r = 0.467* |
| PEVs ($CD42a^+$) | p = 0.031 | p = 0.007 | p = 0.039 | p = 0.002 | p = 0.006 | p = 0.004 | p = 0.006 |
| | *r = 0.34* | *r = 0.42* | *r = 0.33* | *r = 0.47* | *r = –0.43* | *r = 0.441* | *r = 0.426* |
| $TF^+$EVs ($CD142^+$) | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 |
| LEVs ($CD45^+$) | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p = 0.024 | p = 0.049 |
| | | | | | | *r = 0.355* | *r = 0.313* |
| MoEVs ($CD14^+$) | p = 0.001 | p = 0.005 | p > 0.05 | p > 0.05 | p = 0.031 | p = 0.002 | p = 0.005 |
| | *r = 0.51* | *r = 0.44* | | | *r = –0.34* | *r = 0.484* | *r = 0.439* |
| P-selectin ($CD62P^+$) | p = 0.015 | p = 0.004 | p > 0.05 | p = 0.014 | p = 0.015 | p > 0.05 | p = 0.007 |
| | *r = 0.38* | *r = 0.47* | | *r = 0.39* | *r = –0.38* | | *r = 0.420* |
| EEVs ($CD144^+$) | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 |
| E-selectin ($CD62E^+$) | p > 0.05 | p > 0.05 | p > 0.05 | p > 0.05 | p = 0.041 | p > 0.05 | p = 0.028 |
| | | | | | *r = –0.32* | | *r = 0.349* |

*EVs, extracellular vesicles; PS, phosphatidylserine; TF, tissue factor; PEVs, platelet-derived EVs; LEVs, leucocyte-derived EVs; MoEVs, monocyte-derived EVs; EEVs, endothelial EVs; OHP, Overall hemostasis potential; OCP, Overall coagulation potential; OFP, Overall fibrinolytic potential; Max Abs, increase in absorbance from baseline to maximum value.*

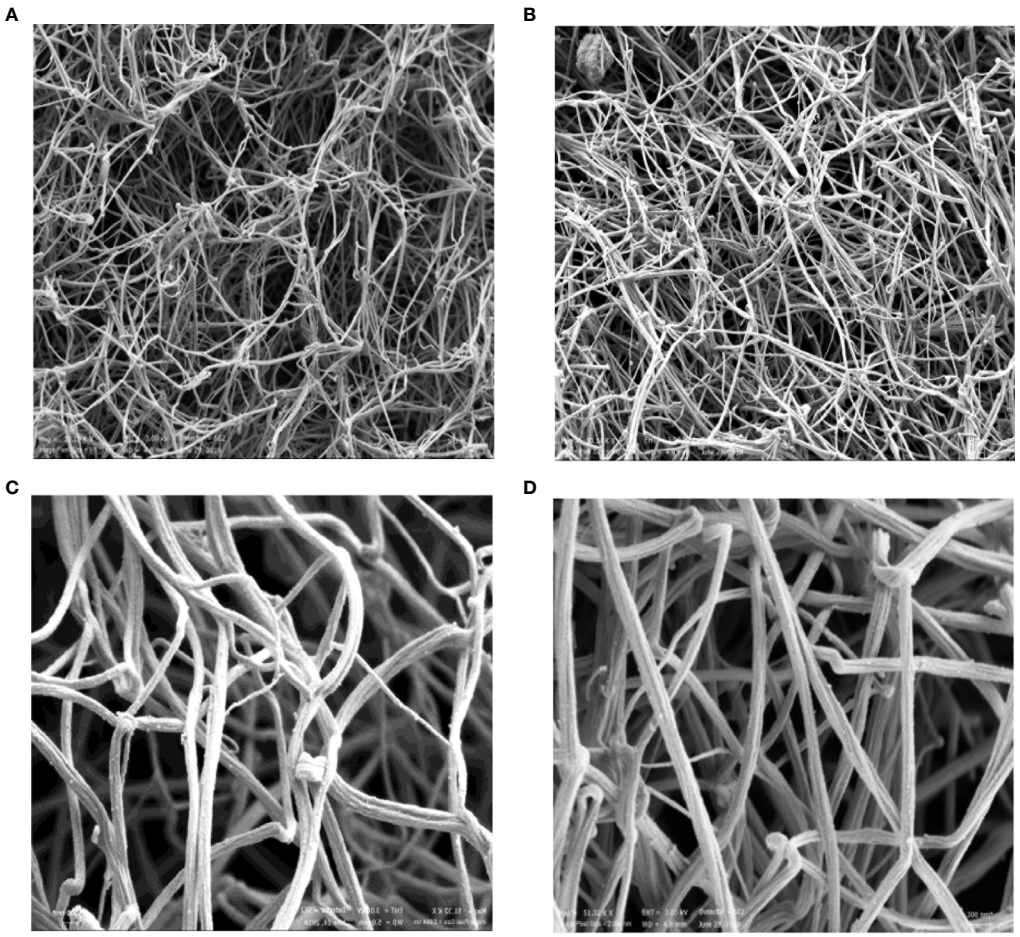


**FIGURE 3** | Scanning electron microscopic images of representative fibrin clots. HC **(A, C)** and RA patient **(B, D)**. The magnification 1 μm was used for **(A, B)** and 300 nm for **(C, D)**. HC, healthy control.

branch points and junctions (24). This finding may suggest intensified fibrin formation around the surface of PEVs, following the activation of platelets and the propagation phase of thrombin generation. Denser fibrin clots formed upon extensive platelet activation and augmented expression of PEVs in RA might therefore be revealed by the increased fibrin turbidity as measured by the Max Abs in our study. Scanning electron microscope pictures of denser fibrin composed of thinner fibers and with smaller intrinsic pores in RA patients confirm this finding, while decreased OFP together with prolonged CLT reflects the reduced fibrinolytic capacity as a consequence. Formatin of fibrin clots with a more prothrombotic phenotype in the presence of PEVs may be in agreement with the work of Knijff-Dutmeret et al. suggesting a possible association between PEVs and the development of CVD in patients with RA (14).

Elevated levels of circulating MoEVs were also found in the RA patients. The interactions between platelets and monocytes are well documented, indicating the accelerated generation of TFs by activated monocytes and the particularly important role of PEVs and MoEVs in this process (36–38). Therefore, our finding of increased TF expression on circulating EVs in RA patients is not surprising. Previously, higher proportions of MoEVs were reported in RA patients with high disease activity compared to patients in remission (39), and we confirmed the association with the inflammatory markers in our cohort. We believe that the elevated concentrations of PEVs together with MoEVs and EVs expressing P-selectin and TF could be potential biomarkers for the inflammatory and procoagulant activity in RA.

Apart from circulating EVs, synovial fluid obtained from the joints of RA patients contains EVs derived from monocytes, granulocytes, T-cells, B-cells, and even platelets (12, 15, 16, 40–43). The major disadvantage of our study is the lack of investigation of EVs in the synovial fluid. The majority of our patients presented with medium-high disease activity as measured by DAS-28 score, but a sufficient amount of synovial fluid was not available for sampling during the study period.

Systemic inflammation in RA is accompanied by activation of the vascular endothelium (44). Still, the levels of EVs expressing E-selectin and endothelium-derived EVs were not significantly

different in plasma from RA patients compared to HC. E-selectin might only be transiently expressed on endothelial cells in RA patients and therefore might be difficult to detect (34). In a study by Viñuela–Berni et al., the administration of immunosuppressive therapy (methotrexate, sulphasalazine, and low-dose glucocorticoids) led to a significant reduction of the plasma levels of $CD62E^+$ EVs (39). Further, Hjeltnes et al. showed that administration of methotrexate or a combination of methotrexate and TNFα inhibitor decreased the serum level of E-selectin (45), and the levels of endothelium-derived EVs levels increased and then decreased after four months of anti-TNFα therapy (46). The expression of endothelium-derived EVs in comparison to PEVs was rather low and might therefore be more sensitive to the ongoing immunosuppressive therapy in the investigated group of patients.

The important limitation of our study is small number of investigated subjects. However, the patients were well characterized for the presence of traditional cardiovascular risk factors and ongoing medications and had moderate to high disease activity, thus enabling an assessment in a real-life setting.

## CONCLUSION

We demonstrate elevated levels of circulating EVs in patients with established RA in relation to inflammatory burden and coagulation activation. Larger studies are needed to confirm these preliminary findings.

## 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 Ethics Committee of the Clinical Center Kragujevac, Serbia. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AS and MV collected samples. AS, IP, and YZ performed the experiments. AS and AA performed statistical analyses. AA and VJ designed the study. AA, VJ, and MV supervised the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES

1. Angelotti F, Parma A, Cafaro G, Capecchi R, Alunno A, Puxeddu I. One Year in Review 2017: Pathogenesis of Rheumatoid Arthritis. *Clin Exp Rheumatol* (2017) 35:368–78.
2. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The Global Burden of Rheumatoid Arthritis: Estimates From the Global Burden of Disease 2010 Study. *Ann Rheum Dis* (2014) 73:1316–22. doi: 10.1136/annrheumdis-2013-204627
3. Dye JR, Ullal AJ, Pisetsky DS. The Role of Microparticles in the Pathogenesis of Rheumatoid Arthritis and Systemic Lupus Erythematosus. *Scand J Immunol* (2013) 78:140–8. doi: 10.1111/sji.12068
4. Smolen JS, Aletaha D, McInnes IB. Rheumatoid Arthritis. *Lancet* (2016) 388:2023–38. doi: 10.1016/S0140-6736(16)30173-8
5. Talsania M, Scofield RH. Menopause and Rheumatic Disease. *Rheum Dis Clin North Am* (2017) 43:287–302. doi: 10.1016/j.rdc.2016.12.011
6. Oliver JE, Silman AJ. Why are Women Predisposed to Autoimmune Rheumatic Diseases? *Arthritis Res Ther* (2009) 11:252. doi: 10.1186/ar2825
7. Sokka T, Toloza S, Cutolo M, Kautiainen H, Makinen H, Gogus F, et al. Women, Men, and Rheumatoid Arthritis: Analyses of Disease Activity, Disease Characteristics, and Treatments in the QUEST-RA Study. *Arthritis Res Ther* (2009) 11(1):R7. doi: 10.1186/ar2591
8. Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al. Rheumatoid Arthritis. *Nat Rev Dis Primers* (2018) 4:18001. doi: 10.1038/nrdp.2018.1
9. Boyer JF, Gourraud PA, Cantagrel A, Davignon JL, Constantin A. Traditional Cardiovascular Risk Factors in Rheumatoid Arthritis: A Meta-Analysis. *Joint Bone Spine* (2011) 78:179–83. doi: 10.1016/j.jbspin.2010.07.016
10. Pisetsky DS, Ullal AJ, Gauley J, Ning TC. Microparticles as Mediators and Biomarkers of Rheumatic Disease. *Rheumatol (Oxford)* (2012) 51:1737–46. doi: 10.1093/rheumatology/kes028
11. Barbati C, Vomero M, Colasanti T, Ceccarelli F, Marcosano M, Miranda F, et al. Microparticles and Autophagy: A New Frontier in the Understanding of Atherosclerosis in Rheumatoid Arthritis. *Immunol Res* (2018) 66:655–62. doi: 10.1007/s12026-018-9053-0
12. Michael BNR, Kommoju V, Kavadichanda Ganapathy C, Negi VS. Characterization of Cell-Derived Microparticles in Synovial Fluid and Plasma of Patients With Rheumatoid Arthritis. *Rheumatol Int* (2019) 39:1377–87. doi: 10.1007/s00296-019-04337-1
13. Connor DE, Exner T, Ma DD, Joseph JE. Detection of the Procoagulant Activity of Microparticle-Associated Phosphatidylserine Using XACT. *Blood Coagul Fibrinolysis* (2009) 20:558–64. doi: 10.1097/MBC.0b013e32832ee915
14. Knijff-Dutmer EA, Koerts J, Nieuwland R, Kalsbeek-Batenburg EM, van de Laar MA. Elevated Levels of Platelet Microparticles are Associated With Disease Activity in Rheumatoid Arthritis. *Arthritis Rheum* (2002) 46:1498–503. doi: 10.1002/art.10312
15. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, et al. Platelets Amplify Inflammation in Arthritis Via Collagen-Dependent Microparticle Production. *Science* (2010) 327:580–3. doi: 10.1126/science.1181928
16. Berckmans RJ, Nieuwland R, Kraan MC, Schaap MC, Pots D, Smeets TJ, et al. Synovial Microparticles From Arthritic Patients Modulate Chemokine and Cytokine Release by Synoviocytes. *Arthritis Res Ther* (2005) 7:R536–44. doi: 10.1186/ar1706
17. Biró E, Nieuwland R, Tak PP, Pronk LM, Schaap MC, Sturk A, et al. Activated Complement Components and Complement Activator Molecules on the Surface of Cell-Derived Microparticles in Patients With Rheumatoid Arthritis and Healthy Individuals. *Ann Rheum Dis* (2007) 66:1085–92. doi: 10.1136/ard.2006.061309
18. van Eijk IC, Tushuizen ME, Sturk A, Dijkmans BA, Boers M, Voskuyl AE, et al. Circulating Microparticles Remain Associated With Complement Activation Despite Intensive Anti-Inflammatory Therapy in Early Rheumatoid Arthritis. *Ann Rheum Dis* (2010) 69:1378–82. doi: 10.1136/ard.2009.118372
19. Rodríguez-Carrio J, Alperi-López M, López P, Alonso-Castro S, Carro-Esteban SR, Ballina-García FJ, et al. Altered Profile of Circulating

Microparticles in Rheumatoid Arthritis Patients. *Clin Sci (Lond)* (2015) 128:437–48. doi: 10.1042/CS20140675
20. Vranic A, Pruner I, Veselinovic M, Soutari N, Petkovic A, Jakovljevic V, et al. Assessment of Hemostatic Disturbances in Women With Established Rheumatoid Arthritis. *Clin Rheumatol* (2019) 38:3005–14. doi: 10.1007/s10067-019-04629-8
21. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO3rd, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Ann Rheum Dis* (2010) 69:1580–8. doi: 10.1136/ard.2010.138461
22. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified Disease Activity Scores That Include Twenty-Eight-Joint Counts. Development and Validation in a Prospective Longitudinal Study of Patients With Rheumatoid Arthritis. *Arthritis Rheum* (1995) 38:44–8. doi: 10.1002/art.1780380107
23. Kirwan JR, Reeback JS. Stanford Health Assessment Questionnaire Modified to Assess Disability in British Patients With Rheumatoid Arthritis. *Br J Rheumatol* (1986) 25:206–9. doi: 10.1093/rheumatology/25.2.206
24. Zong Y, Pruner I, Antovic A, Taxiarchis A, Vila ZP, Soutari N, et al. Phosphatidylserine Positive Microparticles Improve Hemostasis in in-Vitro Hemophilia A Plasma Models. *Sci Rep* (2020) 10(1):7871. doi: 10.1038/s41598-020-64686-x
25. He S, Antovic A, Blombäck M. A Simple and Rapid Laboratory Method for Determination of Haemostasis Potential in Plasma. II. Modifications for Use in Routine Laboratories and Research Work. *Thromb Res* (2001) 103:355–61. doi: 10.1016/s0049-3848(01)00332-2
26. Carter AM, Cymbalista CM, Spector TD, Grant PJ. Euroclot Investigators. Heritability of Clot Formation, Morphology, and Lysis: The EuroCLOT Study. *Arterioscler Thromb Vasc Biol* (2007) 27:2783–9. doi: 10.1161/ATVBAHA.107.153221
27. Lacroix R, Dubois C, Leroyer AS, Sabatier F, Dignat-George F. Revisited Role of Microparticles in Arterial and Venous Thrombosis. *J Thromb Haemost* (2013) 11(Suppl 1):24–35. doi: 10.1111/jth.12268
28. Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-Derived Microparticles in Haemostasis and Vascular Medicine. *Thromb Haemost* (2009) 101:439–51. doi: 10.1160/TH08-08-0521
29. Sellam J, Proulle V, Jüngel A, Ittah M, Miceli Richard C, Gottenberg JE, et al. Increased Levels of Circulating Microparticles in Primary Sjögren's Syndrome, Systemic Lupus Erythematosus and Rheumatoid Arthritis and Relation With Disease Activity. *Arthritis Res Ther* (2009) 11(5):R156. doi: 10.1186/ar2833
30. Horstman LL, Jy W, Jimenez JJ, Bidot C, Ahn YS. New Horizons in the Analysis of Circulating Cell-Derived Microparticles. *Keio J Med* (2004) 53:210–30. doi: 10.2302/kjm.53.210
31. Šibíková M, Živný J, Janota J. Cell Membrane-Derived Microvesicles in Systemic Inflammatory Response. *Folia Biol (Praha)* (2018) 64:113–24.
32. Boilard E, Blanco P, Nigrovic PA. Platelets: Active Players in the Pathogenesis of Arthritis and SLE. *Nat Rev Rheumatol* (2012) 8:534–42. doi: 10.1038/nrrheum.2012.118
33. Xue LJ, Cui BB, Li X, Huang QR, Liu Y, Lin H. Association of Elevated Platelet Microparticles With Disease Activity in Rheumatoid Arthritis. *Sichuan Da Xue Xue Bao Yi Xue Ban* (2017) 48:405–9.
34. Pamuk GE, Vural O, Turgut B, Demir M, Pamuk ON, Cakir N. Increased Platelet Activation Markers in Rheumatoid Arthritis: Are They Related With Subclinical Atherosclerosis? *Platelets* (2008) 19:146–54. doi: 10.1080/09537100701210057
35. Zubairova LD, Nabiullina RM, Nagaswami C, Zuev YF, Mustafin IG, Litvinov RI, et al. Circulating Microparticles Alter Formation, Structure, and Properties of Fibrin Clots. *Sci Rep* (2015) 5:17611. doi: 10.1038/srep17611
36. Rinder HM, Bonan JL, Rinder CS, Ault KA, Smith BR. Activated and Unactivated Platelet Adhesion to Monocytes and Neutrophils. *Blood* (1991) 78:1760–9. doi: 10.1182/blood.V78.7.1760.bloodjournal7871760
37. Villar-Vesga J, Grajales C, Burbano C, Vanegas-García A, Muñoz-Vahos CH, Vásquez G, et al. Platelet-Derived Microparticles Generated *In Vitro* Resemble Circulating Vesicles of Patients With Rheumatoid Arthritis and Activate Monocytes. *Cell Immunol* (2019) 336:1–11. doi: 10.1016/j.cellimm.2018.12.002
38. Silverstein RL, Nachman RL. Thrombospondin Binds to Monocytes-Macrophages and Mediates Platelet-Monocyte Adhesion. *J Clin Invest* (1987) 79:867–74. doi: 10.1172/JCI112896
39. Viñuela-Berni V, Doníz-Padilla L, Figueroa-Vega N, Portillo-Salazar H, Abud-Mendoza C, Baranda L, et al. Proportions of Several Types of Plasma and Urine Microparticles Are Increased in Patients With Rheumatoid Arthritis With Active Disease. *Clin Exp Immunol* (2015) 180:442–51. doi: 10.1111/cei.12598
40. Cloutier N, Tan S, Boudreau LH, Cramb C, Subbaiah R, Lahey L, et al. The Exposure of Autoantigens by Microparticles Underlies the Formation of Potent Inflammatory Components: The Microparticle-Associated Immune Complexes. *EMBO Mol Med* (2013) 5:235–49. doi: 10.1002/emmm.201201846
41. György B, Szabó TG, Turiák L, Wright M, Herczeg P, Lédeczi Z, et al. Improved Flow Cytometric Assessment Reveals Distinct Microvesicle (Cell-Derived Microparticle) Signatures in Joint Diseases. *PloS One* (2012) 7:e49726. doi: 10.1371/journal.pone.0049726
42. Palmer DG, Hogg N, Revell PA. Lymphocytes, Polymorphonuclear Leukocytes, Macrophages and Platelets in Synovium Involved by Rheumatoid Arthritis. A Study With Monoclonal Antibodies. *Pathology* (1986) 18:431–7. doi: 10.3109/00313028609087564
43. Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, et al. Neutrophil-Derived Microvesicles Enter Cartilage and Protect the Joint in Inflammatory Arthritis. *Sci Transl Med* (2015) 7:315ra190. doi: 10.1126/scitranslmed.aac5608
44. Kuuliala A, Eberhardt K, Takala A, Kautiainen H, Repo H, Leirisalo-Repo M. Circulating Soluble E-selectin in Early Rheumatoid Arthritis: A Prospective Five Year Study. *Ann Rheum Dis* (2002) 61:242–6. doi: 10.1136/ard.61.3.242
45. Hjeltnes G, Hollan I, Førre O, Wiik A, Lyberg T, Mikkelsen K, et al. Serum Levels of Lipoprotein(a) and E-selectin are Reduced in Rheumatoid Arthritis Patients Treated With Methotrexate or Methotrexate in Combination With TNF-α-Inhibitor. *Clin Exp Rheumatol* (2013) 31(3):415–21.
46. Barbati C, Vomero M, Colasanti T, Diociaiuti M, Ceccarelli F, Ferrigno S, et al. Tnfα Expressed on the Surface of Microparticles Modulates Endothelial Cell Fate in Rheumatoid Arthritis. *Arthritis Res Ther* (2018) 20:273. doi: 10.1186/s13075-018-1768-8

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# *PADI2* Polymorphisms Are Significantly Associated With Rheumatoid Arthritis, Autoantibodies Serologic Status and Joint Damage in Women from Southern Mexico

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The enzymes of the family peptidylarginine deiminases (PADs) have an important role in the pathogenesis of rheumatoid arthritis (RA) due to their association with the anti-citrullinated protein antibodies (ACPA) production. To evaluate the association between single-nucleotide polymorphisms (SNPs) in the *PADI2* gene and RA susceptibility, related clinical parameters, and the serologic status of autoantibodies in a women population with RA from southern Mexico, a case-control study was conducted (case n=229; control n=333). Sociodemographic characteristics were evaluated, along with clinical parameters, inflammation markers, the levels of ACPAs as anti-cyclic citrullinated peptides (anti-CCPs), anti-modified citrullinated vimentin (anti-MCV), and rheumatoid factor (RF). Genomic DNA was extracted from peripheral blood, and three SNPs of the *PADI2* gene (rs1005753, rs2057094, and rs2235926) were performed by qPCR using TaqMan probes. The data analysis reveals that the carriers of the T allele for rs2057094 and rs2235926 presented an earlier onset of the disease ($\beta$= -3.26; $p$ = 0.03 and $\beta$ = -4.13; $p$ = 0.015, respectively) while the carriers of the T allele for rs1005753 presented higher levels of anti-CCPs ($\beta$= 68.3; $p$ = 0.015). Additionally, the T allele of rs2235926 was associated with a positive RF (OR = 2.90; $p$ = 0.04), anti-MCV (OR = 2.92; $p$ = 0.05), and with the serologic status anti-CCP+/anti-MCV+ (OR = 3.02; $p$ = 0.03), and anti-CCP+/anti-MCV+/RF+ (OR = 3.79; $p$ = 0.004). The haplotypes GTT (OR =1.52; $p$ = 0.027) and TTT (OR = 1.32; $p$ = 0.025) were associated with the presence of RA. In addition, in this study the haplotype TTT is linked to the presence of radiographic joint damage defined by a Sharp-van der Heijde score (SHS) ≥2 (OR = 1.97; $p$ = 0.0021) and SHS ≥3 (OR = 1.94; $p$ = 0.011). The haplotype TTT of SNPs rs1005753, rs2057094, and rs2235926 of the *PADI2* gene confers genetic susceptibility to RA and radiographic joint damage in women from southern Mexico. The evidence reveals that

SNPs of the *PADI2* gene favors the presence of a positive serologic status in multiple autoantibodies and the clinical manifestations of RA at an early onset age.

**Keywords: PADI2, polymorphisms, autoantibodies, radiologic damage, rheumatoid arthritis**

## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease with a variable prevalence amongst populations (1, 2). Clinical manifestations for RA can be from a mild self-limiting arthritis to a progressive multisystemic inflammatory arthritis with high morbidity and mortality (3, 4). In the RA physiopathology, enzymes of family peptidylarginine deiminases (PADs) have an important role in the citrullination of proteins that promote the antibody synthesis against citrullinated proteins (ACPAs) (5–8). The ACPAs positive status has been described as a predictive marker for the severity, the radiological degree (9, 10), joint damage, and the functional disability in patients with RA (11, 12). The growth of ACPAs aimed against citrullinated proteins' epitopes –histones, vimentin and enolase-derived peptides– and fibrinogen is mainly identified during pre-clinical stages of RA. Furthermore, the levels of ACPAs are correlated to the increase of proinflammatory cytokines (TNF-α, IL-6, IL-12p70, IFN-γ, IL-2, and IL-15) and high sensitivity C-reactive protein (hsCRP) (13). In addition, it has been stated that its positive status, along with the rheumatoid factor (RF), promotes the inflammatory process in RA (14).

The expression of the PAD2 and PAD4 isoenzymes have been identified in synovial tissue and fluid in RA patients (15, 16). Although PADs exhibit a limited expression in some tissues, PAD2 is considered the broader expressed isoform (17), and the specificity of its substratum could be related to the clinic phenotype and serologic variability observed in RA patients. It was determined that PAD2 possesses specificity against β/γ-actin, myelin basic protein, histones (H3R26), vimentin, and the glial fibrillary acidic protein (6, 18–21). Meanwhile, for PAD4, the main protein substrates are histones (H2A, H3R2, H3R8, H3R17, H3R26, and H4R3), nuclear lamin C, nucleophosmin/B23, p300/CBP, p21, and inhibitor of growth 4 (6, 22–27). Particularly, high levels of PAD2 have been reported in synovial fluid of RA patients (28–31), as well as their correlation with inflammation markers, the disease's clinical activity, and the anti-CCPs levels (32).

On the other hand, the influence of genetic factors on the modulation of the expression and function of PADs has been proven (8), and, even though the role of single-nucleotide polymorphisms (SNPs) and of a functional haplotype in *PADI4* has been established, few studies that have determined the role of SNPs in *PADI2* in the genetic susceptibility to RA or its association with the serologic status and the clinical parameters that are related to the disease. Lee et al. reported that *PADI2* could be a candidate-like gene for RA (33) and, in some populations, it was determined that SNPs rs2076596 (34), rs1005753 (35), rs2057094, and rs2235926 (29) in the *PADI2* gene are associated with the presence of RA.

This study aims to evaluate the association between SNPs, rs1005753, rs2057094, and rs2235926 of the *PADI2* gene and RA susceptibility as the relation with the clinical parameters, inflammation markers, and the serologic status for antibodies in a women population from southern Mexico.

## MATERIALS AND METHODS

### Subject Selection

A case-control study was carried out in RA patients ($n$ = 229 women). These patients were diagnosed with RA according to the ACR/EULAR criteria 2010 (36). The control group ($n$ = 333 women) was treated for external causes to RA, autoimmune disease, musculoskeletal disease, or cancer in the General Hospital Dr. Raymundo Abarca Alarcón, in Chilpancingo, in the state of Guerrero, Mexico. The case and control subjects were recruited during the period from December 2017 to December 2019. The study was approved by the Research Ethics Committee of the Autonomous University of Guerrero, Mexico (approval code CB-004/2017). All patients agreed to participate and gave their informed consent in writing.

### Clinical Assessment

All of the patients were surveyed to obtain sociodemographic data. The clinical and treatment characteristics were evaluated during the consultation and from the clinical file. In this study "Patients of recent diagnostic" refers to those patients without pharmacological prescription for anti-rheumatic treatment to the date of the sample obtainment.

The rheumatologist performed a clinical evaluation and counted the number of inflamed and painful joints. The patient indicated the level of perception of health status and level of pain perception through a visual analog scale. Rheumatoid arthritis disease activity and the disability level were evaluated through the Disease Activity Score 28 (DAS28), and the Spanish version of the Health Assessment Questionnaire (HAQ-DI), respectively.

To determine joint damage, a radiographic evaluation was performed to observe features of destructive and proliferative changes, and radiological damage. According to Sharp-van der Heijde Score (SHS), this method reviews plain films of 8 proximal interphalangeal joints, 2 interphalangeal thumb joints, 10 metacarpophalangeal joints, and both wrists. The method defines 5 categories: 0 = normal; 1 = asymmetrical or minimal narrowing up to a maximum of 25%; 2 = definite narrowing with loss of up to 50% of the normal space; 3 = definite narrowing with loss of 50-99% of the normal space or subluxation; and, 4 = absence of joint space, presumptive evidence of ankylosis, or complete luxation (37).

## Assay of RA-Related Antibodies and Inflammation Markers

Using a venous blood sample, the erythrocyte sedimentation rate (ESR) was analyzed by the Wintrobe method. Serum samples were used to determine the levels of high sensitivity C- reactive protein (hsCRP) and rheumatoid factor (RF) isotype IgG, using the immunoturbidimetry technique in the automatized reader (COBAS C311, Roche Diagnostics GmbH, Germany). In addition, the anti-cyclic citrullinated peptide (anti-CCPs) and anti-modified citrullinated vimentin (anti-MCV) autoantibodies were measured with a second-generation ELISA kit (DIASTAT anti-CCP Axis-Shield, Dundee, United Kindom; and ORGENTEC Diagnostika GmbH, Mainz, Germany, respectively). Values >20 UI/mL were considered positive for RF, values >5 U/mL for anti-CCPs, and >20 U/mL for anti-MCV, according to the manufacturer instructions.

## DNA Extraction and TaqMan Genotyping

Genomic DNA was extracted from peripheral blood and the SNPs were genotyped by allele discrimination using commercial TaqMan probes (Applied Biosystems, San Francisco, CA, USA). The evaluated SNPs were C_2190445_20 (rs1005753/Intron, cat. 4351379), C_11647256_20 (rs2057094/Intron, Cat.4351379), and C_2190476_1_ (rs2235926/3´UTR, Cat.4351379). These were evaluated using quantitative polymerase chain reaction (qPCR) in a 7300 Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific Inc., Singapore), following the instructions by the manufacturer. The thermal cycling was performed by denaturation at 60°C for 30 sec, followed by 40 cycles of 95°C for 10 min and 95°C for 15 sec, and alignment and extension at 60°C 1 min and 4°C. Genotype analysis was performed through the sequence detection software (SDS) version 2.3 (Applied Biosystems, CA, United States).

## Statistical Analysis

The categorical variables were expressed as numbers and proportions, and they were compared using a Chi-squared test. For quantitative variables, median and percentiles p5-p95th were used according to the Mann Whitney test to compare groups. Allele and genotype frequencies were calculated by direct counting. The differences in the distributions of allele and genotype frequencies between cases and controls, and the associations between clinical characteristics in patients with RA, were performed using a Chi-square test and a logistic regression. The Hardy-Weinberg equilibrium (HWE) was assessed in both groups. The effect of polymorphisms on clinical assessment and serum autoantibodies levels and other clinical parameters was tested using β coefficient and their standard errors, adjusted by age, treatment with disease-modifying anti-rheumatic drugs (DMARDs) and wood smoke exposure. Statistical analysis was carried out using the Stata version 13.0 (StataCorp, College Station, TX, USA). The association and pairwise measure of linkage disequilibrium of the SNPs rs1005753, rs2057094, and rs2235926 of PADI2 was calculated using SHEsis software (38) (**Figure 1**). The association between SNPs and clinical parameters and serologic status was determined using logistic regression models, settling odds ratios (ORs), and 95% confidence intervals (95% CI). Results were considered significant at $p$ <0.05.

# RESULTS

## Demographic Data, Clinical Features, and Serologic Status

The sociodemographic and clinical characteristics between the case and control groups are presented in **Table 1**. The participants with RA displayed a period of evolution of the disease with a median of 7 years, along with a low functional disability HAQ-DI score. At the same time, they also presented a moderate clinical activity and a radiographic joint damage index that refers to the presence of the narrowing of the intra-articular space and bone erosion, even though most of them were pharmacologically treated with DMARDs. Furthermore, the autoantibodies' levels (RF, anti-CCPs, and anti-MCV) were elevated. When comparing the positive serologic status to the antibodies, more than 85% of the subjects were positive, with a predominant positive status for anti-MCV (89.1%). After evaluating the serologic status of combined positivity, it was found that 83.8% were anti-CCPs+/anti-MCV+ and that 75.1% were positive for ACPAs and RF (anti-CCP+/anti-MCV+/RF+) (**Table 2**).

## Frequency of *PADI2* Gene SNPs and Haplotype Analysis

The genotypical distribution of the SNPs rs1005753 (T>G), rs2057094 (T>C), and rs2235926 (T>C) of the *PADI2* gene was in Hardy-Weinberg genetic equilibrium in both studied groups ($p$ > 0.5). The genotypical and allele frequencies are shown in **Table 3**. The T allele of rs2057094 was marginally associated with RA susceptibility (OR = 1.27; 95% CI, 0.99-1.65; $p$ = 0.05), however, the haplotypes TTT (OR=1.32; 95% CI, 1.03-1.67; $p$ = 0.025) and GTT (OR = 1.52; 95% CI, 1.04-2.19; $p$ = 0.027) were significantly associated with RA susceptibility (**Table 4**).

## Association of Clinical Parameters and Serologic Status According to the SNPs and Haplotype of the *PADI2* Gene in RA Patients

In this study, we evaluated the potential effect and the association between *PADI2*'s SNPs and clinical parameters, inflammation markers, and the serologic status in opposition to autoantibodies in RA patients. The presence of the T allele of SNPs rs2057094 and rs2235926 was associated with the presence of the disease's clinical signs at a younger age (β = -3.26; $p$ = 0.03 and β = -4.13; $p$ = 0.02, years respectively), while the presence of the T allele of rs1005753 was associated with the increase of the anti-CCPs levels (β = 68.3; $p$ = 0.01) (**Table 5**).

When analyzing the association between SNPs and the autoantibodies' serologic status, the carriers of the T allele of SNP rs2057094 exhibited a tendency to associate with the presence of positive RF (OR = 2.5; 95% CI, 0.93-6.6; $p$ = 0.06)

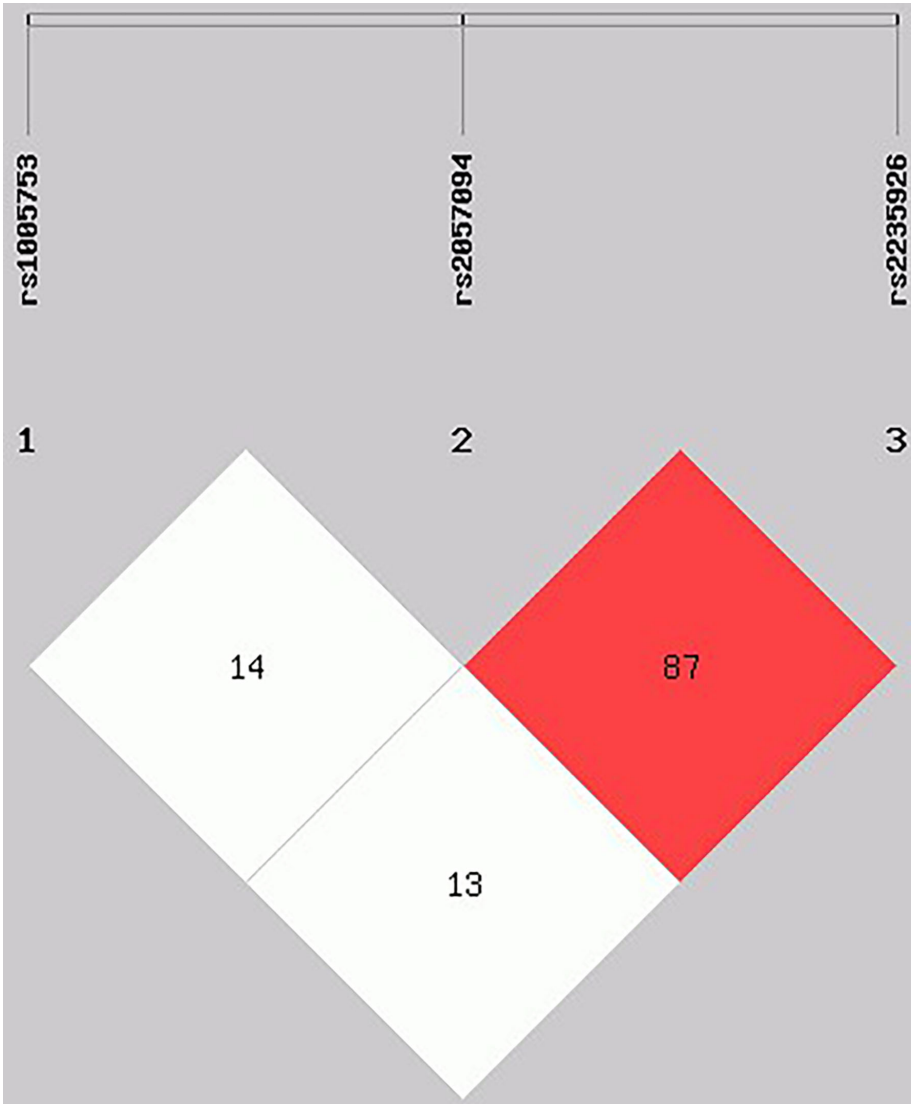


**FIGURE 1** | Linkage disequilibrium (LD) test of *PADI2* gene SNPs in RA patients. Haplotype frequencies and LD were calculated using SHEsis software. Red area represents higher levels of LD. A D′ value of 100 indicates a complete LD between two markers and a D′ value of 0 indicates complete linkage equilibrium.

and with the positive status for ACPAs and RF (anti-CCP+/anti-MCV+/RF+, OR = 2.23; 95% CI, 0.96-5.18; $p = 0.06$). In addition, the genotypes TT+TC of rs2235926 were related to the individual positivity to RF (OR = 2.90; 95% CI, 1.02-8.26; $p = 0.04$), and to anti-MCV (OR = 2.92; 95% CI, 0.96-8.9; $p = 0.05$), as well as with the positive status for anti-CCP+/anti-MCV+ (OR = 3.02; 95% CI, 1.11-8.23; $p = 0.03$) and anti-CCP+/anti-MCV+/RF+ (OR = 3.79; 95% CI, 1.51-9.5; $p = 0.004$) (**Table 6**).

Finally, in the association analysis among haplotypes of *PADI2* gene and the clinical characteristics of the disease, the haplotype TTT was significantly related with the presence of joint damage defined by a SHS score ≥ 2 (OR = 1.97; 95% CI, 1.27-3.05; $p = 0.002$) and SHS ≥3 (OR = 1.94; 95% CI, 1.15-3.19; $p = 0.011$).

## DISCUSSION

Our finds prove the potential role of *PADI2* single-nucleotide variants in the clinical heterogeneity of RA in a women population of southern Mexico. Chang et al. studied a Chinese population and proved that SNPs rs2235926 (OR = 1.57; $p < 0.001$) and rs2057094 (OR = 1.36; $p = 0.003$) conferred RA susceptibility (29). In our study, these SNPs were not associated individually with RA, but they were associated with the presence of high levels of anti-CCPs and a positive status for anti-CCPs+/anti-MCV+/RF+. In the *PADI4* gene, the presence of SNPs and a functional haplotype has been described as a factor for genetic susceptibility to RA in different populations, including the Mexican (39–41). In the Mexican population, the GTG haplotype in *PADI4* was associated with the

**TABLE 1** | Demographics and clinical characteristics in the study population.

| Characteristics | RA *n* = 229 | CS *n* = 333 | *p*-value |
|---|---|---|---|
| **Sociodemographic features** | | | |
| Age, years, median ($P_5$-$P_{95}$)[a] | 44 (24-70) | 50 (25-71) | 0.003 |
| Socioeconomic level, *n* (%)[b] | | | 0.59 |
| Medium | 51 (22.3) | 68 (20.2) | – |
| Low | 178 (77.7) | 265 (79.8) | – |
| Wood smoke exposure, *n* (%)[b] | 139 (60.7) | 138 (41.4) | <0.001 |
| Smoking, *n* (%)[b] | 6 (2.62) | 15 (4.5) | 0.24 |
| **Serological features** | | | |
| RF, IU/mL, median ($P_5$-$P_{95}$)[a] | 182 (0-306) | | |
| Anti-CCP, U/mL, median ($P_5$-$P_{95}$)[a] | 50.8 (0.25-243) | | |
| Anti-MCV, U/mL, median ($P_5$-$P_{95}$)[a] | 83.5 (14-620) | | |
| **Clinical assessment** | | | |
| Disease evolution, years, median ($P_5$-$P_{95}$)[a] | 7 (1-26) | – | – |
| hsCRP, mg/L, median ($P_5$-$P_{95}$)[a] | 7 (0.16-60.2) | – | – |
| ESR, mm/hr, median ($P_5$-$P_{95}$)[a] | 31 (9-56) | 33 (13-55) | 0.03 |
| DAS28-ESR, score, median ($P_5$-$P_{95}$)[a] | 3.71 (1.99-7.67) | – | – |
| DAS28-ESR, *n* (%)[b] | | | |
| Remission | 40 (17.5) | – | – |
| Low activity | 52 (22.8) | – | – |
| Moderate activity | 73 (31.6) | – | – |
| High activity | 64 (28.1) | – | – |
| HAQ-DI, score, median ($P_5$-$P_{95}$)[a] | 0.3 (0.-1.52) | – | – |
| SHS, median ($P_5$-$P_{95}$)[a] | 2 (1-4) | – | – |
| **Current therapy scheme**[b] | | | |
| Recent diagnostic, *n* (%)[b] | 66 (28.8) | – | – |
| Monotherapy DMARDs, *n* (%)[b] | 40 (17.5) | – | – |
| Combination DMARDs, *n* (%)[b] | 123 (53.7) | – | – |

*Anti-CCPs, anti-cyclic citrullinated peptide antibodies; Anti-MCV, Antibodies against modified citrullinate vimentin, CS, control subjects; DAS28, disease activity score 28; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire disability index; hsCRP, high sensibility protein c reactive; RA, rheumatoid arthritis; RF, rheumatoid factor; SHS, Sharp-van der Heijde Score.*

[a]*Data are expressed as the median and percentiles 5th-95th.*

[b]*Data are expressed as the n (%), compared using Chi-square test.*

*p-value < 0.05 was considered statistically significant.*

**TABLE 2** | Serologic pattern status in RA patients.

| Characteristics | *n* = 229 |
|---|---|
| **Positive antibodies** | |
| RF+, >20 IU/mL, *n* (%) | 199 (86.9) |
| Anti-CCP+, >5 U/mL, *n* (%) | 198 (86.45) |
| Anti-MCV+, > 20 U/mL, *n* (%) | 204 (89.1) |
| **ACPA status** | |
| Anti-CCP+/Anti-MCV+, *n* (%) | 192 (83.84) |
| Anti-CCP-/Anti-MCV-, *n* (%) | 19 (8.3) |
| Anti-CCP+/Anti-MCV-, *n* (%) | 6 (2.62) |
| Anti-CCP-/Anti-MCV+, *n* (%) | 12 (5.24) |
| **ACPA/RF status** | |
| Anti-CCP+/Anti-MCV+/RF+, *n* (%) | 172 (75.1) |
| Anti-CCP+/Anti-MCV+/RF-, *n* (%) | 20 (8.73) |
| Anti-CCP-/Anti-MCV+/RF+, *n* (%) | 12 (5.24) |
| Anti-CCP-/Anti- MCV-/RF+, *n* (%) | 11 (4.8) |
| Anti-CCP-/Anti-MCV-/RF-, *n* (%) | 8 (3.5) |
| Anti-CCP+/Anti-MCV-/RF+, *n* (%) | 4 (1.75) |
| Anti-CCP+/Anti-MCV-/RF-, *n* (%) | 2 (0.87) |

*ACPAs, anti-citrullinated protein antibodies; Anti-CCPs, anti-cyclic citrullinated peptide antibodies; Anti-MCV, Antibodies against modified citrullinate vimentin; RA, rheumatoid arthritis; RF, rheumatoid factor.*

*Data are expressed as the n (%).*

RA emergence in ages ≤40 years old and with elevated anti-CCPs levels (40), as well as with anti-MCV antibodies, which are related with the increase of inflammatory cytokines levels and the RA DAS28 score (42). In this study, we found that the T allele of rs2235926 and rs2057094 in *PADI2* was associated with the rise of RA in ages younger than 40 years old. Even though the age of onset for RA is variable, in the Mexican population, the highest point of most incidence fluctuates between 56-65 years old (2), similar to what was described in a Chinese population where it fluctuates between 60-70 years of age (43). It has been described that the citrullination process precedes the appearance of the disease's clinical signs (44, 45), therefore the genetic susceptibility attributed to the *PADIs* genes involved in the protein citrullination process and of its interaction with other genetic and environmental factors could determine the clinical appearance of RA in ages younger than the largest incidence peak of the disease.

Particularly for our population, the observed seropositivity for ACPAs (>80%), as well as the combined positivity for anti-CCP +/anti-MCV+RF+ (75.1%) is high, especially when compared to that described in other populations where seropositivity oscillates between 38% and 53.9% (42, 46). Anti-CCPs positivity is considered a predictive marker for structural joint damage in RA patients (47), and for higher radiographic and inflammation progression (48–50). In this study, the TTT haplotype of the studied SNPs of the *PADI2* gene was associated with a representative score for joint damage, and particularly, the SNP rs2235926 with the ACPAs positivity (anti-CCPs+/anti-MCV+)

**TABLE 3 |** Genotypic and allele frequencies of polymorphisms in *PADI2* gene.

| SNP | RA *n* = 229 | CS *n* = 333 | OR (95% CI), *p*-value |
|---|---|---|---|
| rs1005753 | | | |
| TT, *n* (%) | 126 (55.0) | 195 (58.7) | 1.0* |
| TG, *n* (%) | 88 (38.4) | 121 (36.3) | 1.12 (0.78-1.6), 0.51 |
| GG, *n* (%) | 15 (6.6) | 17 (5.1) | 1.36 (0.65-2.8). 0.40 |
| Allele | | | |
| T, *n* (%) | 340 (74.2) | 511 (76.7) | 1.0* |
| G, *n* (%) | 118 (25.8) | 155 (23.3) | 1.14 (0.86-1.52), 0.33 |
| HWE $X^2$, *p-value* | $X^2$ = 0.005; *p* = 0.94 | $X^2$ = 0.10; *p* = 0.75 | |
| rs2057094 | | | |
| TT, *n* (%) | 106 (46.3) | 120 (36.0) | 1.0* |
| TC, *n* (%) | 93 (40.6) | 166 (49.9) | 0.63 (0.44-0.91), **0.014** |
| CC, *n* (%) | 30 (13.1) | 47 (14.1) | 0.72 (0.42-1.22), 0.22 |
| Allele | | | |
| T, *n* (%) | 305 (66.6) | 406 (61.0) | 1.0* |
| C, *n* (%) | 153 (33.4) | 260 (39.0) | 0.78 (0.60-1.0), **0.05** |
| HWE $X^2$, *p-value* | $X^2$ = 1.74; *p* = 0.18 | $X^2$ = 0.74; *p* = 0.38 | |
| rs2235926 | | | |
| TT, *n* (%) | 100 (43.7) | 135 (40.5) | 1.0* |
| TC, *n* (%) | 107 (46.7) | 156 (46.9) | 0.92 (0.65-1.32), 0.67 |
| CC, *n* (%) | 22 (9.6) | 42 (12.6) | 0.70 (0.39-1.25), 0.23 |
| Allele | | | |
| T, *n* (%) | 307 (67.03) | 426 (63.96) | 1.0* |
| C, *n* (%) | 151 (32.97) | 240 (36.04) | 0.87 (0.67-1.13), 0.28 |
| HWE $X^2$, *p-value* | $X^2$ = 0.74; *p* = 0.38 | $X^2$ = 0.09, *p* = 0.76 | |

*CI, confidence interval CS, control subjects; HWE, Hardy-Weinberg Equilibrium; OR, odds ratio; RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.*
*Data n (%), compared using Chi-square test. Logistic regression calculated OR and 95% CI. *1.0 Reference category. p values were calculated by logistic regression comparisons with the refence category.*
*p-value < 0.05 was considered statistically significant.*
*Bold values represent statistically significant data.*

**TABLE 4 |** Haplotype frequencies of three *PADI2* SNPs in RA and CS.

| Haplotypes | RA *n* = 229 *n* (%) | CS *n* = 333 *n* (%) | OR (95% CI), *p*-value |
|---|---|---|---|
| H1: 111 TTT | 229.83 (50.0) | 288.77 (0.43) | 1.32 (1.03-1.67), **0.025** |
| H2: 112 TTC | 5.62 (0.012) | 42.96 (0.064) | 0.18 (0.07-0.43), **0.00002** |
| H3: 121 TCT | 6.72 (0.015) | 36.52 (0.055) | 0.26 (0.11-0.58), **0.0005** |
| H4: 122 TCC | 97.83 (0.21) | 142.74 (0.21) | 0.99 (0.74-1.32), 0.96 |
| H5: 211 GTT | 62.79 (0.13) | 63.11 (0.09) | 1.52 (1.04-2.19), **0.027** |
| H6: 212 GTC | 6.75 (0.01) | 11.16 (0.01) | ND |
| H7: 221 GCT | 7.66 (0.01) | 37.6 (0.05) | 0.28 (0.12-0.62), **0.0008** |
| H8: 222 GCC | 40.79 (0.09) | 43.14 (0.06) | 1.41 (0.90-2.20), 0.13 |

*CI, confidence interval; CS, control subjects; H, haplotype; ND, not determinate; OR, odds ratio; RA, rheumatoid arthritis; SNPs, single-nucleotide polymorphisms.*
*The SNPs are listed in the order: PADI2 rs1005753_T>G, rs2057094_T>C and rs2235926_T>C. The OR and 95% CI and p values were obtained by SHESIS test.*
*p-value < 0.05 was considered statistically significant.*
*Bold values represent statistically significant data.*

and ACPAs/RF (anti-CCPs+/anti-MCV+/RF+). Furthermore, the seropositivity to multiple autoantibodies of the ACPAs type and RF isotopes is associated with the radiological progression and erosive RA (51), just as with the high levels of C-reactive protein (CRP) and proinflammatory cytokines in RA patients (13, 52–54). In an *in vitro* model, it was proven that the RF from isotype IgM favors the increase of the anti-CCPs levels and the cytokine production, thus suggesting that a positive serologic status for RF and anti-CCPs contributes to the pathogenesis of RA (14), as well as to the clinical diversification of the disease (12). On the other hand, the anti-MCV antibodies are considered better predictors of the disease's severity (55), high clinical activity, and radiographic joint damage and progression (53, 56), given that it increases up to 7.3 times the risk of radiographic progression (10) when compared to anti-CCPs. In our study, the common allele of SNP rs2235926 was associated with the positivity for anti-MCV.

The peptide repertoire that is susceptible to citrullination by PAD2 is more extensive in comparison to PAD4. Assohou-Luty et al. characterized 320 citrullination sites for PAD2 and 178 sites for PAD4, thus proving the specificity variability of these enzymes to their substrate (57). Two main regions have been described in PADs that are involved in the substratum selection, the N-terminal domain, and the cleft of the active site. In PAD4, the Arg-374 contributes to recognizing the substratum and its structural formation, while in PAD2 and PAD3, the amino acid is Gly-374 (58). In this study, we found a relation between positivity to anti-

**TABLE 5** | Effect of *PADI2* SNPs on the clinical characteristics in RA patients.

| Characteristics | rs1005753 | | rs2057094 | | rs2235926 | |
|---|---|---|---|---|---|---|
| | †GG *vs* TT+TG β 95% CI, *p*-value | †TT *vs* GG+TG β 95% CI, *p*-value | †CC *vs* TT+TC β 95% CI, *p*-value | †TT *vs* CC+TC β 95% CI, *p*-value | †CC *vs* TT+TC β 95% CI, *p*-value | †TT *vs* CC+TC β 95% CI, *p*-value |
| Age at diagnosis, years | -1.45 (-5.49, 2.59), 0.48 | 2.11 (0.12, 4.11), **0.04** | -3.26 (-6.2, -0.32), **0.03** | 0.32 (-1.67, 2.32), 0.75 | -4.13 (-7.46, -0.81), **0.02** | 0.04 (-1.96, 2.06), 0.96 |
| ESR, mm/hr | 2.43 (-5.49, 10.35), 0.54 | 1.24 (-2.68, 5.16), 0.53 | 1.94 (-3.85, 7.75), 0.50 | 2.18 (-1.72, 6.9), 0.27 | 3.39 (-3.18, 9.97), 0.31 | 1.89 (-2.04, 5.83), 0.34 |
| DAS28-ESR, score | 0.47 (-0.37, 1.33), 0.27 | 0.18 (-0.24, 0.61), 0.39 | 0.16 (-0.47, 0.80), 0.61 | 0.31 (-0.10, 0.74), 0.14 | 0.09 (-0.63, 0.82), 0.80 | 0.28 (-0.14, 0.71), 0.18 |
| HAQ-DI, score | 0.20 (-0.07, 0.48), 0.15 | -0.05 (-0.19, 0.09), 0.48 | 0.13 (-0.07, 0.34), 0.19 | 0.04 (-0.10, 0.17), 0.60 | 0.15 (-0.08, 0.39), 0.18 | 0.02 (-0.11, 0.16), 0.74 |
| Morning stiffness, min | 3.55 (-44.23, 50.3), 0.88 | -3.13 (-26.4, 20.14), 0.79 | 0.04 (-0.12, 0.21), 0.61 | 21.0 (-2.01, 44.0), 0.07 | 11.0 (-27.9, 49.9), 0.57 | -0.06 (-0.18, 0.05), 0.28 |
| SHS | 0.14 (-0.50, 0.80), 0.65 | -0.35 (-0.68, -0.025), **0.03** | -0.07 (-0.56, 0.42), 0.78 | -0.07 (-0.41, 0.25), 0.63 | -0.36 (-0.92, 0.20), 0.20 | -0.11 (-0.45, 0.22), 0.50 |
| RF, IU/mL | 8.35 (-119.7, 136.4), 0.89 | 15.4 (-50.6, 81.42), 0.64 | 44.0 (-47.4, 135.4), 0.34 | -3.08 (-16.34, 10.2), 0.64 | 88.8 (-14.9, 192.6), 0.09 | 7.71 (-57.4, 72.9), 0.81 |
| Anti-CCP, U/mL | 68.3 (16.3, 120.4), **0.01** | -10.0 (-37.0, 16.86), 0.46 | 1.0 (-35.8, 37.8), 0.95 | -6.76 (-33.7, 20.2), 0.62 | 11.14 (-30.8, 53.1), 0.60 | 3.94 (-23.0, 30.9), 0.77 |
| Anti-MCV, U/mL | 35.54 (-80.0, 151.1), 0.54 | -37.85 (-96.6, 20.9), 0.20 | 0.74 (-44.2, 84.0), 0.98 | -7.08 (-66.8, 52.67), 0.81 | -2.06 (-92.4, 88.2), 0.96 | 6.03 (-53.9, 65.9), 0.84 |

*Anti-CCPs, anti-cyclic citrullinated peptide antibodies; Anti-MCV, Antibodies against modified citrullinate vimentin; CI, confidence interval; DAS28, disease activity score 28; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire disability index; RA, rheumatoid arthritis; RF, rheumatoid factor. SHS, Sharp/van der Heijde Score; SNP, single-nucleotide polymorphism.*

*β, regression coefficient and 95% CI. Model adjusted by age, DMARDs treatment and wood smoke exposure.*

*†Reference category. p-value < 0.05 was considered statistically significant.*

*Bold values represent statistically significant data.*

CCPs, anti-MCV and RF, and SNP rs2235926 of *PADI2*. This polymorphism is found in a 3´untranslated region, however, even though it hasn't been defined, this could influence the post-transcriptional expression of PAD2 and the citrullination of peptides. Nevertheless, PAD2 can citrullinate transcript factors that determine the Th0 cell lineage differentiation, such as GATA3 and RORγt. Citrullination of R300 of GATA3 and 4 arginine residues (R56, R59, R77, and R90) from RORγt, proves it has an effect on the gene regulation and on cell functions (59). This is important because the presence of polymorphisms in *PADI2* could modulate the expression and enzymatic function, therefore relating not only with the serologic positivity to a wide repertoire of antibodies, but also with the activation of naive T cells in response to antigen or "self" antigens, and their subsequent proliferation and differentiation, involved in the pathogenesis and clinical diversification of RA.

Conversely, the enzymatic activity of PADs is mediated by the $Ca^{2+}$ concentrations. In PAD4 five binding sites for $Ca^{2+}$ have been identified. The binding of this element to their sites induces conformational changes that create the active site cleft of the enzyme (60), while in PAD2, the $Ca^{2+}$ binding happens in 6 sites. The activation of the binding sites 3, 4, and 5 control the dependency on $Ca^{2+}$ and on regulatory elements that include a $Ca^{2+}$ switch (61). The citrullination by PAD2 and PAD4 requires $Ca^{2+}$ concentrations in a range of 0.35 to 1.85 mM (30). Vossenar et al. reported that in samples of synovial fluid from RA patients that vimentin can be citrullinated by PAD2 and PAD4 isotypes (28). However, using mass spectrometry, it was found that of 47 sites in fibrinogen that are susceptible to citrullination, 46 of them were citrullinated by PAD2 *in vitro* (62). Furthermore, PAD2 could have more protein citrullination activity such as fibrin, and also it could increase the proinflammatory cytokines' expression (63), indicating the leading role of PAD2 in the pathogenesis of RA related not only with the production of ACPAs but also with the modulation of the inflammatory process. We consider that the identification of the susceptibility polymorphisms in *PADI2* is important from the clinical viewpoint; however, the clinical perspective of our findings related to the presence of a positive serologic status for multiple autoantibodies and the clinical manifestations of RA at an early onset age deserves replication studies amongst populations.

Citrullination of vimentin in joints is crucial during the pathogenesis of RA (64). In an *in vitro* model, it was observed that citrullinated vimentin increases the secretion of TNF-α and IL-1, as well as the expression of PAD4 and RANKL (65). Moreover, during the differentiation process from monocyte to macrophage, the mRNA and PAD2 protein levels increase (66, 67). Likewise, it was observed that the expression of the mRNA of *PADI2* is high in $CD34^+$ cells and that its expression levels are correlated to the Sp1 transcription factor (68), which, interestingly, along with transcription factor Sp3 has an influence over the *PADI2* transcription (69). This proves the important role of PAD2 in the regulation of the expression of proinflammatory cytokines, of genes that promote citrullination, and of differentiation processes and activation of cells involved in joint bone resorption. In this study the haplotype TTT was significantly related with the presence of joint damage defined by a SHS, however other studies are required to clarify the functional role of SNPs in the *PADI2* gene on expression of the protein.

It was proven that the expression of PAD2 is elevated in samples of synovial tissue from RA patients (28–31). Damagaard et al. reported that the levels of PAD2 were high in synovial fluid in RA and that these are higher in patients that are positive to anti-CCPs.

**TABLE 6 |** Association of *PADI2* SNPs on the clinical characteristics in RA patients.

| Characteristics | rs1005753 | | rs2057094 | | rs2235926 | |
|---|---|---|---|---|---|---|
| | †GG *vs* TT+TG OR 95% CI, *p*-value | †TT *vs* GG+TG OR 95% CI, *p*-value | †CC *vs* TT+TC OR 95% CI, *p*-value | †TT *vs* CC+TC OR 95% CI, *p*-value | †CC *vs* TT+TC OR 95% CI, *p*-value | †TT *vs* CC+TC OR 95% CI, *p*-value |
| Age at diagnosis, <40 years | 0.62 (0.08-4.76), 0.65 | 1.42 (0.58-3.46), 0.44 | 1.17 (0.31-4.37), 0.80 | 1.62 (0.67-3.95), 0.28 | 1.73 (0.39-7.67), 0.46 | 1.67 (0.68-4.1), 0.25 |
| DAS28-ESR, >3.2 Units | 1.77 (0.57-5.4), 0.31 | 0.87 (0.49-1.55), 0.65 | 1.05 (0.46-2.4), 0.89 | 1.19 (0.67-2.12), 0.54 | 1.5 (0.57-3.9), 0.40 | 1.31 (0.73-2.34), 0.35 |
| DAS28-ESR, >5.1 Units | 1.91 (0.21-16.8), 0.55 | 0.76 (0.31-1.87), 0.56 | 2.32 (0.54-9.8), 0.25 | 1.30 (0.53-3.2), 0.56 | 2.11 (0.45-9.9), 0.34 | 1.40 (0.59-3.47), 0.46 |
| HAQ-DI, 1-2 Units | 1.78 (0.35-8.9), 0.48 | 0.86 (0.42-1.76), 0.69 | 1.64 (0.44-6.04), 0.45 | 1.38 (0.67-2.8), 0.37 | 5.46 (0.52-11.61), 0.25 | 1.21 (0.59-2.46), 0.59 |
| Radiologic score, SHS >2 | 3.92 (0.76-20.2), 0.10 | 0.40 (0.20-0.81), **0.01** | 0.84 (0.29-2.38), 0.74 | 0.64 (0.32-1.30), 0.22 | 0.33 (0.08-1.34), 0.12 | 0.61 (0.30-1.25), 0.18 |
| Radiologic score, SHS >3 | 1.44 (0.41-5.1), 0.56 | 0.39 (0.19-0.82), 0.01 | 0.74 (0.26-2.15), 0.59 | 0.93 (0.96-1.92), 0.86 | 0.31 (0.07-1.26), 0.10 | 0.80 (0.39-1.0), 0.55 |
| RF+ (>20 IU/mL) | 0.70 (0.13-3.54), 0.67 | 1.16 (0.52-2.54), 0.71 | 2.5 (0.93-6.6), 0.06 | 1.23 (0.56-2.67), 0.60 | 2.90 (1.02-8.26), **0.04** | 1.17 (0.53-2.55), 0.69 |
| Anti-CCP+ (>5 U/mL) | 1.81 (0.45-7.2), 0.39 | 0.98 (0.45-2.12), 0.96 | 1.07 (0.33-3.4), 0.90 | 1.51 (0.70-3.27), 0.28 | 1.55 (0.48-5.0), 0.45 | 1.67 (0.77-3.61), 0.19 |
| Anti-MCV+ (>20 U/mL) | 1.22 (0.25-5.9), 0.80 | 0.60 (0.26-1.40), 0.24 | 1.52 (0.47-4.9), 0.48 | 1.86 (0.79-4.36), 0.15 | 2.92 (0.96-8.9), **0.05** | 2.05 (0.87-4.8), 0.10 |
| Anti-CCP+/Anti-MCV+ | 1.43 (0.36-5.59), 0.60 | 0.83 (0.40-1.69), 0.61 | 1.60 (0.69-4.4), 0.35 | 1.43 (0.70-2.98), 0.32 | 3.02 (1.11-8.23), **0.03** | 1.59 (0.78-3.26), 0.20 |
| Anti-CCP+/Anti-MCV+/FR+ | 0.91 (0.26-3.14), 0.88 | 1.21 (0.65-2.26), 0.52 | 2.23 (0.96-5.18), 0.06 | 1.29 (0.70-2.38), 0.40 | 3.79 (1.51-9.5), **0.004** | 1.32 (0.72-2.43), 0.36 |

*Anti-CCPs, anti-cyclic citrullinated peptide antibodies; Anti-MCV, Antibodies against modified citrullinate vimentin; CI, confidential interval; DAS28, disease activity score 28; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire disability index; RA, rheumatoid arthritis; RF, rheumatoid factor. SHS, Sharp/van der Heijde Score; SNP, single-nucleotide polymorphism.*

*OR, odds ratio and 95% CI. Model adjusted by age, DMARDs treatment and wood smoke exposure.*

*†Reference category. p-value < 0.05 was considered statistically significant.*

*Bold values represent statistically significant data.*

Additionally, the PAD2 levels in synovial fluid correlated with clinical activity, the levels of CRP, anti-CCPs, and leukocyte count, as well as cytokines such as IL-6, IL-8, and IL-10 (32). In an *in vitro* model, it was observed that PAD2 could promote IL-1β, IL-6, and TNF-α production in macrophages, and apoptosis induction when activating the caspases 2, 3, and 9, and, at the same time, when activating cell adhesion by FAK, paxillin, and PAK1 ways, which leads to the increase of inflammation (70). Meanwhile, the presence of anti-PAD2 antibodies is associated with a small number of inflamed joints, with a low prevalence of interstitial lung disease, and slower progression of joint damage (71). However, the current study is not exempt from limitations, including the lack of validation in a second population. Moreover, other studies are required to clarify the functional role of SNPs in the *PADI2* gene.

In conclusion, in a women population from southern Mexico, the TTT haplotype in the *PADI2* gene confers genetic susceptibility to RA and radiographic joint damage related to a positive status to autoantibodies anti-CCP+/anti-MCV+/RF+ and the clinical manifestations of RA at an early onset age.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CB-004/2017. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

IG-G Designed the study and wrote the manuscript. OZ-G, CR-V, and JN-Z recruited the patients, control subjects, and obtained the samples. IG-P and OZ-G data collection. CR-V, IG-P, and RF-V performed the experiments. MR, NC-A, and IP-R provided relevant opinions in the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES


1. Tobón GJ, Youinou P, Saraux A. The Environment, Geo-Epidemiology, and Autoimmune Disease: Rheumatoid Arthritis. *J Autoimmun* (2010) 35(1):10–4. doi: 10.1016/j.jaut.2009.12.009
2. Moreno-Montoya J, Alvarez-Nemegyei J, Sanin LH, Pérez-Barbosa L, Trejo-Valdivia B, Santana N, et al. Association of Regional and Cultural Factors With the Prevalence of Rheumatoid Arthritis in the Mexican Population: A Multilevel Analysis. *J Clin Rheumatol* (2015) 21(2):57–62. doi: 10.1097/RHU.0000000000000223
3. McInnes IB, Schett G. The Pathogenesis of Rheumatoid Arthritis. *N Engl J Med* (2011) 365(23):2205–19. doi: 10.1056/NEJMra1004965
4. Pukšič S, Morovič-Vergles J. Comorbidities in Patients With Rheumatoid Arthritis. *Reumatizam* (2014) 61(2):31–6.
5. Yamada R, Suzuki A, Chang X, Yamamoto K. Citrullinated Proteins in Rheumatoid Arthritis. *Front Biosci* (2005) 10:54–64. doi: 10.2741/1506
6. Darrah E, Rosen A, Giles JT, Andrade F. Peptidylargine Deiminase 2, 3 and 4 Have Distinct Specificities Against Cellular Substrates: Novel Insights Into Autoantigen Selection in Rheumatoid Arthritis. *Ann Rheum Dis* (2012) 71 (1):92–8. doi: 10.1136/ard.2011.151712
7. Romero V, Darrah E, Andrade F. Generation of Distinct Patterns of Rheumatoid Arthritis Autoantigens by Peptidylarginine Deiminase Types 2 and 4 During Perforin-Induced Cell Damage. *Arthritis Rheumatol* (2020) 72 (6):912–8. doi: 10.1002/art.41196
8. Curran AM, Naik P, Giles JT, Darrah E. PAD Enzymes in Rheumatoid Arthritis: Pathogenic Effectors and Autoimmune Targets. *Nat Rev Rheumatol* (2020) 16(6):301–15. doi: 10.1038/s41584-020-0409-1
9. Syversen SW, Gaarder PI, Goll GL, Ødegård S, Haavardsholm EA, Mowinckel P, et al. High Anti-Cyclic Citrullinated Peptide Levels and an Algorithm of Four Variables Predict Radiographic Progression in Patients With Rheumatoid Arthritis: Results From a 10-Year Longitudinal Study. *Ann Rheum Dis* (2008) 67(2):212–7. doi: 10.1136/ard.2006.068247
10. Syversen SW, Goll GL, van der Heijde D, Landewé R, Lie BA, Odegård S, et al. Prediction of Radiographic Progression in Rheumatoid Arthritis and the Role of Antibodies Against Mutated Citrullinated Vimentin: Results From a 10-Year Prospective Study. *Ann Rheum Dis* (2010) 69(2):345–51. doi: 10.1136/ard.2009.113092
11. Scott DL. Prognostic Factors in Early Rheumatoid Arthritis. *Rheumatol (Oxford)* (2000) 39(Suppl 1):24–9. doi: 10.1093/oxfordjournals.rheumatology.a031490
12. Katchamart W, Koolvisoot A, Aromdee E, Chiowchanwesawakit P, Muengchan C. Associations of Rheumatoid Factor and Anti-Citrullinated Peptide Antibody With Disease Progression and Treatment Outcomes in Patients With Rheumatoid Arthritis. *Rheumatol Int* (2015) 35(10):1693–9. doi: 10.1007/s00296-015-3271-8
13. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody Epitope Spreading in the Pre-Clinical Phase Predicts Progression to Rheumatoid Arthritis. *PLoS ONE* (2012) 7(5):e35296. doi: 10.1371/journal.pone.0035296
14. Sokolove J, Johnson DS, Lahey LJ, Wagner CA, Cheng D, Thiele GM, et al. Rheumatoid Factor as a Potentiator of Anti-Citrullinated Protein Antibody-Mediated Inflammation in Rheumatoid Arthritis. *Arthritis Rheumatol* (2014) 66(4):813–21. doi: 10.1002/art.38307
15. Foulquier C, Sebbag M, Clavel C, Chapuy-Regaud S, Al Badine R, Méchin MC, et al. Peptidyl Arginine Deiminase Type 2 (PAD-2) and PAD-4 But Not PAD-1, PAD-3, and PAD-6 are Expressed in Rheumatoid Arthritis Synovium in Close Association With Tissue Inflammation. *Arthritis Rheum* (2007) 56 (11):3541–53. doi: 10.1002/art.22983
16. Kinloch A, Lundberg K, Wait R, Wegner N, Lim NH, Zendman AJ, et al. Synovial Fluid is a Site of Citrullination of Autoantigens in Inflammatory Arthritis. *Arthritis Rheum* (2008) 58(8):2287–95. doi: 10.1002/art.23618
17. Beato M, Sharma P. Peptidyl Arginine Deiminase 2 (PADI2)-Mediated Arginine Citrullination Modulates Transcription in Cancer. *Int J Mol Sci* (2020) 21(4):1351. doi: 10.3390/ijms21041351
18. Lamensa JW, Moscarello MA. Deimination of Human Myelin Basic Protein by a Peptidylarginine Deiminase From Bovine Brain. *J Nuerochem* (1993) 61 (3):987–96. doi: 10.1111/j.1471-4159.1993.tb03612.x
19. Zhang X, Bolt M, Guertin MJ, Chen W, Zhang S, Cherrington BD, et al. Peptidylarginine Deiminase 2-Catalyzed Histone H3 Arginine 26 Citrullination Facilitates Estrogen Receptor α Target Gene Activation. *Proc Natl Acad Sci USA* (2012) 109(33):13331–6. doi: 10.1073/pnas.1203280109
20. Hsu PC, Liao YF, Lin CL, Lin WH, Liu GY, Hung HC. Vimentin is Involved in Peptidylarginine Deiminase 2-Induced Apoptosis of Activated Jurkat Cells. *Mol Cells* (2014) 37(5):426–34. doi: 10.14348/molcells.2014.2359
21. Kim SE, Park JW, Kim MJ, Jang B, Jeon YC, Kim HJ, et al. Accumulation of Citrullinated Glial Fibrillary Acidic Protein in a Mouse Model of Bile Duct Ligation-Induced Hepatic Fibrosis. *PLoS ONE* (2018) 13(8):e0201744. doi: 10.1371/journal.pone.0201744
22. Kan R, Jin M, Subramanian V, Causey CP, Thompson PR, Coonrod SA. Potential Role for PADI-Mediated Histone Citrullination in Preimplantation Development. *BMC Dev Biol* (2012) 12:19. doi: 10.1186/1471-213X-12-19
23. Tanikawa C, Espinosa M, Suzuki A, Masuda K, Yamamoto K, Tsuchiya E, et al. Regulation of Histone Modification and Chromatin Structure by the P53-PADI4 Pathway. *Nat Commun* (2012) 3:676. doi: 10.1038/ncomms1676
24. Hagiwara T, Nakashima K, Hirano H, Senshu T, Yamada M. Deimination of Arginine Residues in Nucleophosmin/B23 and Histones in HL-60 Granulocytes. *Biochem Biophys Res Commun* (2002) 290(3):979–83. doi: 10.1006/bbrc.2001.6303
25. Lee YH, Coonrod SA, Kraus WL, Jelinek MA, Stallcup MR. Regulation of Coactivator Complex Assembly and Function by Protein Arginine Methylation and Demethylimination. *Proc Natl Acad Sci USA* (2005) 102 (10):3611–6. doi: 10.1073/pnas.0407159102
26. Li P, Yao H, Zhang Z, Li M, Luo Y, Thompson PR, et al. Regulation of P53 Target Gene Expression by Peptidylarginine Deiminase 4. *Mol Cell Biol* (2008) 28(15):4745–58. doi: 10.1128/MCB.01747-07
27. Guo Q, Fast W. Citrullination of Inhibitor of Growth 4 (ING4) by Peptidylarginine Deiminase 4 (PAD4) Disrupts the Interaction Between ING4 and P53. *J Biol Chem* (2011) 286(19):17069–78. doi: 10.1074/jbc.M111.230961
28. Vossenaar ER, Radstake TR, van der Heijden A, van Mansum MA, Dieteren C, de Rooij DJ, et al. Expression and Activity of Citrullinating Peptidylarginine Deiminase Enzymes in Monocytes and Macrophages. *Ann Rheum Dis* (2004) 63(4):373–81. doi: 10.1136/ard.2003.012211
29. Chang X, Xia Y, Pan J, Meng Q, Zhao Y, Yan X. PADI2 is Significantly Associated With Rheumatoid Arthritis. *PLoS ONE* (2013) 8(12):e81259. doi: 10.1371/journal.pone.0081259
30. Damgaard D, Senolt L, Nielsen MF, Prujin GJ, Nielsen CH. Demonstration of Extracellular Peptidylarginine Deiminase (PAD) Activity in Synovial Fluid of Patients With Rheumatoid Arthritis Using a Novel Assay for Citrullination of Fibrinogen. *Arthritis Res Ther* (2014) 16(6):498. doi: 10.1186/s13075-014-0498-9
31. Olivares-Martínez E, Hernández-Ramírez DF, Núñez-Álvarez CA, Cabral AR, Llorente L. The Amount of Citrullinated Proteins in Synovial Tissue is Related to Serum Anti-Cyclic Citrullinated Peptide (Anti-CCP) Antibody Levels. *Clin Rheumatol* (2016) 35(1):55–61. doi: 10.1007/s10067-015-3047-2
32. Damgaard D, Senolt L, Nielsen CH. Increased Levels of Peptidylarginine Deiminase 2 in Synovial Fluid From Anti-CCP-Positive Rheumatoid Arthritis Patients: Association With Disease Activity and Inflammatory Markers. *Rheumatol (Oxford)* (2016) 55(5):918–27. doi: 10.1093/rheumatology/kev440
33. Lee YH, Bae SC, Choi SJ, Ji JD, Song GG. Genome-Wide Pathway Analysis of Genome-Wide Association Studies on Systemic Lupus Erythematosus and Rheumatoid Arthritis. *Mol Biol Rep* (2012) 39(12):10627–35. doi: 10.1007/s11033-012-1952-x
34. Freudenberg J, Lee HS, Han BG, Shin HD, Kang YM, Sung YK, et al. Genome-Wide Association Study of Rheumatoid Arthritis in Koreans: Population-Specific Loci as Well as Overlap With European Susceptibility Loci. *Arthritis Rheum* (2011) 63(4):884–93. doi: 10.1002/art.30235
35. Too CL, Murad S, Dhaliwal JS, Larsson P, Jiang X, Ding B, et al. Polymorphism in Peptidylarginine Deiminase Associate With Rheumatoid Arthritis in Diverse Asian Populations: Evidence From MyEIRA Study and Meta-Analysis. *Arthritis Res Ther* (2012) 14(6):R250. doi: 10.1186/ar4093
36. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62(9):2569–81. doi: 10.1002/art.27584

37. vand der Heijde D. How to Read Radiographs According to the Sharp/van Der Heijde Method. *J Rheumatol* (2000) 27(1):261–3.
38. Shi YY, He L. SHEsis, a Powerful Software Platform for Analyses of Linkage Disequilibrium, Haplotype Construction, and Genetic Association at Polymorphism Loci. *Cell Res* (2005) 15(2):97–8. doi: 10.1038/sj.cr.7290272
39. Zavala-Cerna MG, Gonzalez-Montoya NG, Nava A, Gamez-Nava JI, Moran-Moguel MC, Rosales-Gomez RC, et al. PADI4 Haplotypes in Association With RA Mexican Patients, a New Prospect for Antigen Modulation. *Clin Dev Immunol* (2013) 2013:383681. doi: 10.1155/2013/383681
40. Guzmán Guzmán IP, Reyes-Castillo Z, Muñoz-Barrioss S, Ruiz-Noa Y, Martínez-Bonilla GE, Parra-Rojas I, et al. Polymorphisms and Functional Haplotype in PADI4: Further Evidence for Contribution on Rheumatoid Arthritis Susceptibility and Anti-Cyclic Citrullinated Peptide Antibodies in a Western Mexican Population. *Immunol Lett* (2015) 163(2):214–20. doi: 10.1016/j.imlet.2014.10.029
41. Baños-Hernández CJ, Navarro-Zarza JE, Parra-Rojas I, Vázquez-Villamar M, Padilla-Gutiérrez JR, Valle Y, et al. PADI4 Polymorphisms and the Functional Haplotype are Associated With Increased Rheumatoid Arthritis Susceptibility: A Replication Study in a Southern Mexican Population. *Hum Immunol* (2017) 78(9):553–8. doi: 10.1016/j.humimm.2017.05.005
42. Reyes-Castillo Z, Palafox-Sánchez CA, Parra-Rojas I, Martínez-Bonilla GE, del Toro-Arreola S, Ramírez-Dueñas MG, et al. Comparative Analysis of Antibodies Targeting Peptidylarginine Deiminase Type 4, Mutated Citrullinated Vimentin and Cyclic Citrullinated Peptides in Rheumatoid Arthritis: Associations With Cytokine Profiles, Clinical and Genetic Features. *Clin Exp Immunol* (2015) 182(2):119–31. doi: 10.1111/cei.12677
43. Zhang Q, Liu Q, Lin C, Baima Y, Li H, Gong H, et al. The Prevalence of Rheumatoid Arthritis in Middle-Aged and Elderly People Living in Naqu City, Tibet, Autonomous Region of China. *J Orthop Surg Res* (2020) 15(1):338. doi: 10.1186/s13018-020-01883-4
44. Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadel G, Rönnelid J, et al. Antibodies of IgG, IgA and IgM Isotypes Against Cyclic Citrullinated Peptide Precede the Development of Rheumatoid Arthritis. *Arthritis Res Ther* (2011) 13(1):R13. doi: 10.1186/ar3237
45. Svärd A, Kastbom A, Söderlin MK, Reckner-Olsson Å, Skogh T. A Comparison Between IgG- and IgA-Class Antibodies to Cyclic Citrullinated Peptides and to Modified Citrullinated Vimentin in Early Rheumatoid Arthritis and Very Early Arthritis. *J Rheumatol* (2011) 38(7):1265–72. doi: 10.3899/jrheum.101086
46. González-Lopez L, Rocha-Muñoz AD, Ponce-Guarneros M, Flores-Chavez A, Salazar-Paramo M, Nava A, et al. Anti-Cyclic Citrullinated Peptide (Anti-CCP) and Anti-Mutated Citrullinated Vimentin (Anti-MCV) Relation With Extra-Articular Manifestations in Rheumatoid Arthritis. *J Immunol Res* (2014) 2014:536050. doi: 10.1155/2014/536050
47. Kim HH, Kim J, Park SH, Kim SK, Kim OD, Cho JY. Correlation of Anti-Cyclic Citrullinated Antibody With Hand Joint Erosion Score in Rheumatoid Arthritis Patients. *Korean J Intern Med* (2010) 25(2):201–6. doi: 10.3904/kjim.2010.25.2.201
48. Kirino Y, Hama M, Takase-Minegishi K, Kunishita Y, Kishimoto D, Yoshimi R, et al. Predicting Joint Destruction in Rheumatoid Arthritis With Power Doppler, Anti-Citrullinated Peptide Antibody, and Joint Swelling. *Mod Rheumatol* (2015) 25(6):842–8. doi: 10.3109/14397595.2015.1026025
49. Hetland ML, Stengaard-Pedersen K, Junker P, Østergaard M, Ejbjerg BJ, Jacobsen S, et al. Radiographic Progression and Remission Rates in Early Rheumatoid Arthritis-MRI Bone Oedema and Anti-CCP Predicted Radiographic Progression in the 5-Year Extension of the Double-Blind Randomized CIMESTRA Trial. *Ann Rheum Dis* (2010) 69(10):1789–95. doi: 10.1136/ard.2009.125534
50. Joo YB, Park YJ, Park KS, Kim KJ. Association of Cumulative Anti-Cyclic Citrullinated Protein Antibodies With Radiographic Progression in Patients With Rheumatoid Arthritis. *Clin Rheumatol* (2019) 38(9):2423–32. doi: 10.1007/s10067-019-04554-w
51. Vencovský J, Machácek S, Sedová L, Kafková J, Gatterová J, Pesákova V, et al. Autoantibodies can be Prognostic Markers O Fan Erosive Disease in Early Rheumatoid Arthritis. *Ann Rheum Dis* (2003) 62(5):427–30. doi: 10.1136/ard.62.5.427
52. Lu MC, Lai NS, Yu HC, Huang HB, Hsieh SC, Yu CL. Anti-Citrullinated Protein Antibodies Bind Surface-Expressed Citrullinated Grp78 on Monocyte/Macrophages and Stimulate Tumor Necrosis Factor Alpha Production. *Arthritis Rheum* (2010) 62(5):1213–23. doi: 10.1002/art.27386
53. Mansour HE, Metwaly KM, Hassan IA, Elshamy HA, Elbeblawy MM. Antibodies to Mutated Citrullinated Vimentin in Rheumatoid Arthritis: Diagnostic Value, Association With Radiological Damage and Axial Skeleton Affection. *Clin Med Insights Arthritis Musculoskelet Disord* (2010) 3:33–42. doi: 10.4137/cmamd.s4827
54. Salafi F, Carotti M, Ciapetti A, Gasparini S, Filippucci E, Grassi W. Relationship Between Time-Integrate Disease Activity Estimated by DAS28-CRP and Radiographic Progression of Anatomical Damage in Patients With Early Rheumatoid Arthritis. *BMC Musculoskelet Disord* (2011) 12:120. doi: 10.1186/1471-2474-12-120
55. Jilani AA, Mackworth-Young CG. The Role of Citrullinated Protein Antibodies in Redicting Erosive Disease in Rheumatoid Arthritis: A Systematic Literature Review and Meta-Analysis. *Int J Rheumatol* (2015) 2015:728610. doi: 10.1155/2015/728610
56. Barouta G, Katsiari CG, Alexiou I, Liaskos C, Varna A, Bogdanos DP, et al. Anti-MCV Antibodies Predict Radiographic Progression in Greek Patients With Very Early (<3 Months Duration) Rheumatoid Arthritis. *Clin Rheumatol* (2017) 36(4):885–94. doi: 10.1007/s10067-016-3494-4
57. Assohou-Luty C, Raijmakers R, Benckhuijsen WE, Stammen-Vogelzangs J, de Ru A, vann Veelen PA, et al. The Human Peptidylarginine Deiminases Type 2 and Type 4 Have Distinct Substrate Specificities. *Biochim Biophys Acta* (2014) 1844(4):829–36. doi: 10.1016/j.bbapap.2014.02.019
58. Arita K, Shimizu T, Hashimoto H, Hidaka Y, Yamada M, Sato M. Structural Basis for Histone N-Terminal Recognition by Human Peptidylarginine Deiminase 4. *Proc Natl Acad Sci USA* (2006) 103(14):5291–6. doi: 10.1073/pnas.0509639103
59. Sun B, Chang HH, Salinger A, Tomita B, Bawadekar M, Holmes CL, et al. Reciprocal Regulation of Th2 and Th17 Cells by PAD2-Mediated Citrullination. *JCI Insight* (2019) 4(22):e129687. doi: 10.1172/jci.insight.129687
60. Arita K, Hashimoto H, Shimizu T, Nakashima K, Yamada M, Sato M. Structural Basis for $Ca(2^+)$-Induced Activation of Human PAD4. *Nat Struct Mol Biol* (2004) 11(8):777–83. doi: 10.1038/nsmb799
61. Slade DJ, Fang P, Dreyton CJ, Zhang Y, Fuhrmann J, Rempel D, et al. Protein Arginine Deiminase 2 Binds Calcium in an Ordered Fashion: Implications for Inhibitor Design. *ACS Chem Biol* (2015) 10(4):1043–53. doi: 10.1021/cb500933j
62. Sharma M, Damgaard D, Senolt L, Svensson B, Bay-Jensen AC, Nielsen CH, et al. Expanding the Citrullinome of Synovial Fibrinogen From Rheumatoid Arthritis Patients. *J Proteomics* (2019) 208:103484. doi: 10.1016/j.jprot.2019.103484
63. Sanchez-Pernaute O, Filkova M, Gabucio A, Klein M, Maciejewska-Rodrigues H, Ospelt C, et al. Citrullination Enhances the Pro-Inflammatory Response to Fibrin in Rheumatoid Arthritis Synovial Fibroblasts. *Ann Rheum Dis* (2013) 72(8):1400–6. doi: 10.1136/annrheumdis-2012-201906
64. Steendam KV, Tilleman K, Deforce D. The Relevance of Citrullinated Vimentin in the Production of Antibodies Against Citrullinated Proteins and the Pathogenesis of Rheumatoid Arthritis. *Rheumatol (Oxford)* (2011) 50 (5):830–7. doi: 10.1093/rheumatology/keq419
65. Fan LY, He DY, Wang Q, Zong M, Zhang H, Yang H, et al. Citrullinated Vimentin Stimulates Proliferation, Proinflammatory Cytokine Secretion, and PADI4 and RANKL Expression of Fibroblast-Like Synoviocytes in Rheumatoid Arthritis. *Scand J Rheumatol* (2012) 41(5):354–8. doi: 10.3109/03009742.2012.670263
66. Hojo-Nakashima I, Sato R, Nakashima K, Hagiwara T, Yamada M. Dynamic Expression of Peptidylarginine Deiminase 2 in Human Monocytic Leukaemia THP-1 Cells During Macrophage Differentiation. *J Biochem* (2009) 146 (4):471–9. doi: 10.1093/jb/mvp097
67. Lai NS, Yu HC, Tung CH, Huang KY, Huang HB, Lu MC. Increased Peptidylarginine Deiminases Expression During the Macrophage Differentiation and Participated Inflammatory Responses. *Arthritis Res Ther* (2019) 21(1):108. doi: 10.1186/s13075-019-1896-9
68. Nagia T, Matsueda Y, Tomita T, Yoshikawa H, Hirohata S. The Expression of mRNA for Peptidylarginine Deiminase Type 2 and Type 4 in Bone Marrow $CD34^+$ Cells in Rheumatoid Arthritis. *Clin Exp Rheumatol* (2018) 36 (2):248–53.

69. Dong S, Kojima T, Shiraiwa M, Méchin MC, Chavanas S, Serre G, et al. Regulation of the Expression of Peptidylarginine Deiminase Type II Gene (PADI2) in Human Keratinocytes Involves Sp1 and Sp3 Transcription Factors. *J Invest Dermatol* (2005) 124(5):1026–33. doi: 10.1111/j.0022-202X.2005.23690.x
70. Yu HC, Tung CH, Huang KY, Huang HB, Lu MC. The Essential Role of Peptidylarginine Deiminases 2 for Cytokines Secretion, Apoptosis, and Cell Adhesion in Macrophage. *Int J Mol Sci* (2020) 21(16):5720. doi: 10.3390/ijms21165720
71. Darrah E, Giles JT, Davis R, Naik P, Wang H, Koning MF, et al. Autoantibodies to Peptidylarginine Deiminase 2 Are Associated With Less Severe Disease in Rheumatoid Arthritis. *Front Immunol* (2018) 9:2696. doi: 10.3389/fimmu.2018.02696

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# Identifying Immune Cell Infiltration and Effective Diagnostic Biomarkers in Rheumatoid Arthritis by Bioinformatics Analysis

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**Background:** Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder characterized by inflammatory cell infiltration, leading to persistent synovitis and joint destruction. The pathogenesis of RA remains unclear. This study aims to explore the immune molecular mechanism of RA through bioinformatics analysis.

**Methods:** Five microarray datasets and a high throughput sequencing dataset were downloaded. CIBERSORT algorithm was performed to evaluate immune cell infiltration in synovial tissues between RA and healthy control (HC). Wilcoxon test and Least Absolute Shrinkage and Selection Operator (LASSO) regression were conducted to identify the significantly different infiltrates of immune cells. Differentially expressed genes (DEGs) were screened by "Batch correction" and "RobustRankAggreg" methods. Functional correlation of DEGs were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Candidate biomarkers were identified by cytoHubba of Cytoscape, and their diagnostic effectiveness was predicted by Receiver Operator Characteristic Curve (ROC) analysis. The association of the identified biomarkers with infiltrating immune cells was explored using Spearman's rank correlation analysis in R software.

**Results:** Ten significantly different types of immune cells between RA and HC were identified. A total of 202 DEGs were obtained by intersection of DEGs screened by two methods. The function of DEGs were significantly associated with immune cells. Five hub genes (CXCR4, CCL5, CD8A, CD247, and GZMA) were screened by R package "UpSet". CCL5+CXCR4 and GZMA+CD8A were verified to have the capability to diagnose RA and early RA with the most excellent specificity and sensitivity, respectively. The correlation between immune cells and biomarkers showed that CCL5 was positively correlated with M1 macrophages, CXCR4 was positively correlated with memory activated CD4+ T cells and follicular helper T (Tfh) cells, and GZMA was positively correlated with Tfh cells.

**Conclusions:** CCL5, CXCR4, GZMA, and CD8A can be used as diagnostic biomarker for RA. GZMA-Tfh cells, CCL5-M1 macrophages, and CXCR4- memory activated CD4+ T

cells/Tfh cells may participate in the occurrence and development of RA, especially GZMA-Tfh cells for the early pathogenesis of RA.

**Keywords: rheumatoid arthritis, synovial tissues, immune cells infiltration, diagnose biomarker, bioinformatics analysis**

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease (1). The main clinical manifestation of RA is chronic synovitis of the affected joint characterized by persistent synovitis, synovial hyperplasia, and pannus formation, which destroy the bone tissue and gradually lead to the damage of joint function (2, 3). RA is common in middle-aged women aged 40–60 years, with a prevalence rate of 0.5%–2% (4). The underlying mechanism of RA is complex, which is caused by the interaction of genetic, environmental, and immune factors, in which immune factor plays an essential role in the entire process, especially in the early stage (5, 6). Therefore, exploring the diagnostic biomarkers and revealing the immune mechanism of RA is the key to the early prevention and treatment of RA and is the focus of current research.

Immune cells in synovial membrane, including resident and infiltrating immune cells, play a vital role in the occurrence and development of RA, an autoimmune disorder (7). Studies have shown that macrophages play an important role in promoting the development of RA. These cells secrete abundant cytokines, chemokines, and degrading enzymes, which lead to joint inflammation and bone destruction. These cells can cooperate with other immune cells to aggravate the formation of arthritis (8). In the past decade, a great deal of research was conducted to understand the role of T cells, especially activated Th17 and Th1 cells, in RA (9). Autoimmune Th17 cells induce synovial stroma and innate lymphocytes to secrete the cytokine granulocyte-macrophage colony-stimulating factor (MG-CSF) to initiate and enhance autoimmune arthritis in RA (10). In addition, other immune cells, such as dendritic cells (DCs) and natural killer (NK) cells, play an important regulatory role in the pathogenesis of RA (11). However, the immune mechanisms of RA in synovium have not been investigated thoroughly. Therefore, a systematic approach is urgently needed to evaluate the contribution of immune cells and explore key genes related to immune cells.

With the development and widespread use of microarray and high-throughput sequencing technology, bioinformatics analysis can be used to identify novel genes and biomarkers for many diseases, including autoimmune disease (12–14). A previous bioinformatics study suggested that GRB10 and E2f3 could be used as diagnostic markers of osteoarthritis (15). Immune cell infiltration plays an important role in the pathogenesis of many diseases, including tumor and non-tumor diseases. Currently, cell-type identification has been utilized in many diseases; this method estimates the relative subsets of RNA transcripts (CIBERSORT) and analyzes 22 immune cell subsets in complex tissues by normalized bulk transcriptome profiles (16). Thus, this method can help us systematically explore immune cell infiltration in RA synovial tissues.

In this study, CIBERSORT was performed to analyze immune cell infiltration in RA by using five microarray datasets. Wilcoxon test and LASSO regression were conducted to identify the significantly different infiltrates of immune cells in RA and HC. After DEGs were screened, functional correlation was analyzed by GO and KEGG, and hub genes were identified by R package "UpSet." ROC logistic regression was conducted to analyze the predictive of biomarkers. Spearman's rank correlation in R software was also used to analyze the correlation between biomarkers and significantly different infiltrates of immune cells. This work not only systematically analyzed the infiltration of immune cells in the synovial membrane of RA but also screened novel and effective diagnostic biomarkers for RA. Results provide a new method for the diagnosis and treatment of RA.

## MATERIALS AND METHODS

### GEO Dataset Collection

Gene expression profiling of RA was screened using the GEO (http://www.ncbi.nlm.nih.gov/geo) database. The inclusion criteria were as follows: (1) expression profiling by array or high-throughput sequencing of mRNA; (2) availability of the synovial tissues of patients with RA or HC from joint in the datasets; and (3) ten or more synovial specimens in the dataset. Six eligible datasets were selected, including GSE1919, GSE12021, GSE55235, GSE55457, GSE77298, and GSE89408. The details of all data are shown in **Table 1**.

### Data Preprocessing and Study Design

Raw and series matrix files of the five test microarray datasets, namely, GSE1919, GSE12021, GSE55235, GSE55457, and GSE77298, were downloaded. For raw data, probe expression matrix was extracted and normalized by Robust Multiarray Average (RMA) based on R package "affy." Platform annotation file was used to convert the probe expression matrix into a gene expression matrix. For the case of multiple probes corresponding to one gene, the average value was obtained. After five gene matrixes were merged by Perl script, R package "sva" was applied to eliminate heterogeneity caused by different experimental batches and platforms. Finally, we obtained one merging normalized gene expression matrix and used R package "limma" for analysis. For series matrix files, Perl script was used to extract each probe expression matrix one by one and then convert them into gene expression matrices,

**TABLE 1** | Basic information of selected datasets.

| GEO | Platform | Tissue | Samples (number) | | | Experiment | Attribute | Author/Reference |
|---|---|---|---|---|---|---|---|---|
| | | (Homo sapiens) | Total | HC | RA | type | | |
| GSE1919 | GPL91 | Synovium | 10 | 5 | 5 | Array | Test | U. Ungethuem (17) |
| GSE12021 | GPL96 | Synovium | 21 | 9 | 12 | Array | Test | R. Huber (18) |
| GSE55235 | GPL96 | Synovium | 20 | 10 | 10 | Array | Test | D. Woetzel (19) |
| GSE55457 | GPL96 | Synovium | 23 | 10 | 13 | Array | Test | D. Woetzel (19) |
| GSE77298 | GPL570 | Synovium | 23 | 7 | 16 | Array | Test | M.G. Broeren (20) |
| GSE89408 | GPL11154 | Synovium | 180 | 28 | 57/95※ | RNA-Seq | Validation | Y. Guo (21) |

※*Means 57 early RA and 95 established RA in GSE89408 dataset.*

respectively. R package "limma" was used to analyze each gene expression matrix. The validation RNA-Seq dataset, GSE89408, was downloaded by the form of gene expression matrix. R package "edgeR" was employed for subsequent analysis. The flow diagram of this study is shown in **Supplementary Figure S1**.

## Evaluation of Immune Cell Subtype Distribution

CIBERSORT algorithm was performed to evaluate immune cell infiltration in synovial tissues between RA and HC. This algorithm can transform the normalized gene expression matrix into the composition of infiltrating immune cells. After data were submitted to the CIBERSORT web portal (http://CIBERSORT.stanford.edu/), LM22 was used as a reference expression signature with 1000 permutations. The LM22 signature matrix defined 22 infiltrating immune cell components, including subsets of macrophages (M0 macrophages, M1 macrophages, and M2 macrophages), T cells (CD8+ T cells, naïve CD4+ T cells, memory resting CD4+ T cells, memory activated CD4+ T cells, Tfh cells, regulatory T cells, and gamma delta T cells), natural killer (NK) cells (resting NK cells and activated NK cells), mast cells (resting mast cells and activated mast cells), B cells (naïve B cells and memory B cells), dendritic cells (resting dendritic cells and activated dendritic cells), monocytes, plasma cells, neutrophils, and eosinophils. The p-values and root mean squared errors were determined for each expression file in CIBERSORT. Only data with a CIBERSORT p value < 0.05 was filtered and reserved for the following analysis. The output was directly integrated to generate an entire matrix of immune cell fractions. The results from CIBERSORT were visualized using the R packages "corplot", "vioplot", "ggplot2", and "glment".

## Principal Component Analysis (PCA)

Intra-group data repeatability in each group was verified by Pearson's correlation test. The intra-group data repeatability of the dataset was tested by sample clustering analysis. Statistical analysis was performed by R programming language, and the results were presented by R package "ggplot2".

## Screening of DEGs

Two methods were performed to obtain accurate DEGs from multiple microarray datasets. The first method ("Batch correction") merged the downloaded five raw datasets into an expression matrix and then analyzed the DEGs with R package "limma" after batch correction and normalization. The second method ("RobustRankAggreg, RRA") used the R package "limma" to analyze the DGEs of the downloaded gene expression matrices. The DEGs of each dataset were integrated with R package "RobustRankAggreg." DEGs obtained by the two methods were intersected by Venn diagram to extract final DEGs. The threshold points for DEGs were adj.P.Val < 0.05 and |log fold change (FC)| > 1.

## Functional Enrichment Analysis

The gene names of DEGs were converted to gene ID by R package "org.Hs.eg.db". Analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) for DEGs were performed by R package "clusterProfiler." The significantly different GO terms and signal pathways were screened by the threshold p value <0.05 and q value <0.05. The results were visualized by R package "enrichplot" and "ggplot2".

## Screening of Hub Genes

STRING (https://string-db.org) was performed to analyze the PPI network of DEGs with a highly reliable filtering condition (score>0.7). The interaction file (string_interactions.tsv) was downloaded. Perl was conducted to obtain the network file. The cytoHubba of Cytoscape (v 3.7.2) was conducted to score each node gene by top 10 algorithms, namely, MCC (Maximal Clique Centrality), DMNC (Density of Maximum Neighborhood Component), MNC (Maximum Neighborhood Component), Degree, EPC (Edge Percolated Component), BottleNeck, EcCentricity, Closeness, Radiality, and Betweenness. The top 60 node genes scored by each algorithm were used to screen hub genes by R package "UpSet". The RNA-Seq dataset GSE89408 was used to validate hub genes.

## Analysis of the Predictive Value of Biomarkers

ROC analysis was performed to predict the diagnostic effectiveness of biomarkers by SSPA Statistics 23. The area under the ROC curve (AUC) value was utilized to determine the diagnostic effectiveness in discriminating RA from control samples in the GSE89408 dataset.

## Correlation Analysis Between Biomarkers and Immune Cells

The association of the identified gene biomarkers with the levels of infiltrating immune cells was explored using Spearman's rank correlation analysis in R software. The resulting associations were visualized using the chart technique with the "ggplot2" package.

# RESULTS

## Immune Cell Infiltration in RA and Normal Synovial Tissues

Five microarray raw datasets, including 56 RA and 41 normal synovial tissues, were selected for the study of immune cell infiltration. The data before (A and B) and after (C and D) batch correction was presented in **Figure 1**, which indicated that the batch effect of the merged data was removed successfully. A total of 55 RA and 29 normal synovial tissues were found to be eligible for the analysis of CIBERSORT ($p < 0.05$). First, the composition of 22 kinds of immune cells in each sample was presented in a histogram (**Figure 2A**) and a heatmap (**Figure 2B**). In the histogram, the color represents the percentage of different immune cells in each sample, and the sum is 1. In the heatmap, immune cells in each sample are shown with the normalized absolute abundance. The results indicated that M2 macrophages, CD8+ T cells, resting mast cells, naïve B cells, Tfh cells, M0 macrophages, and M1 macrophages were the main infiltrating immune cells. The correlation of 22 types of immune cells in RA synovial tissues was then evaluated (**Figure 2C**). For example, Tfh cells were positively correlated with memory activated CD4+ T cells and M1 macrophages. γδ T cells were positively related to memory activated CD4+ T cells and naïve B cells. Neutrophils were positively associated with resting NK cells, monocytes, and naïve CD4+ T cells. M2 macrophages were negatively correlated with naïve B cells. However, the above correlation of immune cells decreased or disappeared in HC (**Supplementary Figure S2**). Third, based on immune cell infiltration in synovial tissues, we could completely distinguished RA from normal HC by PCA analysis (**Figure 2D**). Two different algorithms, namely, Wilcoxon test and LASSO regression, were used to identify the significantly different infiltrates of immune cells in RA and HC. The results of Wilcoxon test are shown in a violin diagram in **Figure 3A**, which presented 13 types of immune cells with $p < 0.05$. The results of Lasso are presented in **Figures 3B, C**, which contained 12 significantly different types of immune cells. The 10 intersecting immune cells of the two methods were extracted.

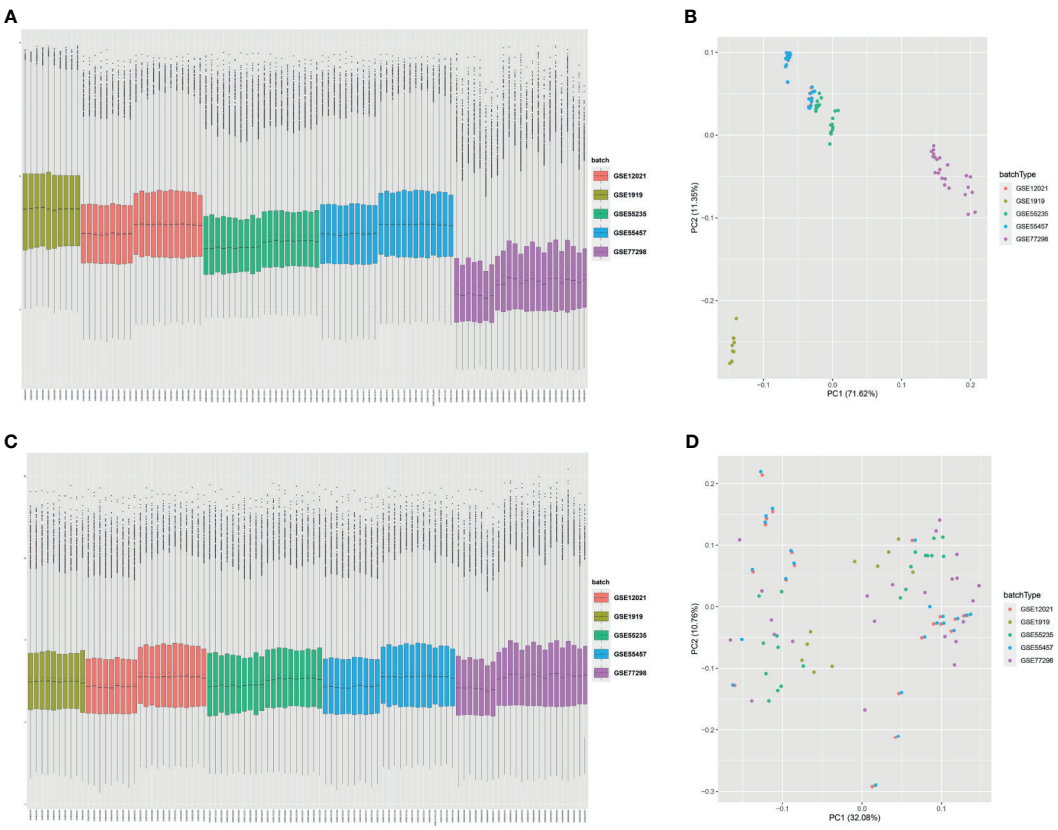


**FIGURE 1** | Data preprocessing. Box plot and principal component analyses were performed to remove batch correction of GSE1919, GSE12021, GSE55235, GSE55457, and GSE77298. **(A, B)** before batch correction and **(C, D)** after batch correction.

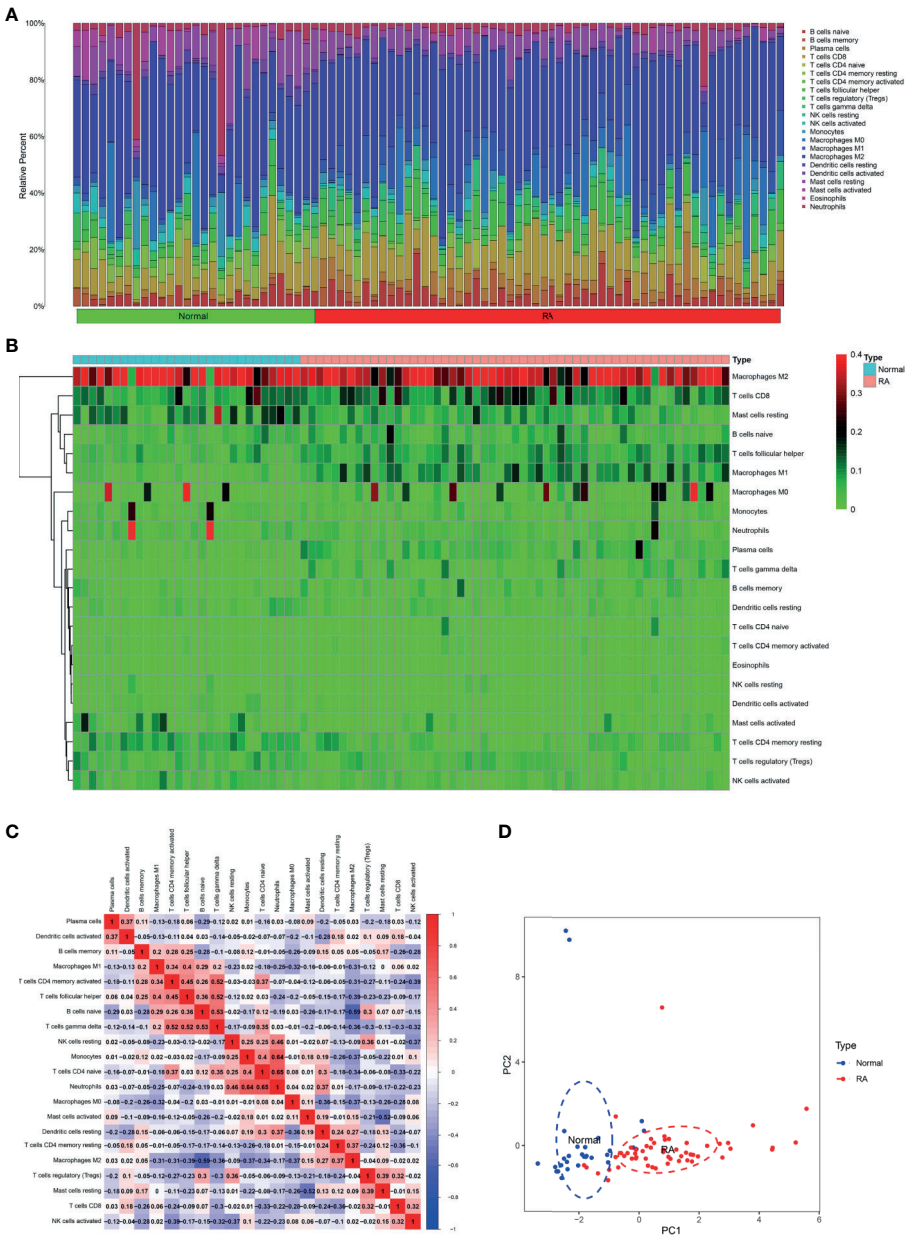


**FIGURE 2** | Immune cell infiltration in RA and normal synovial tissues. The composition of 22 kinds of immune cells in each sample was showed in a histogram **(A)** and a heatmap **(B)**. **(C)** The correlation of 22 types of immune cells in RA synovial tissues was evaluated. Red: positive correlation; blue: negative correlation. **(D)** PCA analysis was performed to classify infiltrating immune cells between RA and normal synovial tissues.

Compared with HC M1 macrophages, Tfh cells, the numbers of memory activated CD4+ T cells, and plasma cells were significantly higher in RA synovial tissues, while those of regulatory T cells, activated dendritic cells, activated NK cells, memory resting CD4+ T cells, resting mast cells, and activated mast cells were significantly lower in RA synovial tissues.

## Identification of DEGs

Five microarray datasets, including 57 RA and 41 normal samples, were used by the two methods to identify DEGs. A total of 360 DEGs were obtained by "Batch correction" and included 335 upregulated and 25 downregulated genes. A total of 461 DEGs were obtained by "RRA" and included 298 upregulated and 163 downregulated genes. Some of DEGs are shown in **Figure 4A**. A total of 202 DEGs, including 179 upregulated and 23 downregulated genes, were obtained through the intersection of the DEGs screened by the two methods (**Figure 4B** and **Supplementary Table S1**). The final DEGs were visualized by the volcano map (**Figure 4C**) and heatmap (**Figure 4D**).

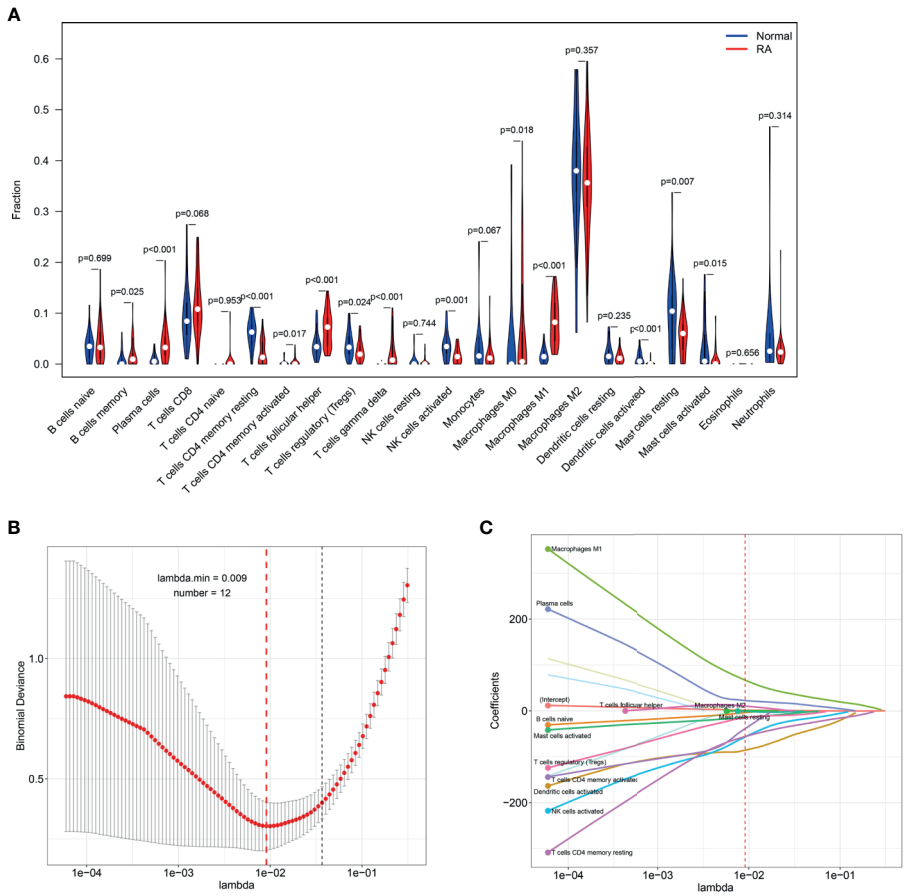


**FIGURE 3** | Identifying the significantly different infiltrates of immune cells in RA. **(A)** Wilcoxon test and **(B, C)** LASSO regression were conducted to analyze the different infiltrates of immune cells in RA and HC.

## Functional Correlation Analysis

After being converted into gene ID, DEGs were analyzed by GO and KEGG. The GO annotations of DEGs consisted of three parts including CC (cellular component), BP (biological process), and MF (Molecular function), which were used to analyze the functional enrichment of DEGs. The DEGs were mainly related to the biological activity of immune cells, such as lymphocyte differentiation, T cell activation, and leukocyte (**Figures 5A, B** and **Supplementary Table S2**). KEGG analysis was conducted to determine the relationship between DEGs and signaling pathway. The DEGs were mainly associated with immune cell-related signaling pathway, such as chemokine signaling pathway, rheumatoid arthritis, and primary immunodeficiency (**Figures 5C, D** and **Supplementary Table S3**). Overall, the function of DEGs were significantly associated with immune cells.

## Identification and Validation of Hub Genes

We obtained the PPI network results of DEGs by STRING (**Supplementary Figure S3**). We then used 10 algorithms to calculate the score of each node gene. Finally, we screened five hub genes (CXCR4, CCL5, CD8A, CD247, and GZMA) by R package "UpSet" and marked them with boxes in **Figure 6A**. The expression levels of the five hub genes were presented by heatmap in merged microarray data (**Figure 6B**). To make the results more reliable, we used the RNA-Seq dataset GSE89408 for validation. The expression levels of CXCR4, CCL5, CD8A, CD247, and GZMA were presented in heatmap (**Figure 7A**). As shown in **Figures 7B–F**, all the five genes had significantly higher expression in RA, both early and established RA, than in the HC ($p < 0.05$). The expression levels of GZMA and CD8A in early RA were significantly higher than those in established RA ($p < 0.05$). Consistent with the above results, the expression of CXCR4, CCL5, CD8A, CD247, and GZMA were also significantly increased in RA compared with osteoarthritis (OA) in dataset GSE89408 (**Figure 7G**). Therefore, GZMA and CD8A might be used as the diagnostic biomarkers for early RA, and CXCR4, CCL5, and CD247 might be used as the diagnostic biomarkers for RA.

## Diagnostic Effectiveness of Biomarkers for RA

The GSE89408 dataset was used to validate the diagnostic effectiveness of the biomarkers for RA by ROC analysis. AUC more than 0.800 was considered as having the capability to diagnose

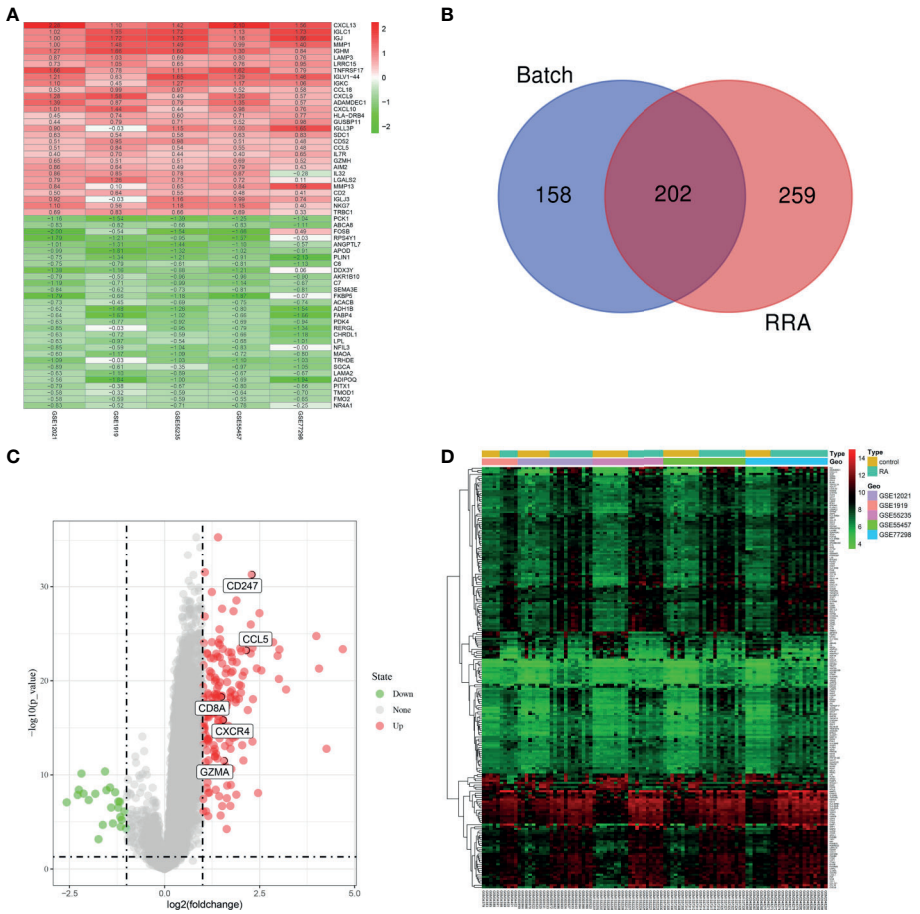


**FIGURE 4** | Identification of DEGs. **(A)** First 30 upregulated and downregulated DEGs of the five datasets determined by "RRA". **(B)** Venn diagram was conducted to obtain the intersection of the DEGs screened by the two methods. The final DEGs were visualized by the volcano map **(C)** and heatmap **(D)**.

RA with excellent specificity and sensitivity. As shown in **Figure 8A**, the AUC values of CCL5, CXCR4, and CD247 were 0.835 (95% CI 0.758–0.913), 0.900 (95% CI 0.847–0.953), and 0.797 (95% CI 0.724–0.871), respectively. Moreover, the combined AUC of CCL5 and CXCR4 reached 0.905 (95% CI 0.852–0.957). As shown in **Figure 8B**, the AUC values of GZMA and CD8A were 0.887 (95% CI 0.819–0.956) and 0.821 (95% CI 0.725–0.918), respectively. The combined AUC of GZMA and CD8A reached 0.900 (95% CI 0.837-0.963). Therefore, CCL5+CXCR4 and GZMA +CD8A had the capability to diagnose RA and early RA with excellent specificity and sensitivity, respectively.

## Correlation Between Biomarkers and Differential Immune Cells in RA

The correlation among four effective biomarkers (CCL5, CXCR4, GZMA, and CD8A) and 10 significantly differential immune cells (M1 macrophages, Tfh cells, memory activated CD4+ T cells, plasma cells, regulatory T cells, activated dendritic cells, activated NK cells, memory resting CD4+ T cells, resting mast cells, and activated mast cells) were analyzed in RA synovial tissues. The correlation results are presented in **Figure 9A**. Significantly related biomarkers and immune cells were screened by $R > 0.40$ and $p < 0.001$. The results indicated that CCL5 was positively correlated with M1 macrophages ($R = 0.47$, $p = 0.00038$), CXCR4 was positively correlated with memory activated CD4+ T cells ($R = 0.44$, $p = 0.00089$) and Tfh cells ($R = 0.70$, $p = 5e\text{-}09$), and GZMA was positively correlated with Tfh ($R = 0.53$, $p = 5e\text{-}05$) (**Figures 9B–E**).

# DISCUSSION

RA is a systemic autoimmune disease characterized by synovitis of joints. Innate and adaptive immune responses play an indispensable role in the pathogenesis of RA. Increasing number of studies have shown that the complex interaction and activation of infiltrating immune cells are key factors in the formation of synovitis and persistent joint damage. Effective biomarkers, especially for the early stage, have not been established due to the significant heterogeneity of RA. Early diagnosis and treatment of RA could effectively prevent the disease progression of 90% patients (22). Therefore, scholars

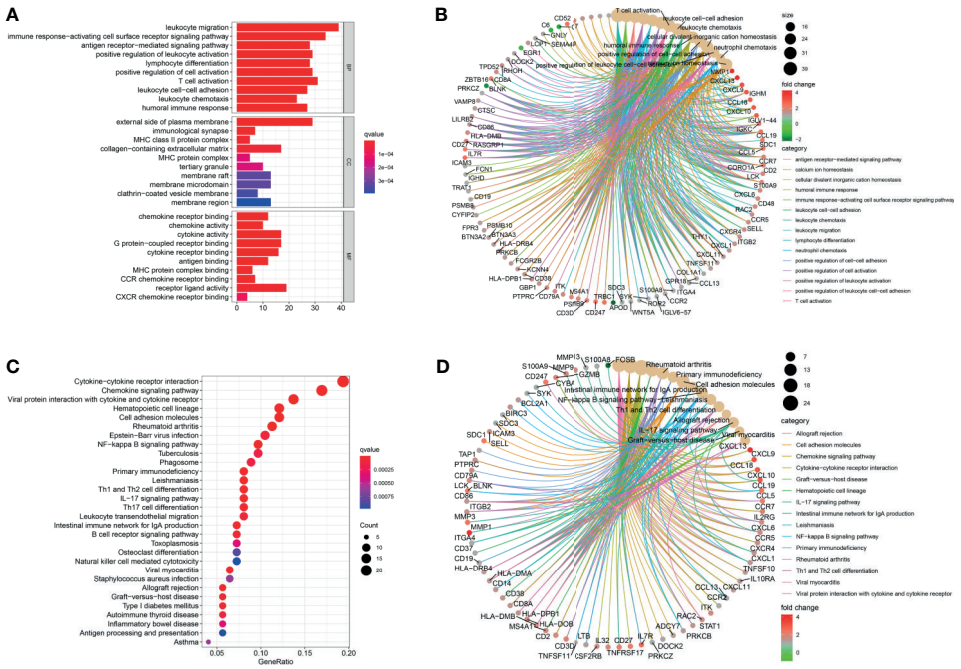


**FIGURE 5** | Functional correlation analysis. DEGs were analyzed by GO and KEGG. **(A, B)** The results of GO were presented by bar plot and circle charts. **(C, D)** The results of KEGG were shown by bubble and circle graphs.

have focused on exploring the immune pathogenesis of RA and searching for novel effective biomarkers. In this study, we used comprehensive, objective, and effective bioinformatics methods to explore the role of immune cell infiltration in the synovium and identify effective diagnostic biomarkers for RA.

Compared with normal control M1 macrophages, the numbers of Tfh cells, memory activated CD4+ T cells, and plasma cells were significantly higher in RA synovial tissues, while those of regulatory T cells, activated dendritic cells, activated NK cells, memory resting CD4+ T cells, resting mast cells, and activated mast cells were significantly lower in RA synovial tissues. Macrophages can be polarized into M1 and M2 macrophages under different conditions due to their high degree of heterogeneity and plasticity. In the synovial environment of RA, macrophages mainly differentiate into type M1, which plays a pro-inflammatory role (23). The pathogenicity of M1 cells in RA is mainly realized by secreting cytokines, which in turn promote inflammation by monocyte/neutrophil recruitment, T cell polarization, and synovial fibroblast proliferation and activation (24). Tfh is a subtype of CD4+T cells, whose main functions are assisting B cells and regulating the production of antibodies (25). Tfh cell surface and secreted molecules, including CXCR5, ICOS (costimulatory molecule), and PD1 (programmed death factor 1), are involved in the development of RA (26, 27). Previous studies suggested that mast cells were aberrantly regulated in RA synovium and were mainly activated by TLRs (toll-like receptors), PAMP (pathogen-associated molecular patterns), and FcγR (Fc gamma receptor) (28, 29). Moreover, dendritic cells, NK cells, and regulatory T cells were demonstrated to play a significant role in the development of RA (30, 31). Therefore, the present results are consistent with previous reports and highlights the importance of these cells in the pathogenesis of RA by bioinformatic analysis.

To improve the accuracy of the results, the DEGs were screened by two independent methods. Further analysis indicated that the function of these DEGs were significantly associated with immune cells. Finally, five hub genes (CXCR4, CCL5, CD8A, CD247, and GZMA) were screened, which were further verified by the validation dataset (GSE89408). Compared with osteoarthritis, all the five hub genes were also significantly higher expressed in RA, which made our result more convincing. Surprisingly, the expression levels of GZMA and CD8A were significantly higher in early RA than in established RA. Therefore, GZMA and CD8A might play an important role in the pathogenesis of early RA, while CXCR4, CCL5, and CD247 might play a vital role in the overall pathogenesis of RA. ROC regression analysis further found that CCL5+CXCR4 and GZMA+CD8A had the capability to diagnose RA and early RA with excellent specificity and sensitivity, respectively.

GZMA is a member of the granzyme family and is mainly secreted by cytotoxic cells (32). In addition to its cytotoxic activity against tumor and virus-infected cells together with perforin, GZMA is involved in innate host during inflammatory and autoimmune disorders in the absence of perforin (33). However, no data have identified the actual function of GZMA. Previous studies reported the elevated expression of GZMA in plasma, synovial fluid, and synovial tissue, indicating that GZMA might be involved in the development of RA (34–36). A recent research further

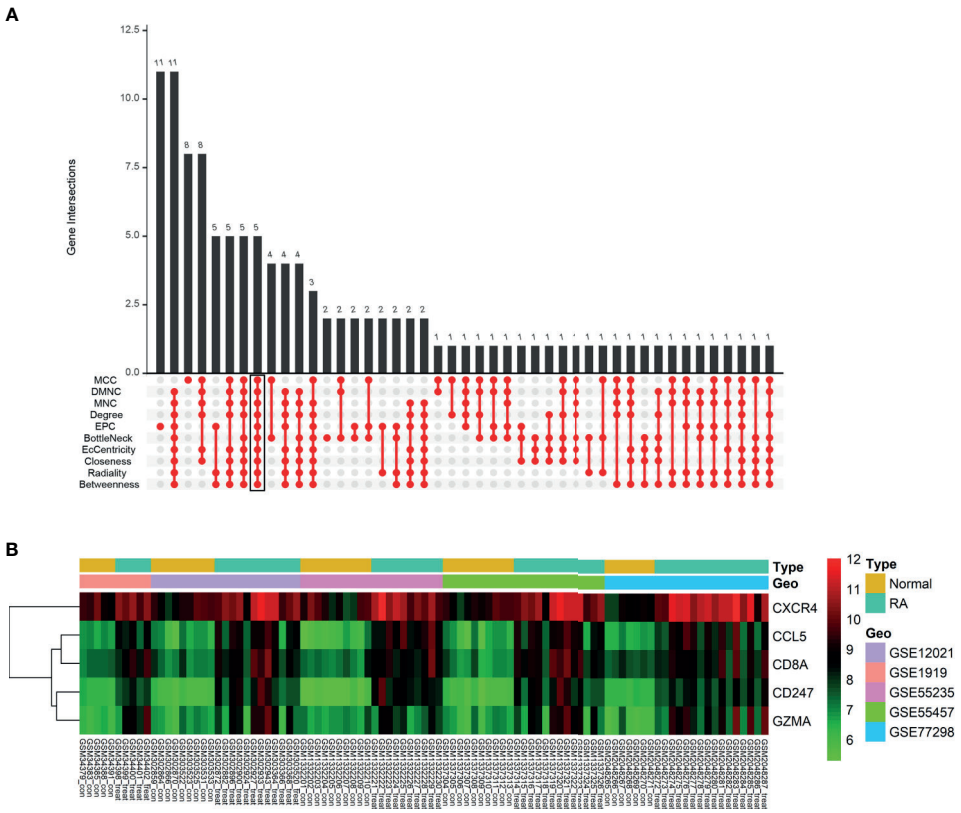


**FIGURE 6** | Identification of hub genes. **(A)** Ten algorithms to screen hub genes by R package "UpSet". **(B)** Expression of five hub genes was presented by heatmap in merged microarray data.

suggested that GZMA contributed to the joint destruction in RA in part by promoting osteoclast differentiation (37). The latest mechanism research showed that GZMA from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells (38). We also found that GZMA was mainly highly expressed in early RA. Therefore, GZMA might be used as a diagnostic biomarker because of its essential role in the pathogenesis in early RA. CD8A encodes the CD8α chain of the dimeric CD8 protein, which is mainly involved in cell-mediated immune defense and T-cell development (39, 40). CD8a could act as diagnosis and prognosis biomarker for many diseases, including tumors and inflammatory diseases (41–44). In the present study, CD8A was significantly overexpressed in RA, especially in early RA. The ROC regression analysis further demonstrated that the combination of GZMA and CD8A had the most excellent specificity and sensitivity for diagnosis of early RA. CCL5, belonging to the C-C chemokine family, is mainly secreted by T lymphocytes, macrophages, certain types of tumor cells, and synovial fibroblasts and mainly induces the recruitment and migration of immune cells to inflammatory sites (45, 46). CXCR4, which belongs to the family of 7-trasmembrance receptors, is involved in many pathophysiological processes by binding to CXCL12 and other non-specific ligands (47). Although numerous studies indicated that CCL5 and CXCR4 played an important role in the progression of many diseases, including chronic liver disease, malignant tumor, and autoimmune disease (48–50), the specific mechanism needs to be further explored. Our study also suggests that CCL5 and CXCR4 are the hub genes of the entire process of RA development and might be used as the diagnose biomarker for this disease.

Given the important role of immune infiltrating cells and hub genes in RA, the correlation among four effective biomarkers (CCL5, CXCR4, GZMA and CD8A) and the top 10 significantly differential immune cells was further investigated in RA. CCL5 was positively correlated with M1 macrophages (R =0.47, p =0.00038), and CXCR4 was positively correlated with memory activated CD4+ T cells (R =0.44, p =0.00089) and Tfh cells (R =0.70, p =5e-09). Previous research revealed that CCL5 could directly activate M1 polarization, and CXCR4 had the ability to active memory activated CD4+ T cells and Tfh cells (51–53). Therefore, CCL5 and CXCR4 may participate in the occurrence and development of RA by regulating corresponding immune cells, which should be verified by further experiments. The most intriguing result was that GZMA was positively correlated with follicular helper T cells (R =0.53, p =5e-05). Studies have elucidated that Tfh cells and GZMA play a crucial role in infection and autoimmune diseases (54–56). M. Perreau et al. showed that HIV-infected individuals had a significantly higher frequency of Tfh cells among total CD4+ T cells compared with non-infected controls (57). Exaggerated

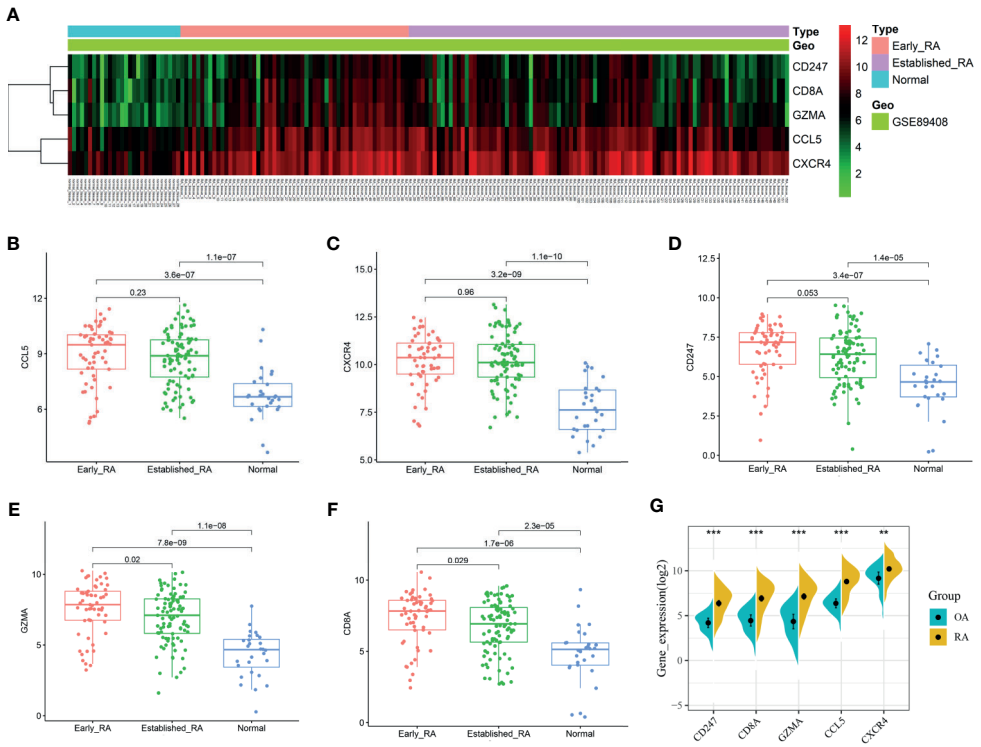


**FIGURE 7** | Validation of hub genes. RNA-Seq dataset GSE89408 was used to validate the expression of CXCR4, CCL5, CD8A, CD247, and GZMA, the results of which were presented as heatmap **(A)**. **(B–F)** Detailed expression of five hub genes in early RA, established RA, and HC. **(G)** Detailed expression of five hub genes in RA and HC. **p < 0.01, ***p < 0.001.

expansion of Tfh cells resulted in self-reactive B cell proliferation, and increased long-lived plasma cell differentiation, as well as an overproduction of pathogenic autoantibodies (58). Repeated exposure to exogenous, endogenous, or symbiotic viruses and bacteria can lead to the high levels of pathogenic autoantibodies, which act as a trigger to promote the occurrence of RA (3). Previous studies have shown that Tfh and GZMA participate in the pathogenesis of RA. Our results also indicated that GZMA was significantly overexpressed in early RA. Therefore, we speculate that infectious agents may play an important role in the early

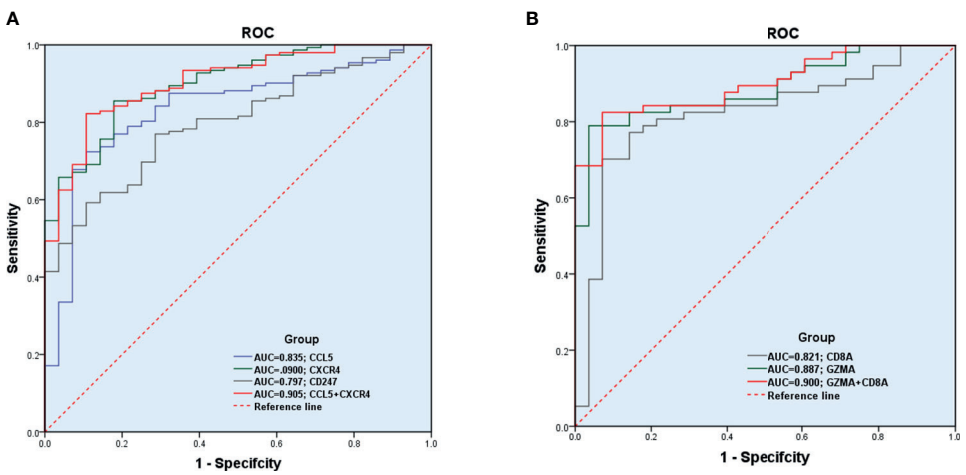


**FIGURE 8** | Diagnostic effectiveness of the biomarkers for RA. **(A, B)** The GSE89408 dataset was used to validate the diagnostic effectiveness of the biomarkers for RA by ROC analysis.

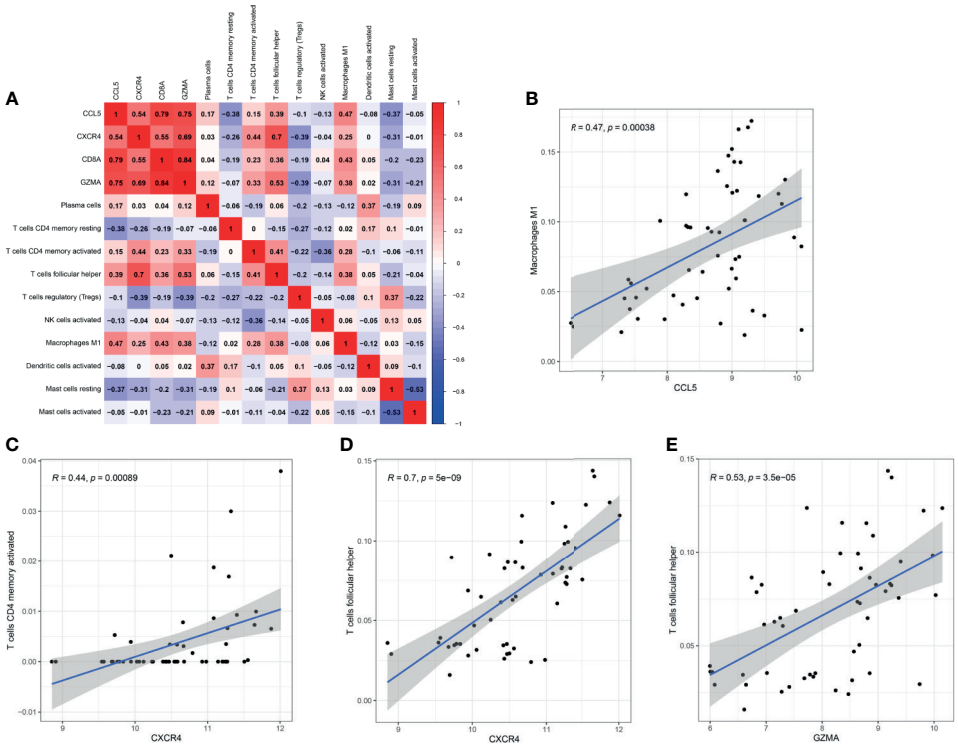


**FIGURE 9** | Correlation between biomarkers and differential immune cells in RA. **(A)** Correlation among four effective biomarkers and 10 significantly differential immune cells. **(B–E)** Significantly related biomarkers and immune cells by R > 0.40 and p < 0.001.

pathogenesis of RA through GZMA-Tfh cells axis, which needs further experimental verification.

This research has some limitations. First, the study lacks clinically relevant information, including the activity of the disease, and the use of drugs for the disease. Second, this study was conducted only from the perspective of gene transcriptome and lacks multi-group trials. Last, only bioinformatics methods were used for data analysis, and subsequent confirmatory experiments *in vivo* and *in vitro* are needed.

In summary, our study not only offers insights into the landscape of immune cells associated with RA but also identifies effective diagnostic biomarkers for RA. GZMA-Tfh cells, CCL5-M1 macrophages, and CXCR4- memory activated CD4+ T cells/Tfh cells may participate in the occurrence and development of RA; in particular, GZMA-Tfh cells may be involved in the early pathogenesis of RA. Therefore, this study may provide a new perspective for the diagnosis and immune cellular-molecular mechanism of RA.

## DATA AVAILABILITY STATEMENT

Publicly available datasets (GSE1919, GSE12021, GSE55235, GSE55457, GSE77298 and GSE89408) were analyzed in this study. All the datasets were obtained from the GEO (http://www.ncbi.nlm.nih.gov/geo) database.

## AUTHOR CONTRIBUTIONS

SZ analyzed and wrote the manuscript. HL designed the experiments and analyzed the data. MX devised the concept and supervised the study. All authors contributed to the article and approved the submitted version.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.726747/full#supplementary-material

**Supplementary Figure 1** | The flow diagram of this study.

**Supplementary Figure 2** | The correlation of 22 types of immune cells in HC synovial tissues was evaluated.

**Supplementary Figure 3** | PPI network results of DEGs by STRING.

## REFERENCES


1. Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al. Rheumatoid Arthritis. *Nat Rev Dis Primers* (2018) 4:18001. doi: 10.1038/nrdp.2018.1
2. Smolen JS, Aletaha D, McInnes IB. Rheumatoid Arthritis. *Lancet* (2016) 388:2023–38. doi: 10.1016/S0140-6736(16)30173-8
3. McInnes IB, Schett G. Pathogenetic Insights From the Treatment of Rheumatoid Arthritis. *Lancet* (2017) 389:2328–37. doi: 10.1016/S0140-6736(17)31472-1
4. Gabriel SE. The Epidemiology of Rheumatoid Arthritis. *Rheum Dis Clin North Am* (2001) 27:269–81. doi: 10.1016/S0889-857X(05)70201-5
5. Karami J, Aslani S, Jamshidi A, Garshasbi M, Mahmoudi M. Genetic Implications in the Pathogenesis of Rheumatoid Arthritis; an Updated Review. *Gene* (2019) 702:8–16. doi: 10.1016/j.gene.2019.03.033
6. Firestein GS, McInnes IB. Immunopathogenesis of Rheumatoid Arthritis. *Immunity* (2017) 46:183–96. doi: 10.1016/j.immuni.2017.02.006
7. Siouti E, Andreakos E. The Many Facets of Macrophages in Rheumatoid Arthritis. *Biochem Pharmacol* (2019) 165:152–69. doi: 10.1016/j.bcp.2019.03.029
8. Elshabrawy HA, Chen Z, Volin MV, Ravella S, Virupannavar S, Shahrara S. The Pathogenic Role of Angiogenesis in Rheumatoid Arthritis. *Angiogenesis* (2015) 18:433–48. doi: 10.1007/s10456-015-9477-2
9. Meednu N, Zhang H, Owen T, Sun W, Wang V, Cistrone C, et al. Production of RANKL by Memory B Cells: A Link Between B Cells and Bone Erosion in Rheumatoid Arthritis. *Arthritis Rheumatol* (2016) 68:805–16. doi: 10.1002/art.39489
10. Hirota K, Hashimoto M, Ito Y, Matsuura M, Ito H, Tanaka M, et al. Autoimmune Th17 Cells Induced Synovial Stromal and Innate Lymphoid Cell Secretion of the Cytokine GM-CSF to Initiate and Augment Autoimmune Arthritis. *Immunity* (2018) 48:1220–32.e5. doi: 10.1016/j.immuni.2018.04.009
11. Yap HY, Tee SZ, Wong MM, Chow SK, Peh SC, Teow SY. Pathogenic Role of Immune Cells in Rheumatoid Arthritis: Implications in Clinical Treatment and Biomarker Development. *Cells* (2018) 7:161. doi: 10.3390/cells7100161
12. Shen-Orr SS, Gaujoux R. Computational Deconvolution: Extracting Cell Type-Specific Information From Heterogeneous Samples. *Curr Opin Immunol* (2013) 25:571–8. doi: 10.1016/j.coi.2013.09.015
13. Qiu L, Liu X. Identification of Key Genes Involved in Myocardial Infarction. *Eur J Med Res* (2019) 24:22. doi: 10.1186/s40001-019-0381-x
14. Zhao X, Zhang L, Wang J, Zhang M, Song Z, Ni B, et al. Identification of Key Biomarkers and Immune Infiltration in Systemic Lupus Erythematosus by Integrated Bioinformatics Analysis. *J Transl Med* (2021) 19:35. doi: 10.1186/s12967-021-02728-2
15. Deng YJ, Ren EH, Yuan WH, Zhang GZ, Wu ZL, Xie QQ. GRB10 and E2F3 as Diagnostic Markers of Osteoarthritis and Their Correlation With Immune Infiltration. *Diagnostics (Basel)* (2020) 10:171. doi: 10.3390/diagnostics10030171
16. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust Enumeration of Cell Subsets From Tissue Expression Profiles. *Nat Methods* (2015) 12:453–7. doi: 10.1038/nmeth.3337
17. Ungethuem U, Haeupl T, Witt H, Koczan D, Krenn V, Huber H, et al. Molecular Signatures and New Candidates to Target the Pathogenesis of Rheumatoid Arthritis. *Physiol Genomics* (2010) 42a:267–82. doi: 10.1152/physiolgenomics.00004.2010
18. Huber R, Hummert C, Gausmann U, Pohlers D, Koczan D, Guthke R, et al. Identification of Intra-Group, Inter-Individual, and Gene-Specific Variances in mRNA Expression Profiles in the Rheumatoid Arthritis Synovial Membrane. *Arthritis Res Ther* (2008) 10:R98. doi: 10.1186/ar2485
19. Woetzel D, Huber R, Kupfer P, Pohlers D, Pfaff M, Driesch D, et al. Identification of Rheumatoid Arthritis and Osteoarthritis Patients by Transcriptome-Based Rule Set Generation. *Arthritis Res Ther* (2014) 16:R84. doi: 10.1186/ar4526
20. Broeren MG, de Vries M, Bennink MB, Arntz OJ, Blom AB, Koenders MI, et al. Disease-Regulated Gene Therapy With Anti-Inflammatory Interleukin-10 Under the Control of the CXCL10 Promoter for the Treatment of Rheumatoid Arthritis. *Hum Gene Ther* (2016) 27:244–54. doi: 10.1089/hum.2015.127
21. Guo Y, Walsh AM, Fearon U, Smith MD, Wechalekar MD, Yin X, et al. CD40L-Dependent Pathway Is Active at Various Stages of Rheumatoid Arthritis Disease Progression. *J Immunol* (2017) 198:4490–501. doi: 10.4049/jimmunol.1601988
22. Aletaha D, Smolen JS. Diagnosis and Management of Rheumatoid Arthritis: A Review. *Jama* (2018) 320:1360–72. doi: 10.1001/jama.2018.13103
23. Di Benedetto P, Ruscitti P, Vadasz Z, Toubi E, Giacomelli R. Macrophages With Regulatory Functions, a Possible New Therapeutic Perspective in Autoimmune Diseases. *Autoimmun Rev* (2019) 18:102369. doi: 10.1016/j.autrev.2019.102369
24. Udalova IA, Mantovani A, Feldmann M. Macrophage Heterogeneity in the Context of Rheumatoid Arthritis. *Nat Rev Rheumatol* (2016) 12:472–85. doi: 10.1038/nrrheum.2016.91
25. Liu R, Zhao P, Zhang Q, Che N, Xu L, Qian J, et al. Adiponectin Promotes Fibroblast-Like Synoviocytes Producing IL-6 to Enhance T Follicular Helper Cells Response in Rheumatoid Arthritis. *Clin Exp Rheumatol* (2020) 38:11–8.
26. Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet ME, Lazaro E, et al. T Follicular Helper Cells in Autoimmune Disorders. *Front Immunol* (2018) 9:1637. doi: 10.3389/fimmu.2018.01637
27. Li Y, Bai S, Zhang J, Shen T, Chen S, Wang Y, et al. [T Follicular Helper Cells and Their Related Molecules in Rheumatoid Arthritis: A Review]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* (2019) 35:281–6.
28. Min HK, Kim KW, Lee SH, Kim HR. Roles of Mast Cells in Rheumatoid Arthritis. *Korean J Intern Med* (2020) 35:12–24. doi: 10.3904/kjim.2019.271
29. Kopicky-Burd JA, Kagey-Sobotka A, Peters SP, Dvorak AM, Lennox DW, Lichtenstein LM, et al. Characterization of Human Synovial Mast Cells. *J Rheumatol* (1988) 15:1326–33.
30. Wehr P, Purvis H, Law SC, Thomas R. Dendritic Cells, T Cells and Their Interaction in Rheumatoid Arthritis. *Clin Exp Immunol* (2019) 196:12–27. doi: 10.1111/cei.13256
31. Schwaneck EC, Renner R, Junker L, Tony HP, Kleinert S, Gernert M, et al. T Cells, Natural Killer Cells, and γδt Cells in a Large Patient Cohort With Rheumatoid Arthritis: Influence of Age and Anti-Rheumatic Therapy. *Scand J Rheumatol* (2020) 49:8–12. doi: 10.1080/03009742.2019.1634755
32. Trapani JA, Smyth MJ. Functional Significance of the Perforin/Granzyme Cell Death Pathway. *Nat Rev Immunol* (2002) 2:735–47. doi: 10.1038/nri911
33. Shimizu K, Yamasaki S, Sakurai M, Yumoto N, Ikeda M, Mishima-Tsumagari C, et al. Granzyme A Stimulates pDCs to Promote Adaptive Immunity *via* Induction of Type I IFN. *Front Immunol* (2019) 10:1450. doi: 10.3389/fimmu.2019.01450
34. Griffiths GM, Alpert S, Lambert E, McGuire J, Weissman IL. Perforin and Granzyme A Expression Identifying Cytolytic Lymphocytes in Rheumatoid Arthritis. *Proc Natl Acad Sci USA* (1992) 89:549–53. doi: 10.1073/pnas.89.2.549
35. Kummer JA, Tak PP, Brinkman BM, van Tilborg AA, Kamp AM, Verweij CL, et al. Expression of Granzymes A and B in Synovial Tissue From Patients With Rheumatoid Arthritis and Osteoarthritis. *Clin Immunol Immunopathol* (1994) 73:88–95. doi: 10.1006/clin.1994.1173
36. Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE. The Levels of Soluble Granzyme A and B are Elevated in Plasma and Synovial Fluid of Patients With Rheumatoid Arthritis (RA). *Clin Exp Immunol* (1999) 116:366–70. doi: 10.1046/j.1365-2249.1999.00881.x
37. Santiago L, Menaa C, Arias M, Martin P, Jaime-Sánchez P, Metkar S, et al. Granzyme A Contributes to Inflammatory Arthritis in Mice Through Stimulation of Osteoclastogenesis. *Arthritis Rheumatol* (2017) 69:320–34. doi: 10.1002/art.39857
38. Zhou Z, He H, Wang K, Shi X, Wang Y, Su Y, et al. Granzyme A From Cytotoxic Lymphocytes Cleaves GSDMB to Trigger Pyroptosis in Target Cells. *Science* (2020) 368:eaaz7548. doi: 10.1126/science.aaz7548
39. Tregaskes CA, Kong FK, Paramithiotis E, Chen CL, Ratcliffe MJ, Davison TF, et al. Identification and Analysis of the Expression of CD8 Alpha Beta and CD8 Alpha Alpha Isoforms in Chickens Reveals a Major TCR-Gamma Delta CD8 Alpha Beta Subset of Intestinal Intraepithelial Lymphocytes. *J Immunol* (1995) 154:4485–94.
40. Xu Q, Chen Y, Zhao WM, Huang ZY, Zhang Y, Li X, et al. DNA Methylation and Regulation of the CD8A After Duck Hepatitis Virus Type 1 Infection. *PloS One* (2014) 9:e88023. doi: 10.1371/journal.pone.0088023
41. Ma K, Qiao Y, Wang H, Wang S. Comparative Expression Analysis of PD-1, PD-L1, and CD8A in Lung Adenocarcinoma. *Ann Transl Med* (2020) 8:1478. doi: 10.21037/atm-20-6486
42. Kristensen LK, Fröhlich C, Christensen C, Melander MC, Poulsen TT, Galler GR, et al. CD4(+) and CD8a(+) PET Imaging Predicts Response to Novel PD-

1 Checkpoint Inhibitor: Studies of Sym021 in Syngeneic Mouse Cancer Models. *Theranostics* (2019) 9:8221–38. doi: 10.7150/thno.37513
43. Kristensen LK, Christensen C, Alfsen MZ, Cold S, Nielsen CH, Kjaer A. Monitoring CD8a(+) T Cell Responses to Radiotherapy and CTLA-4 Blockade Using [(64)Cu]NOTA-CD8a PET Imaging. *Mol Imaging Biol* (2020) 22:1021–30. doi: 10.1007/s11307-020-01481-0
44. Alromaih S, Mfuna-Endam L, Bosse Y, Filali-Mouhim A, Desrosiers M. CD8A Gene Polymorphisms Predict Severity Factors in Chronic Rhinosinusitis. *Int Forum Allergy Rhinol* (2013) 3:605–11. doi: 10.1002/alr.21174
45. Marques RE, Guabiraba R, Russo RC, Teixeira MM. Targeting CCL5 in Inflammation. *Expert Opin Ther Targets* (2013) 17:1439–60. doi: 10.1517/14728222.2013.837886
46. Aldinucci D, Colombatti A. The Inflammatory Chemokine CCL5 and Cancer Progression. *Mediators Inflamm* (2014) 2014:292376. doi: 10.1155/2014/292376
47. Pozzobon T, Goldoni G, Viola A, Molon B. CXCR4 Signaling in Health and Disease. *Immunol Lett* (2016) 177:6–15. doi: 10.1016/j.imlet.2016.06.006
48. Mohs A, Kuttkat N, Reißing J, Zimmermann HW, Sonntag R, Proudfoot A, et al. Functional Role of CCL5/RANTES for HCC Progression During Chronic Liver Disease. *J Hepatol* (2017) 66:743–53. doi: 10.1016/j.jhep.2016.12.011
49. Seo W, Shimizu K, Kojo S, Okeke A, Kohwi-Shigematsu T, Fujii SI, et al. Runx-Mediated Regulation of CCL5 *via* Antagonizing Two Enhancers Influences Immune Cell Function and Anti-Tumor Immunity. *Nat Commun* (2020) 11:1562. doi: 10.1038/s41467-020-15375-w
50. Kawaguchi N, Zhang TT, Nakanishi T. Involvement of CXCR4 in Normal and Abnormal Development. *Cells* (2019) 8;185. doi: 10.3390/cells8020185
51. Li M, Sun X, Zhao J, Xia L, Li J, Xu M, et al. CCL5 Deficiency Promotes Liver Repair by Improving Inflammation Resolution and Liver Regeneration Through M2 Macrophage Polarization. *Cell Mol Immunol* (2020) 17:753–64. doi: 10.1038/s41423-019-0279-0
52. Serre K, Mohr E, Bénézech C, Bird R, Khan M, Caamaño JH, et al. Selective Effects of NF-κb1 Deficiency in $CD4^+$ T Cells on Th2 and TFh Induction by Alum-Precipitated Protein Vaccines. *Eur J Immunol* (2011) 41:1573–82. doi: 10.1002/eji.201041126
53. Yu H, Hu W, Song X, Zhao Y. Immune Modulation of Platelet-Derived Mitochondria on Memory CD4(+) T Cells in Humans. *Int J Mol Sci* (2020) 21:6295. doi: 10.3390/ijms21176295
54. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular Helper T Cells. *Annu Rev Immunol* (2016) 34:335–68. doi: 10.1146/annurev-immunol-041015-055605
55. Schanoski AS, Le TT, Kaiserman D, Rowe C, Prow NA, Barboza DD, et al. Granzyme A in Chikungunya and Other Arboviral Infections. *Front Immunol* (2019) 10:3083. doi: 10.3389/fimmu.2019.03083
56. Arias MA, Jiménez de Bagües MP, Aguiló N, Menao S, Hervás-Stubbs S, de Martino A, et al. Elucidating Sources and Roles of Granzymes A and B During Bacterial Infection and Sepsis. *Cell Rep* (2014) 8:420–9. doi: 10.1016/j.celrep.2014.06.012
57. Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular Helper T Cells Serve as the Major CD4 T Cell Compartment for HIV-1 Infection, Replication, and Production. *J Exp Med* (2013) 210:143–56. doi: 10.1084/jem.20121932
58. Qin L, Waseem TC, Sahoo A, Bieerkehazhi S, Zhou H, Galkina EV, et al. Insights Into the Molecular Mechanisms of T Follicular Helper-Mediated Immunity and Pathology. *Front Immunol* (2018) 9:1884. doi: 10.3389/fimmu.2018.01884

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# SARS-CoV-2 Antibody Isotypes in Systemic Lupus Erythematosus Patients Prior to Vaccination: Associations With Disease Activity, Antinuclear Antibodies, and Immunomodulatory Drugs During the First Year of the Pandemic

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**Objectives:** Impact of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic on individuals with arthritis has been highlighted whereas data on other rheumatic diseases, e.g., systemic lupus erythematosus (SLE), are scarce. Similarly to SLE, severe SARS-CoV-2 infection includes risks for thromboembolism, an unbalanced type I interferon response, and complement activation. Herein, SARS-CoV-2 antibodies in longitudinal samples collected prior to vaccination were analyzed and compared with SLE progression and antinuclear antibody (ANA) levels.

**Methods:** One hundred patients (83 women) with established SLE and a regular visit to the rheumatologist (March 2020 to January 2021) were included. All subjects donated blood and had done likewise prior to the pandemic. SARS-CoV-2 antibody isotypes (IgG, IgA, IgM) to the cell receptor-binding S1-spike outer envelope protein were detected by ELISA, and their neutralizing capacity was investigated. IgG-ANA were measured by multiplex technology.

**Results:** During the pandemic, 4% had PCR-confirmed infection but 36% showed SARS-CoV-2 antibodies of ≥1 isotype; IgA was the most common (30%), followed by IgM (9%) and IgG (8%). The antibodies had low neutralizing capacity and were detected also in prepandemic samples. Plasma albumin ($p = 0.04$) and anti-dsDNA ($p = 0.003$) levels were lower in patients with SARS-CoV-2 antibodies. Blood group, BMI, smoking habits, complement proteins, daily glucocorticoid dose, use of hydroxychloroquine, or

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self-reported coronavirus disease 2019 (COVID-19) symptoms (except fever, >38.5°C) did not associate with SARS-CoV-2 antibodies.

**Conclusion:** Our data from early 2021 indicate that a large proportion of Swedish SLE patients had serological signs of exposure to SARS-CoV-2 but apparently with a minor impact on the SLE course. Use of steroids and hydroxychloroquine showed no distinct effects, and self-reported COVID-19-related symptoms correlated poorly with all antibody isotypes.

**Keywords: COVID-19, lupus (SLE), antibody response, neutralization (effect of), antinuclear antibodies, complement-immunological terms**

## INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has caused disastrous effects worldwide and posed enormous challenges to healthcare. For patients with immune-mediated diseases on continuous treatment with immunosuppressive (or immunomodulatory) drugs, concerns have been raised regarding increased susceptibility to COVID-19 and potentially harmful effects on underlying chronic diseases (1, 2). Recently, the impact of severe COVID-19 on individuals taking disease-modifying antirheumatic drugs (DMARDs) due to inflammatory joint diseases, e.g., rheumatoid arthritis (RA), was demonstrated using Swedish register data (3). Increased risks were mainly linked to comorbidities, and the use of DMARDs (including biologics, such as cytokine-targeted therapies) did not greatly influence the risk of severe COVID-19 infection or death. However, data on other rheumatic conditions, e.g., systemic lupus erythematosus (SLE), are still scarce.

SLE represents a prototype disease of systemic autoimmunity in which immune complexes or cytotoxic antibodies may give rise to tissue damage and organ failure (4). Clinical features and laboratory abnormalities typical of active SLE show several similarities with COVID-19. A dysregulated type I interferon (IFN) system is typical of SLE (5–7). Type I IFNs are key components of the innate and adaptive immune responses to new pathogens, and their pivotal role in antiviral immunity is well established, including unbalanced inflammatory responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (8, 9). Preliminary data suggest that patients with SLE do not have an increased risk of SARS-CoV-2 infection, or severe COVID-19, compared with the general population (2, 10, 11). Still, it cannot be excluded that COVID-19 leads to an increased rate of SLE flares, which has been shown to be the case with other infections or challenges to the immune system (12–14). Furthermore, COVID-19 has been associated with activation of the complement system as well as the development of autoantibodies in hospitalized patients; manifest autoimmune disease related to these newfound autoantibodies and complement consumption has also been observed (15–18). Another feature of COVID-19, resembling SLE and antiphospholipid syndrome (APS), is the increased risk of thromboembolic events (15, 19). Thereto, early in the pandemic, the use of the cornerstone drug for SLE, hydroxychloroquine (HCQ), was suggested to have antiviral effects, but current data do not support its use in COVID-19. The impact on the risk of COVID-19 regarding other drugs used in SLE, e.g., B-cell-targeted therapies, is yet unclear. In multiple sclerosis (MS), the use of rituximab (anti-CD20) is associated with a two to threefold higher risk for severe COVID-19, and the risk increases with the duration of rituximab therapy (20).

To gain an increased understanding of the immune response towards SARS-CoV-2 in patients with SLE, we focused on the following aims: (I) to assess to which extent well-characterized cases with established SLE have been exposed to, and managed to mount an IgG, IgA, or IgM antibody response to, SARS-CoV-2 during the first year of the pandemic (prior to vaccination); (II) to investigate the neutralizing capacity of the detected SARS-CoV-2 antibodies; and to evaluate if the serological signs of COVID-19 were related to (III) progression of SLE or (IV) antinuclear antibody (ANA) levels and (V) the use of immunomodulatory drugs. To address these questions, we took advantage of a previously described cohort of Swedish SLE patients with longitudinal follow-up (21).

## MATERIALS AND METHODS

### Subjects

The study population consisted of 100 patients (83 females, 17 males) with established SLE who had a regular physical visit to the Rheumatology unit at Linköping University Hospital, Sweden, from March 2020 to January 2021. All patients fulfilled the 1982 American College of Rheumatology (ACR) and/or the 2012 Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE and had previously been included in the prospective follow-up program KLURING (a Swedish acronym for *Clinical LUpus Register In North-eastern Gothia*) at the Department of Rheumatology, Linköping University Hospital, as described in detail (21). SLE disease activity was assessed using SLE disease activity index-2000 (SLEDAI-2K) and physician's global assessment (PGA) (22). Irreversible organ damage, required to have been persistent for ≥6 months, was recorded annually by SLICC/ACR damage index (SDI), which encompasses damage in 12 defined organ systems (23). Health-related quality of life (HRQoL) was obtained using the EuroQoL-5 dimensions (EQ-5D) (24).

The participating patients donated blood consecutively at their regular visit during the pandemic and had done likewise at another visit to the Rheumatology unit prior to the pandemic. Thus, a corresponding prepandemic serum sample (from August 2015 to November 2019) was available from each participating subject. All serum samples were stored at −70°C until analysis. Ongoing pharmacotherapy, including daily prednisolone dose was registered at each visit. Detailed characteristics of the study population are shown in **Table 1**.

## Routine Laboratory Measurements and Autoantibody Analyses

Blood cell counts, plasma creatinine, creatine kinase, complement protein 3 (C3), C4, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and urinalysis were measured as part of a clinical routine both at the prepandemic and the pandemic visits. In addition, IgG-ANA fine specificities, including antidouble-stranded DNA (dsDNA) and 13 additional autoantibodies, were analyzed by FIDIS™ Connective Profile,

**TABLE 1** | Characteristics of the 100 included patients with SLE.

| | Prepandemic (n = 100) | Pandemic (n = 100) |
|---|---|---|
| *Background variables* | | |
| Females (*n*) | 83 | 83 |
| Age at blood sampling [*mean years* (range years)] | 48.7 (19–87) | 51.3 (20–90) |
| SLE duration at sampling [*mean years* (range years)] | 12.5 (0–42) | 15.1 (1–47) |
| Caucasian ethnicity (*n*) | 86 | 86 |
| Ever smoker (former or current) (*n*) | 42 | 49 |
| Body mass index [*mean kg/m*$^2$ (range)] | 26.6 (17.0–42.7) | 27.1 (17.1–45.7) |
| *Disease variables* | | |
| SLEDAI-2K [*mean score* (range)] | 2.4 (0–16) | 1.9 (0–24) |
| SLICC/ACR damage index (SDI) [*mean score* (range)] | 1.1 (0–6) | 1.3 (0–8) |
| Physician's global assessment [*mean score* (range)] | 0.4 (0–3) | 0.3 (0–2) |
| EQ–5D (*mean score* (range)) | 0.66 (–0.24–1) | 0.69 (–0.48–1) |
| Erythrocyte sedimentation rate [*mean mm/h* (range)] | 20.0 (2–108) | 18.3 (1–106) |
| C-reactive protein [*mean mg/L* (range)] | 6.8 (2.5–172) | 6.1 (2.5–166) |
| Complement protein 3 [*mean g/L* (range)] | 1.1 (0.4–1.8) | 0.98 (0.5–1.9) |
| Complement protein 4 [*mean g/L* (range)] | 0.19 (0.02–0.55) | 0.18 (0.02–0.53) |
| Albumin [*mean g/L* (range)] | NE | 39.1 (28–50) |
| Anti-dsDNA antibody levels [*mean IU/ml* (range)] | 95.6 (2–900) | 101.3 (0–1081) |
| Blood group 0* (*n*) | 38 | 38 |
| Blood group A (*n*) | 37 | 37 |
| Blood group B (*n*) | 11 | 11 |
| Blood group AB (*n*) | 4 | 4 |
| Rh+ (*n*) | 78 | 78 |
| Rh– (*n*) | 12 | 12 |
| *Pharmacotherapy* | | |
| Hydroxychloroquine (*n*) | 77 | 73 |
| Azathioprine (*n*) | 8 | 8 |
| Mycophenolate mofetil (*n*) | 21 | 23 |
| Rituximab (*n*) | 2 | 3 |
| Cyclophosphamide (*n*) | 1 | 0 |
| Sirolimus (*n*) | 4 | 3 |
| Belimumab (*n*) | 0 | 4 |
| Daily prednisolone dose (*mean mg*) | 4.5 (0–30) | 3.5 (0–15) |
| *1982 American College of Rheumatology classification criteria (ACR-82)* | | |
| Cases meeting ≥4 ACR-82 criteria (*n*) | 80# | 82# |
| Number of fulfilled ACR-82 criteria [*mean* (range)] | 4.7 (3–9) | 4.8 (3–9) |
| 1. Malar rash (*n*) | 33 | 35 |
| 2. Discoid rash (*n*) | 11 | 12 |
| 3. Photosensitivity (*n*) | 48 | 48 |
| 4. Oral ulcers (*n*) | 14 | 14 |
| 5. Arthritis (*n*) | 78 | 80 |
| 6. Serositis (*n*) | 29 | 29 |
| 7. Renal disorder (*n*) | 34 | 34 |
| 8. Neurologic disorder (*n*) | 10 | 10 |
| 9. Hematologic disorder (*n*) | 58 | 62 |
| 10. Immunological disorder (*n*) | 57 | 61 |
| 11. Antinuclear antibody† (*n*) | 99 | 99 |

*NE, not estimated.*

**Blood group data available for 90 participants.*

*#All patients that did not fulfil ACR-82 met the 2012 SLICC classification criteria.*

*†Positive by indirect immunofluorescence microscopy on HEp-2 cells.*

Solinium software version 1.7.1.0 (Theradiag, Croissy-Beaubourg, France) in February 2021 at the Clinical Immunology Laboratory, Linköping University Hospital in collected sera from both visits (25). This addressable laser bead assay (ALBIA) measures autoantibodies to Ro52/SSA, Ro60/SSA, La/SSB, Smith antigen (Sm), Smith/ribonucleoprotein (Sm/RNP), U1RNP, scleroderma 70 kD antigen (Scl-70), dsDNA, histone, ribosomal P protein (RibP), centromere protein B (CENP-B), polymyositis/systemic sclerosis complex (PmScl), histidyl-tRNA synthetase (Jo-1), and proliferating cell nuclear antigen (PCNA). According to the manufacturer's instructions, a cutoff for each antibody specificity at 40 IU/ml was applied. Sera collected prior to and during the pandemic were analyzed in parallel to avoid interassay variation.

## Self-Reported Symptoms Associated With COVID-19

Using a questionnaire, patients were interviewed by telephone regarding COVID-19-associated symptoms during the study period: fever >38.5°C, headache, hypogeusia, hyposmia, cough, dyspnea, sore throat, rhinorrhea, myalgia, fatigue, diarrhea, nausea, vomiting, abdominal pain, deep vein thrombosis, and pulmonary embolism.

## Review of Medical Records

Digital medical records were reviewed with respect to confirmed COVID-19 by detection of SARS-CoV-2 RNA in the respiratory tract, hospitalization, and severity of illness category according to the National Institute of Health, and blood group according to the Rhesus (Rh) and the AB0 group system, respectively.

## SARS-CoV-2 PCR Assay

The RealTime SARS-CoV-2 assay using nasopharyngeal swab specimens was performed at the Clinical Microbiology Laboratory, Linköping University Hospital, according to the Emergency Use Authorization product insert (26). Tests were considered negative if no genome had been detected over 44 cycles.

## SARS-CoV-2 Antibody Assay

Enzyme-linked immunosorbent assays (ELISA) were mainly performed as described elsewhere (27, 28), with a slight modification as presented below. SARS-CoV-2 S1-spike protein (Wuhan strain, 2019) was used as soluble antigen on 96-well microplates (0.5 µg/ml) in PBS (Sino Biological, Eschborn, Germany). The antigen coated plates were blocked for 60 min at 37°C with 5% fat-free milk buffer. Sera from the SLE cases and patients with previously confirmed COVID-19 infection, as well as from positive and negative controls, were diluted in PBS-Tween 20 (0.05%) with 2.5% fat-free milk buffer. Serum dilutions were added to the coated plate wells and incubated 90 min at 37°C. Conjugates against antihuman IgG-HRP (BioRad, Richmond, CA, USA), antihuman IgM (Abcam, NordicBiosite, Täby, Sweden), or antihuman IgA-HRP (Nordic BioSite, Täby, Sweden) were added to separate wells with diluted serum samples and incubated 90 min at 37°C. Finally, 0.003% $H_2O_2$/o-phenylene diamine substrate (Sigma-Aldrich, St. Louis, MA, USA; 0.4 mg/ml) was added, and the plates were kept in darkness at room temperature for 30 min before 2.5 M $H_2SO_4$ was used as stop solution. Plates were read at an optical density (OD) of 490 nm. A standard curve was used to determine arbitrary units (AU), estimating quantitative levels of SARS-CoV-2-specific antibodies. Cutoff for positive tests was defined as an OD above the 3rd standard deviation of samples from healthy donors collected before the pandemic. All SLE samples were analyzed in parallel and blinded regarding whether they had been collected before or during the pandemic.

## Inhibition Assays

Serum samples with strong IgA and/or IgM SARS-CoV-2 reactivity as judged by our assay were used for blocking experiments. The sera were preincubated 1 h at 37°C with increasing concentrations (0.1, 1, and 10 µg/L) of SARS-CoV-2 S1-spike protein or with irrelevant antigens: influenza A or bovine serum albumin (BSA). The samples were thereafter treated and analyzed as described above.

## Commercial IgA SARS-CoV-2 Antibody ELISA

To cross-validate the in-house ELISA, we tested the prepandemic and pandemic samples with an FDA-approved *in vitro* diagnostic ELISA kit (IgA anti-SARS-CoV-2 ELISA, EI-2606-9601A, EUROIMMUN AG, Kriens, Switzerland) which provides a semiquantitative *in vitro* determination of human antibodies of the immunoglobulin class IgA against SARS-CoV-2 in serum, EDTA, heparin, or citrate plasma (29). The assay was performed according to the manufacturer's instruction. Briefly, all the reagents were brought to room temperature approximately 30 minutes before use. One hundred microliters of the diluted samples (prepandemic and pandemic) was transferred to the individual microplate wells and incubated for 60 min at 37°C. Immediately after sample incubation, the microplate was washed with washing buffer three times, leaving the wash buffer in each well for 30 to 60 s per washing cycle, followed by 100 µl incubation of enzyme conjugate for 30 min at 37°C. Another round of washing as described earlier was carried out. One hundred microliters of substrate solution was added and incubated for another 30 min this time at room temperature and away from direct sunlight. The reaction was stopped by adding 100 µl of stop solution into each well and finally photometric measurement of the color intensity was recorded at 450 nm wavelength and a reference wavelength at 650 nm. The extinction of the calibrator (provided in the kit) defines the upper limit of the reference range of noninfected persons (cutoff) recommended by the manufacturer.

Semiquantitative results were calculated using a ratio of the extinction of the control or patient sample over the extinction of the calibrator. The ratio was estimated according to the following formula:

$$\text{Ratio} = \text{Extinction of the control or patient sample} / \text{Extinction of calibrator}$$

The manufacturer recommended the following interpretation of the results:

Ratio < 0.8 = Negative

Ratio ≥ 0.8– < 1.1 = Borderline

Ratio ≥ 1.1 = Positive

## Microneutralization Assay

Serum neutralization assay was performed as described elsewhere (30). Briefly, heat-inactivated serum including positive and negative controls was serially diluted in twofold steps from 1:4, 1:8, 1:16 until 1:1,024 in MEM-2% HI fetal calf serum (FCS). Serum dilutions was added 75 µl/well in duplicate wells (96-well flat well cell culture plate). Virus (SARS-CoV-2, 2020-nCoV [SARS-CoV-2-Iso_LiU-Human-2020-03-04-Swe]) was added 75 µl/well at a concentration of 100–130 PFU/ml and incubated for 1 h at 37°C 5% $CO_2$. After incubation, the serum/virus mixture was added onto wells with $5 \times 10^4$ Vero E6 cells/well in 100 µl MEM with 2% HI FSC. The plates were kept at 37°C 5% $CO_2$ for 96 h before examined in the microscope for ratio of healthy cells vs virus-induced cell cytotoxic effect (CPE) areas. Cells were fixed for 30 min and stained with 0.1% crystal violet. Serum dilutions that showed 50% inhibition of CPE was given as the neutralization titer.

## Inhibition of S1-Spike Protein-Binding to ACE2 Cell Receptor *In Vitro*

Analysis was performed as previously described (31). In brief, 96-well microplates were coated with 1 µg/ml recombinant human ACE2-protein (in PBS pH 7.4) over night at 4°C. Heat-inactivated serum samples diluted 1:50, 1:150, and 1:450 were mixed with 1 µg/ml soluble recombinant S1-spike protein in duplicate microwells in 100 µl/well and incubated at 37°C for 30 min before being transferred to the ACE2-coated microwells. Plates were incubated for 1 h at 37°C. Plates were washed with PBS-Tween 20 (0.05%). HRP-labeled antihuman S1-spike monoclonal antibody was added (100 µl/well) and incubated for 1 h at 37°C. Plates were washed and substrate o-phenylene diamine/0.003% $H_2O_2$ was added as substrate and incubated at RT for 20 min. Substrate reaction was stopped with 100 µl/well 2.5 M $H_2SO_4$ before plates were read at optical density (OD) 490 nm. Controls with no serum and human serum lacking S1-spike binding was used as negative controls, and human serum with high SARS-CoV-2 neutralization titer (1,024) was used as positive control. Serum samples with 50% of more inhibition of ACE2-S1-spike protein-binding OD 490 nm reactivity was considered a significantly inhibiting serum titer.

## Avidity Assay

Avidity analysis of serum immunoglobulin binding to viral antigens by ELISA was performed as previously reported (32).

## Statistics

For comparisons of biomarker levels between groups, the Mann-Whitney *U*-test was used. Associations between seropositivity (categorical variable) and self-reported symptoms, SLE phenotypes (ACR criteria), and disease activity were examined with the $\chi^2$ test, or Fisher's exact test when appropriate ($n \leq 5$). When antibody levels were compared before and during the pandemic, Wilcoxon-matched paired signed rank test was used. Spearman's correlation was employed for all correlation analyses. $p$-Values ≤0.05 were considered statistically significant. Statistical analyses were performed using the SPSS software ver. 26.0.0.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism ver. 8.4.3 (GraphPad Software Inc., San Diego, CA). Graphs were created using GraphPad Prism ver. 8.4.3 (GraphPad Software).

## Ethical Considerations

This study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The study protocol was approved by the regional ethics review board in Linköping (Decision number M75-08/2008).

# RESULTS

## SARS-CoV-2 IgG, IgA, and IgM Antibodies Pre- and During the Pandemic

In total, four patients (4%) had confirmed COVID-19 during the study period and one of them was hospitalized in the intensive care unit for 42 days and needed mechanical ventilation for 29 days. The cycle threshold (Ct) values of the four positive samples were 16.79, 31.83, 31.94, and 35.92. During the study period, 26 patients tested negative at least once.

Specific blood group, according to the AB0 and Rh systems, did not associate with either confirmed COVID-19 or presence of SARS-CoV-2 antibodies. A history of SARS-CoV-2 RNA positivity during the pandemic did not associate with presence of any of the SARS-CoV-2 antibody isotypes. During the pandemic, 36% had detectable SARS-CoV-2 antibodies of ≥1 isotype; IgA was the most common (30%), followed by IgM (9%) and IgG (8%). No significant gender difference was detected in SARS-CoV-2 antibodies. However, trends were found with higher percentage of men being positive for both IgG ($p = 0.10$) and ≥1 isotype ($p = 0.11$). SLE duration and age at sampling were not associated with SARS-CoV-2 antibody status. As illustrated in **Figures 1A–C**, SARS-CoV-2 IgG was significantly higher during than prior to the pandemic, whereas the IgA and IgM isotypes did not differ significantly. Several patients showed detectable SARS-CoV-2 antibody levels during the prepandemic period.

Self-reported respiratory symptoms (cough, dyspnea, sore throat, rhinorrhea) did not associate with presence of any SARS-CoV-2 antibody isotype. However, self-reported fever (>38.5°C) associated significantly with the presence of SARS-CoV-2 IgG ($p = 0.03$) during the pandemic but not with IgA or IgM. None of the other self-reported symptoms (headache, hypogeusia, hyposmia, myalgia, fatigue, diarrhea, nausea,

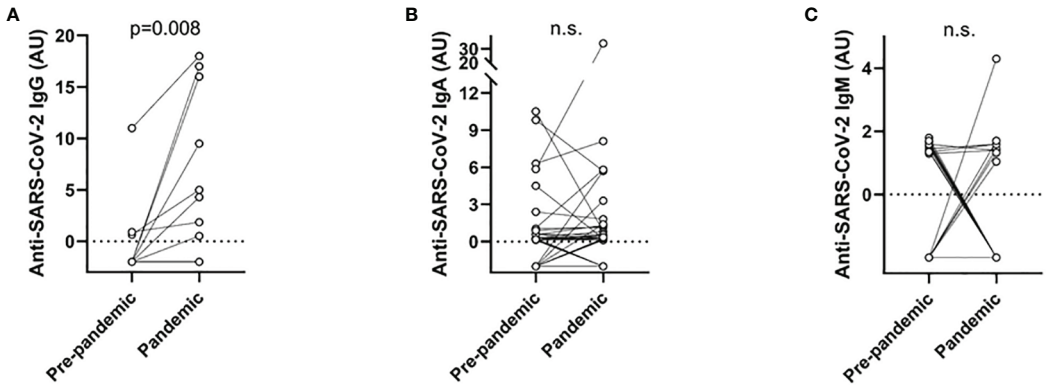


**FIGURE 1** | Arbitrary units (AU) of SARS-CoV-2 antibodies in the sera of the 100 included patients with SLE demonstrated for each isotype: IgG **(A)**, IgA **(B)**, and IgM **(C)** before and during the pandemic. Only SARS-CoV-2 IgG levels were significantly higher during the pandemic compared with prepandemic samples. n.s., not significant.

vomiting, abdominal pain, deep vein thrombosis, and pulmonary embolism) associated with antibody findings.

## Dose-Dependent Reduction of IgA/IgM Reactivity by Preincubation With S1-Spike Protein

Inhibition of SARS-CoV-2 IgA and IgM reactivity by preincubation with S1-spike protein or irrelevant proteins was evaluated in a selected collection of serum samples yielding strong reactivity in the assays described above. As illustrated in paired samples from three patients in **Supplementary Figure S1A–F**, a dose-dependent reduction in antibody binding was achieved for both IgA and IgM following preincubation with S1-spike protein. For IgA, inhibition of >50% was observed in 11 of 13 (85%) samples and >80% in 8/13 (62%). For IgM, inhibition of >50% was demonstrated in nine of 10 samples (90%) and >80% in seven of 10 (70%).

## Agreement Between the In-House IgA Assay and EUROIMMUN SARS-CoV-2 IgA ELISA

The level of agreement between the in-house IgA assay and the commercial SARS-CoV-2 IgA ELISA was evaluated in 50 paired samples originating from 25 patients of the study population. As demonstrated in **Table 2**, the differences between prepandemic and pandemic samples were strongly correlated ($p = 0.0001$). The correlation between the assays in prepandemic samples was also highly significant ($p = 0.005$) whereas it did not reach statistical significance in the pandemic samples. The concordance (defined in **Table 2**) was fair, 76% for all samples. The entire data set of the commercial SARS-CoV-2 IgA ELISA is shown in **Supplementary Figure S2A–G**.

## Neutralizing Capacity of Detected SARS-CoV-2 Antibody Isotypes

In a selected serum sample collection (20 serum samples, originating from 13 patients), where evident ELISA-seropositive immunoglobulin reactivity against the recombinant S1-spike protein was observed, the virus-neutralizing activity *in vitro* was investigated. All tested serum samples had no, or very low neutralizing reactivity; below eight in neutralizing serum titer *in vitro* with the used virus (**Table 3**). The positive control showed neutralizing titer of 1,024 and negative controls were negative (<8). The same sera were subsequently tested in a S1-spike- and recombinant ACE2 receptor-binding inhibition

**TABLE 2** | Correlation analyses and concordance between results of the IgA in-house S1 spike assay and the EUROIMMUN IgA ELISA.

| | Number of samples | Percentage positive by assay | Concordance* | Spearman's rho | *p*-Value |
|---|---|---|---|---|---|
| **Prepandemic to pandemic: change between paired samples** (in-house, AU, *vs*. EUROIMMUN, ratio) | 25x2 | N/A | N/A | 0.78 | 0.0001 |
| **Prepandemic samples compared** (in-house, AU, *vs*. EUROIMMUN, ratio) | 25 | | 72% | 0.54 | 0.005 |
| *In-house* | | 19/25 (76%) | | | |
| *EUROIMMUN* | | 12/25 (48%) | | | |
| **Pandemic serum samples compared** (in-house, AU, *vs*. EUROIMMUN, ratio) | 25 | | 80% | 0.36 | 0.07 |
| *In-house* | | 22/25 (88%) | | | |
| *EUROIMMUN* | | 15/25 (60%) | | | |
| **All samples compared** (in-house, AU, *vs*. EUROIMMUN, ratio) | 50 | | 76% | 0.47 | 0.0005 |
| *In-house* | | 41/50 (82%) | | | |
| *EUROIMMUN* | | 27/50 (54%) | | | |

*AU, arbitrary units; N/A, not applicable.*

**The sum of double-positive samples and double-negative samples, divided by the total number of samples, multiplied by 100.*

**TABLE 3** | Serological virus-inhibition analysis of S1 ELISA-reactive sera.

| Serum samples | Number of samples | NT (>8) | S1-ACE2 block (>25%) | AI (median) | | |
|---|---|---|---|---|---|---|
| | | | | IgG | IgA | IgM |
| SARS-CoV-2 positive: SLE | 20 (13 patients) | 0/20 | 1/20 | 0.63 | 0.22 | 0.21 |
| Negative control | 8 | 0/8 | 0/8 | <0.1 | <0.1 | <0.1 |
| Positive control | 4 | 4/4 | 4/4 | 0.99 | 0.84 | N/A |

*NT, neutralization assay; AI, avidity index; N/A, not applicable.*

assay *in vitro*. The inhibiting capacity was low, shown to be between 5% and 42% (median 10%, range 0%–42%). Serum from SARS-CoV-2 seropositive controls showed >90% inhibition (91%–96%) and negative control sera <15% inhibition (median 6%, range 0%–14%). Serum immunoglobulin avidity was tested against the recombinant S1 protein with ELISA-positive IgG, IgA, and IgM serum samples, and the avidity index (AI) of reactivity among the patient sera were strongest with the IgG isotype (median 0.63, range 0.5–0.83); avidity index of the IgA and IgM isotypes were considerably lower (for IgA, median was 0.23, range 0.19–0.26; for IgM, median 0.21, range 0.18–0.27). The positive control serum showed median IgG avidity index of 0.99, and for IgA, a median avidity of 0.81 was observed.

## SARS-CoV-2 Antibodies in Relation to SLE Phenotypes, HRQoL, and Disease Activity

Regarding SLE phenotypes, photosensitivity (ACR criterion 3) associated inversely with presence of SARS-CoV-2 IgA ($p$ = 0.05). Immunological disorder (ACR criterion 10) associated inversely with both presence of SARS-CoV-2 IgM ($p$ = 0.01) and with any SARS-CoV-2 antibody isotype ($p$ = 0.03). Limited accrual of organ damage was observed (see SDI, **Table 1**); individuals who acquired damage were not SARS-CoV-2 antibody positive to a higher extent than those with unchanged SDI.

Global SLE disease activity assessed at the two visits did not differ significantly, either by assessment of SLEDAI-2K (**Figure 2A**) or PGA. Systemic inflammation detected by CRP and ESR

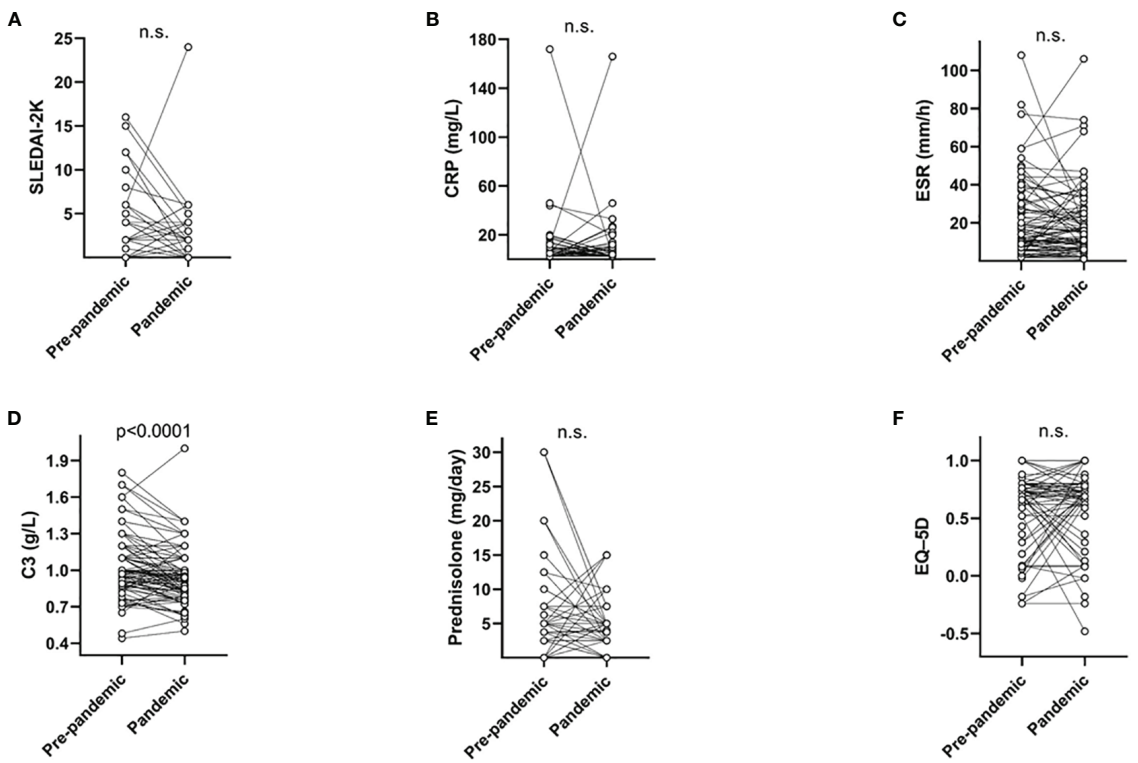


**FIGURE 2** | Global SLE disease activity (SLEDAI-2K) **(A)**, C-reactive protein (CRP) **(B)**, erythrocyte sedimentation rate (ESR) **(C)**, Complement protein 3 (C3) **(D)**, daily prednisolone dose **(E)**, and EuroQoL-5 dimensions (EQ–5D) **(F)** shown for the 100 included patients with SLE before and during the pandemic. Only the C3 levels were significantly lower during the pandemic compared with prepandemic samples. n.s., not significant.

were also similar (**Figures 2B, C**). However, signs of increased complement consumption were observed with significantly decreased C3 levels (**Figure 2D**) whereas the reduction of C4 did not meet statistical significance. The daily use of prednisolone did not differ and neither did the HRQoL assessed by the EQ–5D (**Figures 2E, F**). Plasma albumin ($p$ = 0.04) and anti-dsDNA ($p$ = 0.003) levels during the pandemic were lower in patients with positive SARS-CoV-2 antibodies (of at least one isotype) compared with negative cases.

## ANA Levels Pre- and During the Pandemic *vs*. SARS-CoV-2 Antibodies

None of the samples contained antibodies to Jo-1 or Scl-70. Antibodies to Ro52/SSA, Ro60/SSA, Sm, dsDNA, histone, RibP,

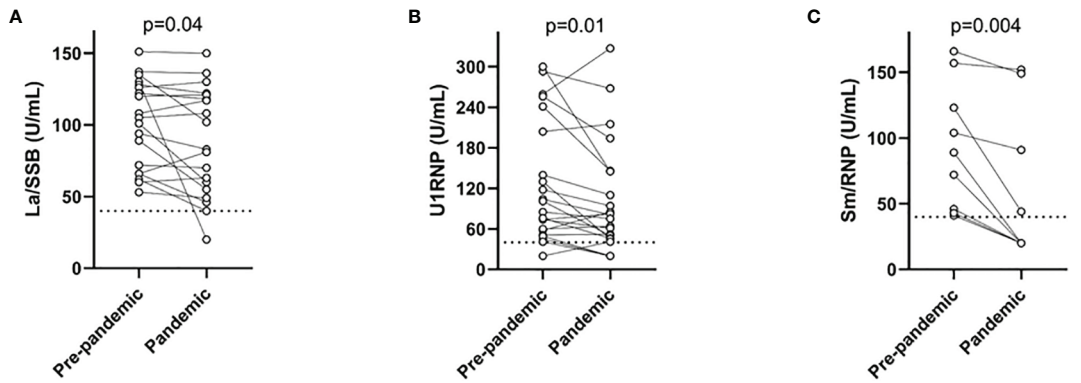


**FIGURE 3** | Levels of three antinuclear antibodies (ANA) targeting extrachromosomal antigens demonstrated before and during the pandemic. Significantly lower levels of La/SSB **(A)**, U1RNP **(B)**, and Sm/RNP **(C)** were found during the pandemic compared with prepandemic samples. To increase the readability, only patients with at least one sample above the cutoff of the assay (before or during the pandemic) are shown.

**TABLE 4** | Associations between ANA specificities and SARS-CoV-2 antibody isotypes in SLE patients before and during the pandemic.

| | | *SLE: prepandemic (n = 100)* | | | *SLE: pandemic (n = 100)* | | |
|---|---|---|---|---|---|---|---|
| **SARS-CoV-2 isotypes** | | **IgG (*n* = 4)** | **IgA (*n* = 31)** | **IgM (*n* = 13)** | **IgG (*n* = 8)** | **IgA (*n* = 30)** | **IgM (*n* = 9)** |
| Ro52/SSA | + | 1/33 | 5/33 | 5/33 | 1/29 | 6/29 | 1/29 |
| | – | 3/67 | 26/67 | 8/67 | 7/71 | 24/71 | 8/71 |
| Ro60/SSA | + | 1/35 | 7/35 | 4/35 | 1/30 | 8/30 | 2/30 |
| | – | 3/65 | 24/65 | 9/65 | 7/70 | 22/70 | 7/70 |
| La/SSB | + | 0/19 | 5/19 | 4/19 | 0/17 | 5/17 | 3/17 |
| | – | 4/81 | 26/81 | 9/81 | 8/83 | 25/83 | 6/83 |
| Sm | + | 1/3 | 0/3 | 0/3 | 1/2 | 1/2 | 0/2 |
| | – | 3/97 | 31/97 | 13/97 | 7/98 | 29/98 | 9/98 |
| Sm/RNP | + | 0/9 | 3/9 | 1/9 | 0/4 | 2/4 | 0/4 |
| | – | 4/91 | 28/91 | 12/91 | 8/96 | 28/96 | 9/96 |
| U1RNP | + | 1/22 | 6/22 | 2/22 | 2/19 | 5/19 | 1/19 |
| | – | 3/78 | 25/78 | 11/78 | 6/81 | 25/81 | 8/81 |
| dsDNA | + | 0/41 | 16/41 | 5/41 | 0/37 | 6/37 | 0/37 |
| | – | 4/59 | 15/59 | 8/59 | 8/63 | 24/63 | 9/63 |
| RibP | + | 0/3 | 0/3 | 0/3 | 1/5 | 2/5 | 0/5 |
| | – | 4/97 | 31/97 | 13/97 | 7/95 | 28/95 | 9/95 |
| CENP-B | + | 0/3 | 0/3 | 0/3 | 0/4 | 1/4 | 1/4 |
| | – | 4/97 | 31/97 | 13/97 | 8/96 | 29/96 | 8/96 |
| PmScl | + | 0/4 | 2/4 | 1/4 | 0/5 | 2/5 | 1/5 |
| | – | 4/96 | 29/96 | 12/96 | 8/95 | 28/95 | 8/95 |
| PCNA | + | 0/5 | 2/5 | 2/5 | 0/3 | 1/3 | 0/3 |
| | – | 4/95 | 29/95 | 11/95 | 8/97 | 29/97 | 9/97 |
| Histone | + | 0/9 | 3/9 | 1/9 | 0/10 | 0/10 | 0/10 |
| | – | 4/91 | 28/91 | 12/91 | 8/90 | 30/90 | 9/90 |

$p$ = 0.008
$p$ = 0.02
$p$ = 0.03
Division by zero

CENP-B, PmScl, and PCNA were not statistically different when prepandemic and pandemic samples of antibody-positive individuals were compared. However, ANA targeting three extrachromosomal antigens decreased significantly during the pandemic (**Figures 3A–C**). As demonstrated, lower values were achieved for most samples regarding La/SSB where 13/19 (68.4%) samples decreased, for U1RNP 15/22 (68.2%) samples decreased, and for Sm/RNP 9/9 (100%) samples decreased. However, when also taking the intra-assay variation of the method into account: 6/19 (31.6%) La/SSB-positive patients decreased and none increased; 8/22 (36.4%) U1RNP-positive patients decreased and 3/22 (13.6%) increased; and 4/9 (44.4%) Sm/RNP-positive patients decreased and none increased.

**Table 4** illustrates the associations between each ANA specificity and presence of SARS-CoV-2 antibody isotypes before and during the pandemic. Presence of anti-Sm was significantly associated with SARS-CoV-2 IgG in both prepandemic ($p$ = 0.008) and pandemic ($p$ = 0.03) samples. Presence of anti-dsDNA was inversely associated with SARS-CoV-2 IgA only during the pandemic ($p$ = 0.02).

## SARS-CoV-2 Antibodies in Relation to Immunomodulatory Drugs

During the pandemic, potential associations between SARS-CoV-2 antibodies and ongoing immunomodulatory drugs were investigated. Presence of SARS-CoV-2 IgA was associated with the use of mycophenolate mofetil ($p$ = 0.04), but none of the other DMARDs were significantly associated with the presence of SARS-CoV-2 antibodies.

# DISCUSSION

Serological testing is a cornerstone in our understanding of infections and immune responses. A reliable immunoassay should be both sensitive and specific and perform well not only among healthy individuals but also in patient groups with immune-mediated diseases (33). Subsequently, the need of reliable SARS-CoV-2 antibody assays is extensive and crucial for opening societies after lockdowns and permit traveling. Furthermore, as the vaccination programs against COVID-19 progress worldwide, the requirement of credible SARS-CoV-2 antibody assays (to demonstrate a successful vaccine response) will probably remain over the next couple of years.

The present study was primarily undertaken to extend our knowledge of the adaptive immune response against SARS-CoV-2 in patients with established SLE, of which the great majority (92%) were taking immunosuppressive or immunomodulatory drugs. Such treatments, e.g., glucocorticoids (used by 66% during the pandemic) or mycophenolate mofetil (23%), could potentially result in decreased ability to recover from the infection, establish immunity or respond to vaccinations (34). In contrast though, a recent paper from the USA showed that most patients with SLE and confirmed COVID-19 were able to produce and maintain an IgG response despite the use of a variety of DMARDs, providing reassurance about the efficacy and durability of humoral immunity and possible protection against reinfection with SARS-CoV-2 (35). However, Saxena et al. did not have prepandemic samples available for comparison and did not report potential associations with ANA (35). However, studying the humoral immune response to SARS-CoV-2 in SLE is not only of relevance due to potential effects of pharmacotherapies, the pathogenesis of SLE is characterized by B-cell hyperactivity and reflected by the large amount of autoantibodies described (36). In addition, similarities between severe COVID-19 and SLE, such as risks for thromboembolism, an unbalanced type I IFN response and complement activation have been observed, resulting in implementation of regular use of anticoagulation and glucocorticoids in the clinical management of patients with COVID-19 (6, 7). A major strength of this study was that the SLE patients were their own controls as prepandemic samples were available and analyzed in parallel with samples collected during the pandemic.

Our data from early 2021, obtained prior to the introduction of vaccines, indicate that a large proportion of Swedish patients with SLE had serological signs of exposure to SARS-CoV-2, seemingly with poor correlation to COVID-19-related symptoms. To validate the reliability of our in-house assay, a commercially available and FDA-approved diagnostic IgA SARS-CoV-2 antibody kit was used. The assays showed a reassuring concordance in both prepandemic and pandemic samples (72%–80%), and the inhibition tests yielded dose-dependent reduction of IgA/IgM antibody reactivity following preincubation with S1-spike protein. Still, almost all analyzed antibody-positive samples showed a low neutralizing capacity indicating low-affinity antibodies with uncertain protective effect against SARS-CoV-2. Interestingly, SARS-CoV-2 antibodies (particularly IgA and IgM) were also detected in corresponding samples collected ahead of the pandemic. Whether this represents an entirely unspecific "sticky" immunoglobulin response, exposure to previous corona viruses or signs of interference with autoantibodies in the immunoassays remains an open question. Since SLE is a condition characterized by a broad repertoire of circulating autoantibodies, it is not unlikely that this group of patients in general may be more prone to produce antibodies targeting various antigens, including coronaviruses, even in the absence of COVID-19. In line with this, it was recently demonstrated that certain infections (e.g., malaria, schistosomiasis, and dengue) may yield unreliable results in rapid diagnostic COVID-19 antibody tests (37). Some betacoronaviruses have been described as capable of inducing ELISA and Western blot cross-reactive anti-SARS-CoV-2 serum responses, but in general not cross-neutralizing antibodies (38–40). The coronavirus 229E appears to be an exception (41, 42). In our study, the SARS-CoV-2 IgG isotype was less often found in prepandemic samples and was in addition the only antibody that significantly associated with self-reported symptoms (body temperature >38.5°C) during the pandemic. A meta-analysis recently concluded that blood group A may be associated with a higher risk of severe SARS-CoV-2 infection compared with group 0, which usually associates with a lower

risk (43). We could not demonstrate any clear associations of specific blood group (according to the AB0 and Rh systems) with PCR-confirmed COVID-19, or with presence of SARS-CoV-2 antibodies, but larger study populations are probably needed to confirm such associations. Neither did we find any clear differences regarding SARS-CoV-2 antibody positivity related to sex, age, or SLE duration. In contrast, an age-related serum half-life of anti-SARS-CoV-2 IgM and IgG has previously been reported in the general population and severity of the infection may also be of relevance (44, 45). An obvious limitation of the study is that the proportion of subjects infected by SARS-CoV-2 is unknown. In Sweden, PCR testing of individuals with symptoms of infection was not introduced in routine for the general population until June of 2020 (46). Thus, the four patients (4%) with PCR-confirmed COVID-19 during the study period indisputably represent an underestimation, especially since the infection may well pass without symptoms.

Presence of rheumatoid factor often challenges the specificity of immunoassays and is, in general, a common suspect of false positive tests. This was recently highlighted also for different immunoassays of serological SARS-CoV-2 testing in patients with chronic inflammatory diseases, whereof most of the investigated samples originated from patients with MS and RA (47). A subgroup of rituximab-treated SLE patients was included, but rheumatoid factor did not seem to have a major impact on the SARS-CoV-2 test results in SLE. Herein, we further extended the knowledge by analyzing ANA and their potential interference with SARS-CoV-2 (shown in **Table 4**). Solely, the presence of anti-Sm showed a significant association with SARS-CoV-2 IgG in prepandemic as well as pandemic samples. Furthermore, anti-dsDNA was inversely associated with SARS-CoV-2 IgA during the pandemic and samples positive for any SARS-CoV-2 antibody isotype contained significantly lower levels of anti-dsDNA. Thus, possibly except for anti-Sm, our data indicate that the presence of specific SLE autoantibodies does not interfere with detection of SARS-CoV-2 IgG.

Regardless of SARS-CoV-2 antibody status, we observed a significant decrease during the pandemic of three specific autoantibodies targeting extrachromosomal antigens (La/SSB, U1RNP, and Sm/RNP). These reductions remained significant also when the variation of the method was considered and did not coincide with additional use of immunosuppression in the patients with declining antibody levels. Overall, clinical disease activity assessed by SLEDAI-2K and PGA remained stable. Neither did the systemic inflammation (ESR or CRP) differ over time. However, C3 decreased significantly during the pandemic and a similar trend was observed for C4. The latter usually represents increased activation of the complement pathway following immune complex deposition (48, 49). If accompanied by positive anti-dsDNA in patients with SLE, this observation is referred to as "serologically active clinically quiescent" (50). The findings of diminishing ANA levels in pandemic, compared with prepandemic, samples were unexpected. Potential reasons could be increased isolation during the pandemic and meticulous adherence to advice of social distancing that overall might have led to less infections and challenges to the immune system (13, 14).

Some limitations deserve to be mentioned. The size of the study population was limited, especially as only 4% tested positive with PCR. Due to the lack of general testing in Sweden during the beginning of the pandemic, the percentage of truly infected subjects remains unknown. Thus, associations between "confirmed" infection, laboratory variables and clinical parameters should be interpreted with caution. Furthermore, we only evaluated the humoral and not the cell-mediated immune response to SARS-CoV-2. Ethnicity of the study population constitutes another limitation. Mainly Caucasian patients were enrolled, and as ethnicity is well known to affect SLE severity, extrapolation of our results to other populations should be done with caution (51). In contrast, a major strength is the Swedish healthcare system, which is public, tax funded, and offers universal access. This significantly reduces the risk of selection bias and ensures a high coverage of cases. The well-characterized cohort of SLE patients followed by a limited number of experienced rheumatologists at a single tertiary referral center constituted another strength of the study. Finally, the SARS-CoV-2 antibodies were not only quantified but their neutralizing capacity was also evaluated.

To conclude, we show that a large proportion of Swedish SLE patients have serological signs of exposure to SARS-CoV-2 prior to vaccination but apparently with a minor impact on the SLE course. SARS-CoV-2 antibodies, particularly IgA and IgM, had low neutralizing capacity and were detected also in samples obtained prior to the pandemic. Except for anti-Sm, specific SLE autoantibodies did not associate with SARS-CoV-2 IgG. The use of steroids and DMARDs showed no distinct effects on the ability to mount an antibody response to SARS-CoV-2 and self-reported COVID-19 symptoms (except for fever) correlated poorly with all detected antibody isotypes.

## 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 study protocol was approved by the regional ethics review board in Linköping (Decision number M75-08/2008). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JS: drafting the manuscript and revising it critically for important intellectual content; access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; study conception and design; data validation; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. MA: drafting the manuscript and revising it critically for important intellectual content; acquisition of data, analysis, and interpretation of data;

and approval of the final version to be published. MF: drafting the manuscript and revising it critically for important intellectual content; access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. YZ: drafting the manuscript and revising it critically for important intellectual content; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. LS: drafting the manuscript and revising it critically for important intellectual content; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. CD: drafting the manuscript and revising it critically for important intellectual content; study conception and design; data validation; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. JH: drafting the manuscript and revising it critically for important intellectual content; study conception and design; data validation; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. CS: writing the original draft; drafting the manuscript and revising it critically for important intellectual content; access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; study conception and design; data validation; acquisition of data, analysis, and interpretation of data; and approval of the final version to be published. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724047/full#supplementary-material

## REFERENCES

1. Akiyama S, Hamdeh S, Micic D, Sakuraba A. Prevalence and Clinical Outcomes of COVID-19 in Patients With Autoimmune Diseases: A Systematic Review and Meta-Analysis. *Ann Rheum Dis* (2020) annrheumdis–2020-218946. doi: 10.1136/annrheumdis-2020-218946
2. Favalli EG, Gerosa M, Murgo A, Caporali R. Are Patients With Systemic Lupus Erythematosus at Increased Risk for COVID-19? *Ann Rheum Dis* (2021) 80(2):e25. doi: 10.1136/annrheumdis-2020-217787
3. Bower H, Frisell T, Di Giuseppe D, Delcoigne B, Ahlenius GM, Baecklund E, et al. Impact of the COVID-19 Pandemic on Morbidity and Mortality in Patients With Inflammatory Joint Diseases and in the General Population: A Nationwide Swedish Cohort Study. *Ann Rheum Dis* (2021) 80(8):1086–93. doi: 10.1136/annrheumdis-2021-219845
4. Kaul A, Gordon C, Crow MK, Touma Z, Urowitz MB, van Vollenhoven R, et al. Systemic Lupus Erythematosus. *Nat Rev Dis Primers* (2016) 2:16039. doi: 10.1038/nrdp.2016.39
5. Ronnblom L, Leonard D. Interferon Pathway in SLE: One Key to Unlocking the Mystery of the Disease. *Lupus Sci Med* (2019) 6(1):e000270. doi: 10.1136/lupus-2018-000270
6. Oon S, Wilson NJ, Wicks I. Targeted Therapeutics in SLE: Emerging Strategies to Modulate the Interferon Pathway. *Clin Transl Immunol* (2016) 5(5):e79. doi: 10.1038/cti.2016.26
7. Ramos-Casals M, Brito-Zeron P, Mariette X. Systemic and Organ-Specific Immune-Related Manifestations of COVID-19. *Nat Rev Rheumatol* (2021) 17 (6):315–32. doi: 10.1038/s41584-021-00608-z
8. Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, et al. Impaired Type I Interferon Activity and Inflammatory Responses in Severe COVID-19 Patients. *Science* (2020) 369(6504):718–24. doi: 10.1126/science.abc6027
9. Gaziano L, Giambartolomei C, Pereira AC, Gaulton A, Posner DC, Swanson SA, et al. Actionable Druggable Genome-Wide Mendelian Randomization Identifies Repurposing Opportunities for COVID-19. *Nat Med* (2021) 27 (4):668–76. doi: 10.1038/s41591-021-01310-z
10. Ramirez GA, Gerosa M, Beretta L, Bellocchi C, Argolini LM, Moroni L, et al. COVID-19 in Systemic Lupus Erythematosus: Data From a Survey on 417 Patients. *Semin Arthritis Rheum* (2020) 50(5):1150–7. doi: 10.1016/j.semarthrit.2020.06.012
11. Gartshteyn Y, Askanase AD, Schmidt NM, Bernstein EJ, Khalili L, Drolet R, et al. COVID-19 and Systemic Lupus Erythematosus: A Case Series. *Lancet Rheumatol* (2020) 2(8):e452–e4. doi: 10.1016/S2665-9913(20)30161-2
12. Raghavan S, Gonakoti S, Asemota IR, Mba B. A Case of Systemic Lupus Erythematosus Flare Triggered by Severe Coronavirus Disease 2019. *J Clin Rheumatol* (2020) 26(6):234–5. doi: 10.1097/RHU.0000000000001531
13. Nived O, Sturfelt G, Wollheim F. Systemic Lupus Erythematosus and Infection: A Controlled and Prospective Study Including an Epidemiological Group. *Q J Med* (1985) 55(218):271–87.
14. Wahren-Herlenius M, Dorner T. Immunopathogenic Mechanisms of Systemic Autoimmune Disease. *Lancet* (2013) 382(9894):819–31. doi: 10.1016/S0140-6736(13)60954-X
15. Zuo Y, Estes SK, Ali RA, Gandhi AA, Yalavarthi S, Shi H, et al. Prothrombotic Autoantibodies in Serum From Patients Hospitalized With COVID-19. *Sci Transl Med* (2020) 12(570):eabd3876. doi: 10.1126/scitranslmed.abd3876
16. Fujii H, Tsuji T, Yuba T, Tanaka S, Suga Y, Matsuyama A, et al. High Levels of Anti-SSA/Ro Antibodies in COVID-19 Patients With Severe Respiratory Failure: A Case-Based Review: High Levels of Anti-SSA/Ro Antibodies in COVID-19. *Clin Rheumatol* (2020) 39(11):3171–5. doi: 10.1007/s10067-020-05359-y
17. Risitano AM, Mastellos DC, Huber-Lang M, Yancopoulou D, Garlanda C, Ciceri F, et al. Complement as a Target in COVID-19? *Nat Rev Immunol* (2020) 20(6):343–4. doi: 10.1038/s41577-020-0320-7
18. Dieker J, Berden JH, Bakker M, Briand JP, Muller S, Voll R, et al. Autoantibodies Against Modified Histone Peptides in SLE Patients Are Associated With Disease Activity and Lupus Nephritis. *PloS One* (2016) 11 (10):e0165373. doi: 10.1371/journal.pone.0165373

19. Kollias A, Kyriakoulis KG, Lagou S, Kontopantelis E, Stergiou GS, Syrigos K. Venous Thromboembolism in COVID-19: A Systematic Review and Meta-Analysis. *Vasc Med* (2021) 26(4):415–25. doi: 10.1177/1358863X21995566
20. Sormani MP, De Rossi N, Schiavetti I, Carmisciano L, Cordioli C, Moiola L, et al. Disease-Modifying Therapies and Coronavirus Disease 2019 Severity in Multiple Sclerosis. *Ann Neurol* (2021) 89(4):780–9. doi: 10.1002/ana.26028
21. Ighe A, Dahlstrom O, Skogh T, Sjowall C. Application of the 2012 Systemic Lupus International Collaborating Clinics Classification Criteria to Patients in a Regional Swedish Systemic Lupus Erythematosus Register. *Arthritis Res Ther* (2015) 17:3. doi: 10.1186/s13075-015-0521-9
22. Griffiths B, Mosca M, Gordon C. Assessment of Patients With Systemic Lupus Erythematosus and the Use of Lupus Disease Activity Indices. *Best Pract Res Clin Rheumatol* (2005) 19(5):685–708. doi: 10.1016/j.berh.2005.03.010
23. Gladman D, Ginzler E, Goldsmith C, Fortin P, Liang M, Urowitz M, et al. The Development and Initial Validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for Systemic Lupus Erythematosus. *Arthritis Rheum* (1996) 39(3):363–9. doi: 10.1002/art.1780390303
24. Dolan P. Modeling Valuations for EuroQol Health States. *Med Care* (1997) 35 (11):1095–108. doi: 10.1097/00005650-199711000-00002
25. Enocsson H, Wirestam L, Dahle C, Padyukov L, Jonsen A, Urowitz MB, et al. Soluble Urokinase Plasminogen Activator Receptor (suPAR) Levels Predict Damage Accrual in Patients With Recent-Onset Systemic Lupus Erythematosus. *J Autoimmun* (2020) 106:102340. doi: 10.1016/j.jaut.2019.102340
26. Abbot. *Alinity M SARS-CoV-2 AMP Kit (EUA). Instructions for Use: Package Insert. Abbott Molecular*. Abbott Park, IL: Manufacturer's Instructions from Abbott Molecular (2019) p. 09N78–095.
27. Yu J, Tostanoski LH, Peter L, Mercado NB, McMahan K, Mahrokhian SH, et al. DNA Vaccine Protection Against SARS-CoV-2 in Rhesus Macaques. *Science* (2020) 369(6505):806–11. doi: 10.1126/science.abc6284
28. Chi X, Yan R, Zhang J, Zhang G, Zhang Y, Hao M, et al. A Neutralizing Human Antibody Binds to the N-Terminal Domain of the Spike Protein of SARS-CoV-2. *Science* (2020) 369(6504):650–5. doi: 10.1126/science.abc6952
29. Beavis KG, Matushek SM, Abeleda APF, Bethel C, Hunt C, Gillen S, et al. Evaluation of the EUROIMMUN Anti-SARS-CoV-2 ELISA Assay for Detection of IgA and IgG Antibodies. *J Clin Virol* (2020) 129:104468. doi: 10.1016/j.jcv.2020.104468
30. Haveri A, Smura T, Kuivanen S, Osterlund P, Hepojoki J, Ikonen N, et al. Serological and Molecular Findings During SARS-CoV-2 Infection: The First Case Study in Finland, January to February 2020. *Euro Surveill* (2020) 25 (11):2000266. doi: 10.2807/1560-7917.ES.2020.25.11.2000266
31. Byrnes JR, Zhou XX, Lui I, Elledge SK, Glasgow JE, Lim SA, et al. Competitive SARS-CoV-2 Serology Reveals Most Antibodies Targeting the Spike Receptor-Binding Domain Compete for ACE2 Binding. *mSphere* (2020) 5 (5):e00802-20. doi: 10.1128/mSphere.00802-20
32. Korhonen MH, Brunstein J, Haario H, Katnikov A, Rescaldani R, Hedman K. A New Method With General Diagnostic Utility for the Calculation of Immunoglobulin G Avidity. *Clin Diagn Lab Immunol* (1999) 6(5):725–8. doi: 10.1128/CDLI.6.5.725-728.1999
33. Galipeau Y, Greig M, Liu G, Driedger M, Langlois MA. Humoral Responses and Serological Assays in SARS-CoV-2 Infections. *Front Immunol* (2020) 11:610688. doi: 10.3389/fimmu.2020.610688
34. Furer V, Rondaan C, Agmon-Levin N, van Assen S, Bijl M, Kapetanovic MC, et al. Point of View on the Vaccination Against COVID-19 in Patients With Autoimmune Inflammatory Rheumatic Diseases. *RMD Open* (2021) 7(1): e001594. doi: 10.1136/rmdopen-2021-001594
35. Saxena A, Guttmann A, Masson M, Kim MY, Haberman RH, Castillo R, et al. Evaluation of SARS-CoV-2 IgG Antibody Reactivity in Patients With Systemic Lupus Erythematosus: Analysis of a Multi-Racial and Multi-Ethnic Cohort. *Lancet Rheumatol* (2021) 3(8):e585–e94. doi: 10.1016/S2665-9913 (21)00114-4
36. Yaniv G, Twig G, Shor DB, Furer A, Sherer Y, Mozes O, et al. A Volcanic Explosion of Autoantibodies in Systemic Lupus Erythematosus: A Diversity of 180 Different Antibodies Found in SLE Patients. *Autoimmun Rev* (2015) 14 (1):75–9. doi: 10.1016/j.autrev.2014.10.003
37. Vanroye F, Bossche DVD, Brosius I, Tack B, Esbroeck MV, Jacobs J. COVID-19 Antibody Detecting Rapid Diagnostic Tests Show High Cross-Reactivity When Challenged With Pre-Pandemic Malaria, Schistosomiasis and Dengue Samples. *Diagnostics (Basel)* (2021) 11(7):1163. doi: 10.3390/diagnostics 11071163
38. Ruetalo N, Businger R, Althaus K, Fink S, Ruoff F, Hamprecht K, et al. Neutralizing Antibody Response in Non-Hospitalized SARS-CoV-2 Patients. *medRxiv* (2020). doi: 10.1101/2020.08.07.20169961
39. Robbiani DF, Gaebler C, Muecksch F, Lorenzi JCC, Wang Z, Cho A, et al. Convergent Antibody Responses to SARS-CoV-2 in Convalescent Individuals. *Nature* (2020) 584(7821):437–42. doi: 10.1038/s41586-020-2456-9
40. Long QX, Tang XJ, Shi QL, Li Q, Deng HJ, Yuan J, et al. Clinical and Immunological Assessment of Asymptomatic SARS-CoV-2 Infections. *Nat Med* (2020) 26(8):1200–4. doi: 10.1038/s41591-020-0965-6
41. Song G, He WT, Callaghan S, Anzanello F, Huang D, Ricketts J, et al. Cross-Reactive Serum and Memory B-Cell Responses to Spike Protein in SARS-CoV-2 and Endemic Coronavirus Infection. *Nat Commun* (2021) 12(1):2938. doi: 10.1038/s41467-021-23074-3
42. Tso FY, Lidenge SJ, Pena PB, Clegg AA, Ngowi JR, Mwaiselage J, et al. High Prevalence of Pre-Existing Serological Cross-Reactivity Against Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) in Sub-Saharan Africa. *Int J Infect Dis* (2021) 102:577–83. doi: 10.1016/j.ijid.2020.10.104
43. Goel R, Bloch EM, Pirenne F, Al-Riyami AZ, Crowe E, Dau L, et al. ABO Blood Group and COVID-19: A Review on Behalf of the ISBT COVID-19 Working Group. *Vox Sang* (2021). doi: 10.1111/vox.13076
44. Zhang K MZ, Yang L, Kang W, Yin Y, Lau JYN. Significant Reduction of Humoral Response to SARS-CoV-2 4 Months After the Diagnosis of COVID-19. *Precis Clin Med* (2021) 4(1):73–6. doi: 10.1093/pcmedi/pbaa036
45. Ibarrondo FJ, Fulcher JA, Goodman-Meza D, Elliott J, Hofmann C, Hausner MA, et al. Rapid Decay of Anti-SARS-CoV-2 Antibodies in Persons With Mild Covid-19. *N Engl J Med* (2020) 383(11):1085–7. doi: 10.1056/ NEJMc2025179
46. Tegnell A. The Swedish Public Health Response to COVID-19. *APMIS* (2021) 129(7):320–3. doi: 10.1111/apm.13112
47. Kharlamova N, Dunn N, Bedri SK, Jerling S, Almgren M, Faustini F, et al. False Positive Results in SARS-CoV-2 Serological Tests for Samples From Patients With Chronic Inflammatory Diseases. *Front Immunol* (2021) 12:666114. doi: 10.3389/fimmu.2021.666114
48. Sjowall C, Wettero J, Bengtsson T, Askendal A, Almroth G, Skogh T, et al. Solid-Phase Classical Complement Activation by C-Reactive Protein (CRP) is Inhibited by Fluid-Phase CRP-C1q Interaction. *Biochem Biophys Res Commun* (2007) 352(1):251–8. doi: 10.1016/j.bbrc.2006.11.013
49. Mathsson L, Ahlin E, Sjowall C, Skogh T, Ronnelid J. Cytokine Induction by Circulating Immune Complexes and Signs of in-Vivo Complement Activation in Systemic Lupus Erythematosus are Associated With the Occurrence of Anti-Sjogren's Syndrome A Antibodies. *Clin Exp Immunol* (2007) 147 (3):513–20. doi: 10.1111/j.1365-2249.2006.03313.x
50. Steiman AJ, Gladman DD, Ibanez D, Urowitz MB. Prolonged Serologically Active Clinically Quiescent Systemic Lupus Erythematosus: Frequency and Outcome. *J Rheumatol* (2010) 37(9):1822–7. doi: 10.3899/jrheum.100007
51. Arnaud L, Tektonidou MG. Long-Term Outcomes in Systemic Lupus Erythematosus: Trends Over Time and Major Contributors. *Rheumatology (Oxford)* (2020) 59(Suppl5):v29–38. doi: 10.1093/rheumatology/keaa382


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# ImmunosuppressiveTherapies Differently Modulate Humoral- and T-Cell-Specific Responses to COVID-19 mRNA Vaccine in Rheumatoid Arthritis Patients

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**Objective:** To assess in rheumatoid arthritis (RA) patients, treated with different immunosuppressive therapies, the induction of SARS-CoV-2-specific immune response after vaccination in terms of anti-region-binding-domain (RBD)-antibody- and T-cell-specific responses against spike, and the vaccine safety in terms of clinical impact on disease activity.

**Methods:** Health care workers (HCWs) and RA patients, having completed the BNT162b2-mRNA vaccination in the last 2 weeks, were enrolled. Serological response was evaluated by quantifying anti-RBD antibodies, while the cell-mediated response was evaluated by a whole-blood test quantifying the interferon (IFN)-γ-response to spike peptides. FACS analysis was performed to identify the cells responding to spike stimulation. RA disease activity was evaluated by clinical examination through the DAS28crp, and local and/or systemic clinical adverse events were registered. In RA patients, the ongoing therapeutic regimen was modified during the vaccination period according to the American College of Rheumatology indications.

**Results:** We prospectively enrolled 167 HCWs and 35 RA patients. Anti-RBD-antibodies were detected in almost all patients (34/35, 97%), although the titer was significantly

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reduced in patients under CTLA-4-inhibitors (median: 465 BAU/mL, IQR: 103-1189, p<0.001) or IL-6-inhibitors (median: 492 BAU/mL, IQR: 161-1007, p<0.001) compared to HCWs (median: 2351 BAU/mL, IQR: 1389-3748). T-cell-specific response scored positive in most of RA patients [24/35, (69%)] with significantly lower IFN-γ levels in patients under biological therapy such as IL-6-inhibitors (median: 33.2 pg/mL, IQR: 6.1-73.9, p<0.001), CTLA-4-inhibitors (median: 10.9 pg/mL, IQR: 3.7-36.7, p<0.001), and TNF-α-inhibitors (median: 89.6 pg/mL, IQR: 17.8-224, p=0.002) compared to HCWs (median: 343 pg/mL, IQR: 188-756). A significant correlation between the anti-RBD-antibody titer and spike-IFN-γ-specific T-cell response was found in RA patients (rho=0.432, p=0.009). IFN-γ T-cell response was mediated by $CD4^+$ and $CD8^+$ T cells. Finally, no significant increase in disease activity was found in RA patients following vaccination.

**Conclusion:** This study showed for the first time that antibody-specific and whole-blood spike-specific T-cell responses induced by the COVID-19 mRNA-vaccine were present in the majority of RA patients, who underwent a strategy of temporary suspension of immunosuppressive treatment during vaccine administration. However, the magnitude of specific responses was dependent on the immunosuppressive therapy administered. In RA patients, BNT162b2 vaccine was safe and disease activity remained stable.

**Keywords: COVID-19, mRNA vaccine, rheumatoid arthritis, whole blood, T cell response, antibody response, DMARD (disease modifying anti-rheumatic drug), biological therapy**

## INTRODUCTION

The COronaVIrus Disease-2019 (COVID-19) pandemic caused by the Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) has recently emerged as a new human-to-human transmissible disease with a serious global health impact and still difficult clinical management (1–3).

Mass vaccination is the single most effective public health measure for controlling the COVID-19 pandemic, and a global effort to develop and distribute an effective vaccine produced important containment results. Several data are currently available about efficacy of mRNA platform vaccines, namely BNT162b2 and mRNA-1273 vaccines, in inducing strong antibody and cell-mediated immune responses in naïve healthy individuals (4–6). The ability to elicit a coordinated induction of both humoral- and cell-mediated arms is fundamental for a more effective fighting of SARS-CoV-2 infection (7, 8).

Currently available data suggest that patients with autoimmune inflammatory rheumatic diseases have a slightly higher prevalence of SARS-CoV-2 infections, risk of hospitalization, and death from COVID-19 than the general population, and they have been considered a priority target group for vaccine administration (9, 10). However, considering the immunologic dysregulation and the immunosuppressive treatments frequently adopted in these patients, some concerns have arisen regarding vaccine efficacy and safety.

Recently, some encouraging data on mRNA vaccination in rheumatoid arthritis (RA) patients have emerged from few small and one large prospective observational multicenter study evaluating the immunogenicity and safety of the BNT162b2 mRNA vaccine compared to control subjects without rheumatic diseases (11–13). Overall, these studies show that the antibody response to BNT162b2 vaccine is immunogenic in the majority of patients with RA (86-100%), but delayed and reduced compared to controls. Although the results on the impact of the immunosuppressive therapy on vaccine immunogenicity are not homogenous, most of studies suggest that rituximab followed by abatacept, mycophenolate mofetil, corticosteroids (CCS), and methotrexate (MTX) can induce a significant reduction of seropositive rate and antibody levels (14). These data are crucial to optimize the management of RA patients and to improve vaccine safety and effectiveness, but they need to be confirmed and supplemented by additional real-world studies and by the evaluation of the T-cell-specific response.

This study aimed to assess in RA patients treated with different immunosuppressive therapies the induction of a specific immune response after SARS-CoV-2 vaccination in terms of anti-region-binding-domain (RBD)-antibody- and T-cell-specific responses against spike, and the safety of vaccination in terms of clinical impact on disease activity. A cohort of health care workers (HCWs) was used as a healthy control group.

## MATERIALS AND METHODS

### Study Population

Participants were enrolled from two parallel prospective studies conducted at the National Institute for Infectious Diseases (INMI) Lazzaro Spallanzani and approved by the INMI Ethical Committee. The approved studies evaluated the immune

response to SARS-CoV-2 vaccination in both HCWs enrolled at INMI (approval number 297/2021) and in rheumatologic patients enrolled at Sant'Andrea Hospital in Rome (approval number 318/2021). All HCWs and rheumatologic patients received the BNT162b2-mRNA vaccine. Inclusion criteria for the enrollment of rheumatologic patients were: a diagnosis of RA according to the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) 2010 criteria (15), having completed the two-dose schedule of the mRNA vaccine in the last 2 weeks, being on treatment with a biological drug (except anti-CD20) with or without MTX or other disease modifying anti-rheumatic drugs (DMARD), with only DMARD, with anti-Janus kinase (JAK) or low dosage of CCS (prednisone <7.5 mg/day or methylprednisolone <6 mg/day). Written, informed consent was required to consecutively enroll patients and controls.

## Study Procedures

Clinical, demographic data and the use of medication were collected at the time of enrollment (T0) and after 2 weeks from the second dose (T1) (**Table 1**). RA disease activity was evaluated by clinical examination at T0 and T1 through the DAS28crp. At T1, blood samples were collected and local and/or systemic clinical adverse events were registered.

RA patients were stratified according to drug treatments in four groups: TNF-α-inhibitors with or without DMARD, IL-6-inhibitors with or without DMARD/CCS, CTLA-4-inhibitors with or without DMARD/CCS, and DMARD with or without CCS. The lymphocyte count of the RA patients was performed within one week from the samples' collection taken for the immune-based assays.

A convenient sample of 167 individuals was included as healthy controls from the cohort of vaccinated HCWs at INMI L. Spallanzani (4, 16).

## IFN-γ Whole-Blood Assay

Whole-blood (600 μL) was stimulated with a pool of peptides covering the sequence of SARS−CoV−2 spike protein (SARS−CoV−2 PepTivator® Prot_S1, Prot_S, and Prot_S+, Miltenyi Biotec, Germany) in a 48-well flat-bottom plate (17). The PepTivator® Peptide Pools used were constituted by peptides of 15 amino acid length with an 11 amino acid overlap. After 20-24 hours of incubation at 37°C (5% $CO_2$), plasma was harvested and stored at -80°C until use. IFN-γ levels were quantified in the plasma samples using an automatic ELISA (ELLA, protein simple). IFN-γ values of the stimulated samples were subtracted from the unstimulated-control value. The detection limit of this assay was 0.17 pg/mL.

## Peripheral Blood Mononuclear Cells (PBMCs) and *In Vitro* Stimulation

PBMCs, isolated from HCWs (n=7) and RA patients (n=15), were thawed, counted, assessed for viability, and rested for 2-4 hours at 37°C in RPMI supplemented with 1% L-glutamine, 1% penicillin/streptomycin (Euroclone S.p.A, Italy), and 10% heat-inactivated FBS. For antigen-specific T-cell stimulation, PBMCs were seeded at a concentration of $2.5 \times 10^6$ cells/mL in a final volume of 200 μL in a 96-multiwell flat-bottom plate (COSTAR, Sigma Aldrich), and stimulated with spike peptide pool at 1 μg/mL or Staphylococcal Enterotoxin B (SEB) at 200 ng/mL, used as a positive control. We added anti-CD28 and anti-CD49d monoclonal antibodies (BD Biosciences San Jose, USA) to

**TABLE 1** | Demographical and clinical characteristics of the 202 enrolled subjects.

| Characteristics | | RA patients | HCWs | P value |
|---|---|---|---|---|
| **N (%)** | | 35 (17.3) | 167 (82.7) | |
| **Age median (IQR)** | | 59 (55–65) | 42 (32–53) | <0.0001* |
| **Male N (%)** | | 8 (22.9) | 48 (28.7) | 0.298§ |
| **Origin N (%)** | **West Europe** | 31 (88.6) | 165 (98.8) | 0.0050§ |
| | **East Europe** | 2 (5.8) | 2 (1.2) | |
| | **Africa** | 1 (2.8) | 0 (0) | |
| | **Sud America** | 1 (2.8) | 0 (0) | |
| **Rheumatologic treatment N (%)** | **TNF-α-inhibitors +/- DMARD** | 7 (20) | – | |
| | **IL-6-inhibitors+/-DMARD/CCS** | 8 (22.9) | – | |
| | **CTLA-4-inhibitors +/-DMARD/CCS** | 13 (37.1) | – | |
| | **DMARD +/- CCS** | 7 (20) | – | |
| **Disease activity** | **DAS28crp T0** | 3.2 (2.5-3.5) | – | 0.732* |
| **median (IQR)** | **DAS28crp T1** | 3.2 (2.0-3.5) | – | |
| **Therapy** | **Years** | 4.9 (1.9-8.0) | – | |
| **Lymphocytes count N (%)** | | 32 (91.4) | 0 (0) | |
| **Lymphocytes count N (%)** | **TNF-α-inhibitors +/- DMARD** | 7 (21.9) | – | 0.067# |
| **Median x10$^3$/μL (IQR)** | | 1.97 (1.07-4.01) | | |
| | **IL-6-inhibitors+/-DMARD/CCS** | 7 (21.9) | – | |
| | | 1.44 (0.74-1.71) | | |
| | **CTLA-4-inhibitors +/-DMARD/CCS** | 11 (34.3) | – | |
| | | 2.07 (1.75-2.84) | | |
| | **DMARD +/- CCS** | 7 (21.9) | – | |
| | | 1.37 (1.26-1.86) | | |

*DMARD, disease modifying antirheumatic drugs; CCS, corticosteroids; RA, rheumatoid arthritis; DAS28, disease activity score 28; N, number; IQR, interquartile range; *Mann-Whitney U-statistic test; §Chi-square test; #Kruskal-Wallis test.*

co-stimulate cells at a final concentration of 1 μg/mL each. After 1h of incubation at 37°C (5% $CO_2$), a Golgi plug (BD Biosciences) at 1 μL/mL was added to cell cultures to inhibit cytokine secretion and to allow intracellular molecule detection by flow cytometry. After 16-24 h, cells were stained as described in the following.

## T-Cell Subpopulations and Intracellular IFN-γ Detection

Stimulated PBMCs were stained with fluorochrome-conjugated antibodies prepared in Brilliant Stain Buffer (BD Biosciences) (see **Supplementary Figure S1** for gating strategy). The Cytofix/Cytoperm solution kit (BD Biosciences) was used for the intracellular IFN-γ staining, according to the manufacturer's instructions (see **Supplementary Table S1** for the list of antibodies and reagents used). Dead cells were excluded from the analysis by side/forward scatter gating and then by Fixable Viability stain 700 (BD Biosciences). At least 100,000 lymphocytes from each sample were gated (except for three samples, two from the unstimulated conditions and one from the SEB condition which were gated with 80,000 events). Samples were acquired on a BD Lyric (BD Biosciences) cytometer and data were analyzed by the FlowJo software (version 10, Tree Star). IFN-γ-mediated T-cell response was considered positive when: i) the frequency of the SARS-COV-2 peptide-stimulated PBMCs was at least twofold higher compared to the unstimulated control; and ii) at least 10 events were present within the IFN-γ gate (18).

## Anti-SARS-CoV-2 Specific IgG Evaluation

The humoral response to vaccination was assessed by quantifying the anti-Nucleoprotein-IgG and the anti-RBD-IgG (Architect® i2000sr Abbott Diagnostics, Chicago, IL). Anti-N-IgG were expressed as arbitrary units (AU)/mL and values were considered positive when ≥ 1.4. Anti-RBD-IgG were expressed as binding arbitrary units (BAU)/mL and values were considered positive when ≥ 7.1.

## Statistical Analysis

Data were analyzed using GraphPad (GraphPad Prism 8 XML ProjecT). Categorical variables were reported as count and proportion, whereas continuous variables, including IFN-γ levels and anti-RBD titers, were reported as median and interquartile range (IQR). Results were evaluated by non-parametric statistical inference tests. The comparisons among groups were evaluated using the Kruskal-Wallis test, whereas the Mann-Whitney U-test with Bonferroni correction was used for pairwise comparisons. The Chi-squared test was used for categorical variables. Correlations of demographic, clinical, and laboratory variables with antibody and S-specific T-cell response, as well as between-assay correlations, were assessed by non-parametric Spearman's Rank test. Spearman's $r_{ho}$>0.7 was considered high correlation, 0.7 <$r_{ho}$>0.5 moderate correlation, and $r_{ho}$<0.5 low correlation. Two-tailed p-values were considered significant if <0.05, except for subgroup analyses by type of rheumatologic-specific treatment, where a correction for multiplicity was applied according to the Bonferroni method, yielding to a significant two-tailed p-value threshold of 0.0125 (α/4).

# RESULTS

## Demographic and Clinical Characteristics of the Enrolled Subjects

We prospectively enrolled 202 vaccinated subjects from whom 35 were RA patients and 167 were HCWs. Significant differences were found with respect to age (p<0.0001) and origin (p=0.005), but not for sex between the two groups (**Table 1**).

The RA cohort consisted of 7 patients under treatment with TNF-α-inhibitors with or without DMARD, 8 treated with IL-6-inhibitors with or without DMARD/CCS, 13 under CTLA-4-inhibitors with or without DMARD/CCS, and 7 under only DMARD (5 patients were receiving MTX, 1 salazopyrin, and 1 hydroxychloroquine) with or without CCS. At vaccination, the median treatment duration for TNF-α-inhibitors with or without DMARD was 2.9 years (IQR: 1.3-11), for IL-6-inhibitors with or without DMARD/CCS 6.1 years (IQR: 4.9-7.6), for CTLA-4-inhibitors with or without DMARD/CCS 6 years (IQR: 1.9-10), and for DMARD with or without CCS 2.2 years (IQR: 1.9-4.9).

## Monitoring of Disease Activity at Baseline and After the Second Dose of Vaccination in RA Patients

In RA patients, the ongoing therapeutic regimen was modified during the vaccination period according to the ACR indications (19). In particular, MTX and JAK-inhibitors were stopped for one week after the first and second dose, whereas abatacept, the CTLA-4 inhibitor, was stopped one week before and after the first dose only.

No significant increase of disease activity was found at T1 compared with baseline values [T0: median 2.9, IQR (2.4-3.5) *vs* T1: median 3.1, IQR (2.0-3.5), p=0.759]. No severe adverse reactions were observed in vaccinated patients. Mild, transient, systemic, and local side effects, mainly pain at the injection location, mild fever, arthromyalgia, and fatigue, were reported by 18 patients (46%).

## Antibody-Specific Response in Vaccinated Individuals

Humoral response was evaluated by measuring the anti-RBD antibodies, while the natural infection was excluded by the detection of anti-N-antibodies. Both HCWs and RA patients were naïve for SARS-CoV-2 infection, as confirmed by the undetectable levels of anti-N antibodies (data not shown). A detectable anti-RBD antibody response was observed in all HCWs (100%) and in all RA patients, except for one individual (97.1%). However, the magnitude of the HCWs response was significantly higher than that of RA patients under CTLA-4 and IL-6 inhibitors with or without DMARD/CCS (p<0.0001 in both groups). Differently, no significant differences were found for the anti-RBD antibody response of patients under TNF-α-inhibitors with or without DMARD (p=0.273) and DMARD with or without CCS (p=0.421) (**Figure 1A**). The response to vaccination can naturally wane with age. Older age may have an impact on the magnitude of the

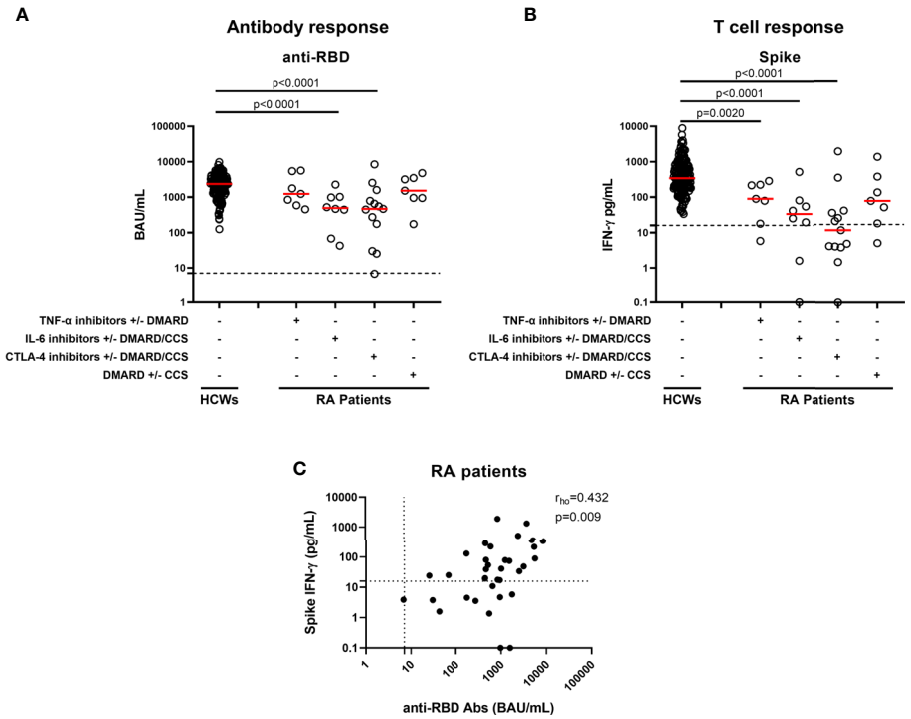


**FIGURE 1** | Antibody and T-cell responses elicited by SARS-CoV-2 vaccination in RA patients. Evaluation of antibody response **(A)** and IFN-γ response to spike antigen **(B)** in 167 HCWs and 35 RA patients stratified according to drug treatment in four groups: TNF-α inhibitors with or without DMARD (n=7), IL-6 inhibitors with or without DMARD/CCS (n=8), CTLA-4 inhibitors with or without DMARD/CCS (n=13), and DMARD with or without CCS (n=7). Correlation across humoral and cell-mediated immunity in RA patients **(C)** is shown. SARS-CoV-2 specific anti-RBD Abs were quantified in plasma or sera samples. Anti-RBD-IgG were expressed as binding arbitrary units (BAU)/mL and values ≥ 7.1 were considered positive. IFN-γ levels were shown as median after subtracting the background. Dashed lines identify the cut-off of each test (spike 16 pg/mL, anti-RBD 7.1 BAU/mL). Each black dot represents one sample, and the red horizontal lines represent the median. Statistical analysis was performed using the Mann-Whitney U-test with Bonferroni correction, and $p \leq 0.0125$ was considered significant. Correlations between assays were assessed by non-parametric Spearman's rank tests. A two-sided p-value <0.05 was considered statistically significant. CCS, corticosteroids; DMARD, disease modifying anti-rheumatic drugs; RA, rheumatoid arthritis; Abs, antibodies; RBD, receptor-binding-domain; HCWs, health care workers.

humoral response (19). Therefore, among the HCWs we selected a group (n=50) who were age-matched [age median: 56, IQR (53–61)] with the RA cohort. We confirmed the results described in **Figure 1A** and we suggest that, more than the age, the RA related-therapies are likely responsible for the reduced specific-antibody response (**Supplementary Figure S2A**).

## SARS-CoV-2-S-Specific T-Cell Response in Vaccinated Individuals

All HCWs showed an IFN-γ-S-specific T-cell response, evaluated by the whole blood platform (3, 10). Contrarily, significant different proportions of responders were found in RA patients under both CTLA-4 and IL-6-inhibitors with or without DMARD/CCS therapy, compared to HCWs (p=0.0018 and p<0.0001, respectively) (**Table 2**). Moreover, the quantitative responses were significantly different among groups (p<0.0001) (**Figure 1B**). In particular, the IFN-γ-S-specific levels were significantly lower in RA patients under TNF-α-inhibitors with or without DMARD, IL-6-inhibitors with or without DMARD/CCS, and CTLA-4-inhibitors with or without DMARD/CCS therapy than those in HCWs (p=0.0020, p<0.0001, p<0.0001, respectively). In contrast, no significant difference was found between the IFN-γ-S-specific response of patients treated with DMARD with or without CCS compared to that of HCWs (p=0.016), albeit the IFN-γ levels were lower than those of HCWs. These data were confirmed comparing the S-specific T-cell response of RA patients with that of age-matched HCWs (n=50) [age median: 56, IQR (53–61)] (**Supplementary Figure S2B**).

## Correlation Between Anti-RBD Antibody Titer, S-Specific T-Cell Response, and Lymphocyte Number

We then focused on the correlation between the two arms of the immune response. A significant correlation between anti-RBD-antibody titer and SARS-CoV-2-S-specific IFN-γ T-cell response was found in HCWs (3). Similarly, a significant moderate correlation was observed in RA patients (rho=0.432, p=0.009)

**TABLE 2** | Serological and T-cell specific response.

| Characteristics | | | | RA patients | HCWs | P value | |
|---|---|---|---|---|---|---|---|
| | | | N (%) | 35 (17.3) | 167 (82.7) | | |
| **Antibody response** | **Qualitative response** | **Anti-RBD abs responders N (%)** | | 34 (97) | 167 (100) | | 0.028§ |
| | | **Anti-RBD abs responders within the subgroups N (%)** | **TNF-α-inhibitors +/- DMARD** | 7/7 (100) | – | 0.627§ | >0.999§ |
| | | | **IL-6- inhibitors +/-DMARD/CCS** | 8/8 (100) | – | | >0.999§ |
| | | | **CTLA-4- inhibitors +/-DMARD/CCS** | 12/13 (92.3) | – | | 0.072§ |
| | | | **DMARD +/- CCS** | 7/7 (100) | – | | >0.999§ |
| | **Quantitative response** | **Anti-RBD abs BAU/mL Median (IQR)** | | 784.7 (441–1763) | 2351 (1389–3748) | | <0.0001* |
| | | | **TNF-α-inhibitors +/- DMARD** | 1239 (589–5426) | – | <0.0001# | 0.273* |
| | | | **IL-6-inhibitors +/-DMARD/CCS** | 492 (161–1007) | – | | <0.0001* |
| | | | **CTLA-4-inhibitors +/-DMARD/CCS** | 465 (103–1189) | – | | <0.0001* |
| | | | **DMARD +/- CCS** | 1526 (943–3471) | – | | 0.421* |
| **Spike specific IFN-γ T cell response** | **Qualitative response** | **Anti-S responders N (%)** | | 24 (69) | 167 (100) | | <0.0001§ |
| | | **Anti-S responders within the subgroups N (%)** | **TNF-α-inhibitors +/- DMARD** | 6/7 (86) | – | 0.165§ | 0.040§ |
| | | | **IL-6-inhibitors +/-DMARD/CCS** | 6/8 (75) | – | | 0.0018§ |
| | | | **CTLA-4-inh-bitors +/-DMARD/CCS** | 6/13 (46) | – | | <0.0001§ |
| | | | **DMARD +/- CCS** | 6/7 (86) | – | | 0.040§ |
| | **Quantitative response** | **Anti-S IFN-γ pg/mL Median (IQR)** | | 34 (4.7-130) | 343 (188–756) | | <0.0001* |
| | | | **TNF-α-inhibitors +/- DMARD** | 89.6 (17.8-224) | – | <0.0001# | 0.0020* |
| | | | **IL-6-inhibitors +/-DMARD/CCS** | 33.2 (6.1-73.9) | – | | <0.0001* |
| | | | **CTLA-4-inhibitors +/-DMARD/CCS** | 10.9 (3.7-36.7) | – | | <0.0001* |
| | | | **DMARD +/-CCS** | 74.6 (17.2-364) | – | | 0.016* |

*DMARD, disease modifying antirheumatic drugs; CCS, corticosteroids; RA, rheumatoid arthritis; N, number; IQR, interquartile range; abs, antibodies; RBD, receptor-binding-domain; S, spike; §Chi-square test; *Mann-Whitney U-statistic test; #Kruskal-Wallis test.*

(**Figure 1C**). Differently, there was no correlation between the lymphocyte number and anti-RBD antibody titer (rho=0.325, p=0.069) or T-specific response (rho=0.151, p=0.409) (data not shown). Further, we analyzed in the RA cohort the impact of age, gender, or years of therapy to identify potential factors affecting the qualitative and quantitative immune responses. None of these variables showed a significant impact on the humoral- or T-cell-specific responses (**Supplementary Tables S2, S3**).

## IFN-γ Response to Spike Is Mainly Mediated by $CD4^+$ T Cells

To assess which T-cell subset among $CD4^+$ or $CD8^+$ T cells was responsible for the SARS-CoV-2-S-specific response, the IFN-γ-S-specific T-cell frequency was analyzed by flow cytometry after stimulation of the PBMCs with the spike peptide pool. To this aim, we used PBMCs isolated from 7 HCWs and 15 RA patients. Among the RA subjects, we selected those characterized by good specific antibody and T-cell responses (**Figure 2**). In particular, we selected 5 RA patients under TNF-α-inhibitors with or without DMARD, 3 patients under CTLA-4-inhibitors with or without DMARD/CCS, 3 patients under IL-6-inhibitors with or without DMARD/CCS, and 4 DMARD with or without CCS-treated patients. We show that the IFN-γ response is mediated by $CD4^+$ T cells following *in vitro* stimulation with spike peptide pool compared to the unstimulated control, both in HCWs and in RA patients (**Figures 2A, B**). The $CD4^+$ T-cell response was scored positive in all HCWs (7/7, 100%) (**Figure 2E**) and in most RA patients (12/15, 80%) (**Figure 2E**). The IFN-γ response was mediated also by $CD8^+$ T cells in most HCWs (5/7, 71%) and in a portion of the RA patients tested (3/15, 20%) (**Figures 2C, D**). In the HCWs, the magnitude of the specific response was higher in $CD4^+$ T cells (median: 0.28%, IQR: 0.19-0.42) (**Figure 2E**) compared to $CD8^+$ T cells (median: 0.058%, IQR: 0.00-0.14) (**Figure 2E**). Similarly, in RA patients the $CD4^+$ T cells showed a higher specific response (median: 0.17%, IQR: 0.05-0.24) (**Figure 2E**) compared to the $CD8^+$ T cells (median: 0.00%, IQR:

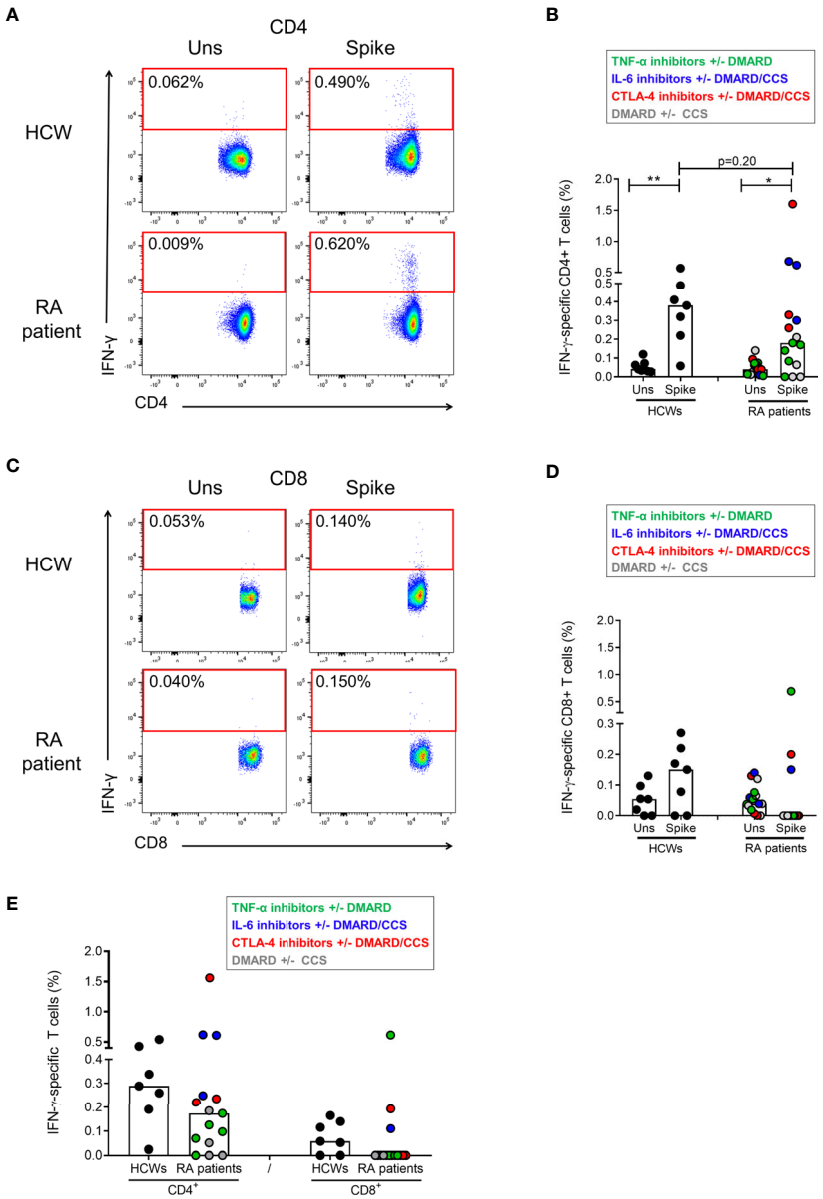


**FIGURE 2** | Characterization of the IFN-γ-S-specific T-cell response by flow cytometry. PBMCs from HCWs (n=7) and RA patients (n=15) were *in vitro* stimulated for 24 h with spike peptide pool and the frequency of IFN-γ-specific T cells was evaluated by flow cytometry. **(A)** Plots show the frequency of IFN-γ in a representative HCW subject and RA patient within the $CD4^+$ T-cell subpopulations. **(B)** $CD4^+$ T-cell-specific response compared to the unstimulated condition is shown in HCWs and RA patients. **(C)** Plots show the frequency of IFN-γ in a representative HCW subject and RA patient within the $CD8^+$ T-cell subpopulations. **(D)** $CD8^+$ T-cell-specific response compared to the unstimulated condition is shown in HCWs and RA patients. **(E)** Frequency of the $CD4^+$ and $CD8^+$ T-cell response (after subtraction of the unstimulated-condition value) is shown in HCWs and RA patients. Each dot represents a different HCW or RA individual and black lines represent the median. RA patients were color-coded, and each color corresponds to a different administered treatment, as shown in the figure legend. Statistical analysis was performed using the Mann-Whitney test and p-value was considered significant if ≤0.05. *p = 0.012 and **p = 0.004. IFN, interferon; RA, rheumatoid arthritis; HCWs, health care workers.

0.00-0.00) (**Figure 2E**). All HCWs and RA patients responded to SEB, used as a positive control, confirming the absence of an impairment of the cytokine production (**Supplementary Figures S3A, B**). Interestingly, in the RA cohort, the $CD8^+$ T-cell mediated response to SEB was significantly higher compared to that observed in HCWs (p=0.001) (**Supplementary Figure S3B**).

## DISCUSSION

Mass vaccination is a crucial public health measure for limiting COVID-19 pandemic especially in fragile populations such as RA patients. Here, we show the results of the immune response to BNT162b2 vaccine in RA patients that were vaccinated based on

the ACR indications (20) with an interruption of MTX and JAK-inhibitors 1 week after the first and second vaccine dose, or with an interruption of abatacept for 1 week before and after the first dose.

In RA patients, BNT162b2 vaccine showed a good safety profile and disease activity remained stable with no patient experiencing a disease relapse. The vaccine induced an antibody-specific response in almost all patients (97%), although the titer was significantly reduced in those under CTLA-4-inhibitors (abatacept) or IL-6-inhibitors compared to HCWs. Concomitantly, spike-specific T-cell response was evaluated and scored positive in 69% of RA patients vs. 100% of HCWs with significantly lower levels in those under a biological therapy compared to HCWs, particularly in patients under CTLA-4-inhibitors or IL-6-inhibitors. The response to vaccination may decrease with age. However, the present finding was confirmed when comparing the results of a group of age-matched HCWs with the RA cohort suggesting that the lower magnitude of both RBD-antibody and T-cell responses was not due to the older age of patients but likely to the RA-treatment. Based on these results, we confirm that COVID-19 vaccination is immunogenic and safe, also in patients under immunosuppressive therapies, although the specific immune responses were present at a lower magnitude compared to the healthy population.

Recently, it has been shown that individuals with immune-mediated inflammatory diseases treated with MTX have up to a 62% rate of specific-immune response to BNT162b2 mRNA vaccine (14), whereas those under cytokine-inhibitors have levels similar to those of healthy controls (greater than 90%) (11, 13, 14). The different results reported here on the impact of immunosuppressant drugs on the humoral response may be associated with the therapeutic strategy adopted to optimize vaccine immunogenicity. Indeed, as already seen for seasonal influenza vaccination (21), the 1-week interruption of MTX after the first and second vaccine dose may have reduced the negative impact on antibody production previously shown (11, 14). This strategy was useful also for those under CTLA-4-inhibitors, as demonstrated by the reassuring proportion of patients mounting an anti-RBD-specific response rate, 92% here *vs*. 62% of previous reports (11, 14), although with a significant decreased antibody titer compared to that observed in the HCWs. Furthermore, notably, this brief "window of therapy interruption" did not affect the RA disease activity, as shown by the DAS28crp that remained stable throughout the vaccination period.

On the other hand, the strategy of interrupting abatacept administration limited to the first dose was not satisfactory to provide the induction of the T-cell-specific response, which is known to be impaired by the drug itself (22), as shown here by the 46% positive responder rate. Based on these results and considering that abatacept blocks the T-cell activation by binding with high-affinity CD80/CD86 molecules thus interfering with the co-stimulation signals delivered through the antigen presenting cells (23), it may be reasonable to extend its interruption also at the second vaccine dose. This approach may improve the induction of a specific immune response especially at the T-cell level.

Interestingly, IL-6-inhibitors had a higher negative impact on the magnitude of antibody- and T-cell-specific Responses compared to TNF-α-inhibitors. This is likely due to the important effect of IL-6 in controlling the survival, population expansion, and maturation of B cells and plasmablasts acting on the follicular helper T cells, a specialized subset of $CD4^+$ T cells that localize to B cell follicles, where they promote B cell proliferation and immunoglobulin class switching (24, 25). IL-6 is also important for the T-cell memory response (24, 26).

Flow cytometry analysis showed that the *in vitro* T-cell response to SARS-CoV-2 spike peptides is mediated by $CD4^+$ and $CD8^+$ T cells in both HCWs and RA patients. Notably, $CD4^+$ T-cell frequency was higher compared to that observed for the $CD8^+$ T cells. These data agree with the results from the HCWs cohorts (5, 27–31) and the COVID-19 convalescent subjects (17, 32). Remarkably, $CD8^+$ T-cell response was lower in the RA cohort compared to that observed in the HCWs. Due to the small sample size of samples included in the flow cytometry analysis, we cannot associate the low frequency of the $CD8^+$ T cell response observed in RA patients to the different therapeutic regimens.

Some limitations of this study need to be considered. First, it was a single center study with a low number of recruited patients that may limit the impact of the study, especially for the comparison of the effects of vaccination among different immunosuppressive therapies. However, the enrolled patients are representative of RA patients under different therapies, and they were well characterized, both clinically and immunologically. Second, the evaluation of the immune responses was performed at a single time point post-vaccination, and the assay used to detect the T-cell response was based on the measurement of a single cytokine (IFN-γ) differently from published studies assessing additional T-helper 1 cytokines (4). However, it was shown that the IFN-γ-specific T-cell response correlates with RBD-antibody titers (4); therefore this cytokine may be considered as a robust parameter to detect T-cell-specific response induced after vaccination.

Importantly, one of the strengths of this study is the more accurate assessment of the humoral immune response using specific anti-RBD-IgG against the total spike protein compared to the previous published work, where IgG antibody titers against only S1 were evaluated (14). In addition, we characterized the T-cell response in terms of $CD4^+$ or $CD8^+$ T-cell involvement. The assays used in the present study to detect SARS-CoV-2 specific response are easy and highly reproducible (17, 33–38), and therefore are compatible with the routine monitoring of vaccinated individuals (4, 5, 16). Indeed, the T-cell response was detected by a whole blood assay, whose platform is similar to current tests measuring T-cell-specific responses against *M. tuberculosis* in both immune-competent and immune-suppressed subjects (39–42).

To the best of our knowledge, this is the first study evaluating both humoral and cellular immune responses to BNT162b2 vaccine in RA patients, who underwent a temporary suspension of immunosuppressive treatment during vaccine administration. For the optimal management of RA patients, clinicians need to consider both the risk of disease relapse and

that of a decreased vaccine immunogenicity. These findings suggest that holding treatment with MTX and abatacept at the first and second vaccine dose can be considered a useful practice in clinically stable patients. To draw definite conclusions, these results need to be confirmed in a larger population adopting a similar therapeutic strategy suspension, and future studies are needed to further evaluate the longevity of humoral and T-cell responses following vaccination.

## DATA AVAILABILITY STATEMENT

The raw data generated and/or analyzed within the present study are available in our institutional repository (rawdata.inmi.it), subject to registration. The data can be found by selecting the article of interest from a list of articles ordered by year of publication. No charge for granting access to data is required. In the event of a malfunction of the application, the request can be sent directly by e-mail to the Library (biblioteca@inmi.it).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the National Institute of Infectious Diseases "Lazzaro Spallanzani" IRCCS (approval numbers 297/2021 and 318/2021). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

DG and EN wrote the project to be submitted to the Ethical Committtee. DG, EN, AP, BL, CA, and CC conceived and designed the study. Experiments were performed by AA, SM, DL, EC, GG, VV, AS, FR, AMGA, SNF, and CF performed the flow cytometry analysis. AA and SL performed the statistical analysis. AP, BL, GC, RR, SS, GN, GM, CP, and SV enrolled patients and collected clinical data. AP, AA, DG, BL, VP, MC, GI, and EN drafted the article or revised it critically. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.740249/full#supplementary-material

## REFERENCES

1. Cantini F, Goletti D, Petrone L, Najafi Fard S, Niccoli L, Foti R. Immune Therapy, or Antiviral Therapy, or Both for COVID-19: A Systematic Review. *Drugs* (2020) 80:1929–46. doi: 10.1007/s40265-020-01421-w
2. Goletti D, Cantini F. Baricitinib Therapy in Covid-19 Pneumonia - An Unmet Need Fulfilled. *N Engl J Med* (2021) 384:867–9. doi: 10.1056/NEJMe2034982
3. Picchianti Diamanti A, Rosado MM, Pioli C, Sesti G, Laganà B. Cytokine Release Syndrome in COVID-19 Patients, A New Scenario for an Old Concern: The Fragile Balance Between Infections and Autoimmunity. *Int J Mol Sci* (2020) 21:3330. doi: 10.3390/ijms21093330
4. Agrati C, Castilletti C, Goletti D, Meschi S, Sacchi A, Matusali G, et al. Coordinate Induction of Humoral and Spike Specific T-Cell Response in a Cohort of Italian Health Care Workers Receiving BNT162b2 mRNA Vaccine. *Microorganisms* (2021) 9:1315. doi: 10.3390/microorganisms9061315
5. Angyal A, Longet S, Moore S, Payne RP, Harding A, Tipton T, et al. T-Cell and Antibody Responses to First BNT162b2 Vaccine Dose in Previously SARS-CoV-2-Infected and Infection-Naive UK Healthcare Workers: A Multicentre, Prospective, Observational Cohort Study. *SSRN* (2021). doi: 10.2139/ssrn.3812375
6. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med* (2021) 384:403–16. doi: 10.1056/NEJMoa2035389
7. Rydyznski Moderbacher C, Ramirez SI, Dan JM, Grifoni A, Hastie KM, Weiskopf D, et al. Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and Associations With Age and Disease Severity. *Cell* (2020) 183:996–1012.e19. doi: 10.1016/j.cell.2020.09.038.
8. Dan JM, Mateus J, Kato Y, Hastie KM, Yu ED, Faliti CE, et al. Immunological Memory to SARS-CoV-2 Assessed for Up to 8 Months After Infection. *Science* (2021) 371:eabf4063. doi: 10.1126/science.abf4063
9. Landewé RB, Machado PM, Kroon F, Bijlsma HW, Burmester GR, Carmona L, et al. EULAR Provisional Recommendations for the Management of Rheumatic and Musculoskeletal Diseases in the Context of SARS-CoV-2. *Ann Rheum Dis* (2020) 79:851–8. doi: 10.1136/annrheumdis-2020-217877
10. Belleudi V, Rosa AC, Poggi FR, Armuzzi A, Nicastri E, Goletti D, et al. Direct and Indirect Impact of COVID-19 for Patients With Immune-Mediated

Inflammatory Diseases: A Retrospective Cohort Study. *J Clin Med* (2021) 10:2388. doi: 10.3390/jcm10112388
11. Furer V, Eviatar T, Zisman D, Peleg H, Paran D, Levartovsky D, et al. Immunogenicity and Safety of the BNT162b2 mRNA COVID-19 Vaccine in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases and in the General Population: A Multicentre Study. *Ann Rheum Dis* (2021). doi: 10.1136/annrheumdis-2021-220647
12. Geisen UM, Berner DK, Tran F, Sümbül M, Vullriede L, Ciripoi M, et al. Immunogenicity and Safety of Anti-SARS-CoV-2 mRNA Vaccines in Patients With Chronic Inflammatory Conditions and Immunosuppressive Therapy in a Monocentric Cohort. *Ann Rheum Dis* (2021). doi: 10.1136/annrheumdis-2021-220496.
13. Simon D, Tascilar K, Fagni F, Krönke G, Kleyer A, Meder C, et al. SARS-CoV-2 Vaccination Responses in Untreated, Conventionally Treated and Anticytokine-Treated Patients With Immune-Mediated Inflammatory Diseases. *Ann Rheum Dis* (2021). doi: 10.1136/annrheumdis-2021-220461.
14. Haberman RH, Herati RS, Simon D, Samanovic M, Blank RB, Tuen M, et al. Methotrexate Hampers Immunogenicity to BNT162b2 mRNA COVID-19 Vaccine in Immune-Mediated Inflammatory Disease. *medRxiv* (2021). doi: 10.1101/2021.05.11.21256917.
15. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham C, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62:2569–81. doi: 10.1002/art.27584
16. Puro V, Castilletti C, Agrati C, Goletti D, Leone S, Agresta A, et al. Impact of Prior Influenza and Pneumoccocal Vaccines on Humoral and Cellular Response to SARS-CoV-2 BNT162b2 Vaccination. *Vaccines (Basel)* (2021) 9:615. doi: 10.3390/vaccines9060615
17. Aiello A, Najafi Fard S, Petruccioli E, Petrone L, Vanini V, Farroni C, et al. Spike is the Most Recognized Antigen in the Whole-Blood Platform in Both Acute and Convalescent COVID-19 Patients. *Int J Infect Dis* (2021) 106:338–47. doi: 10.1016/j.ijid.2021.04.034.
18. Roederer M. How Many Events is Enough? Are You Positive? *Cytometry A* (2008) 73:384–5. doi: 10.1002/cyto.a.20549
19. Schwarz T, Tober-Lau P, Hillus D, Helbig ET, Lippert LJ, Thibeault C, et al. Delayed Antibody and T-Cell Response to BNT162b2 Vaccination in the Elderly, Germany. *Emerg Infect Dis* (2021) 27:2174–8. doi: 10.3201/eid2708.211145
20. Curtis JR, Johnson SR, Anthony DD, Arasaratnam RJ, Baden LR, Bass AR, et al. American College of Rheumatology Guidance for COVID-19 Vaccination in Patients With Rheumatic and Musculoskeletal Diseases: Version 2. *Arthritis Rheumatol* (2021) 73(8):e30–45. doi: 10.1002/art.41877
21. Park JK, Lee YJ, Shin K, Ha YJ, Lee EY, Song YW, et al. Impact of Temporary Methotrexate Discontinuation for 2 Weeks on Immunogenicity of Seasonal Influenza Vaccination in Patients With Rheumatoid Arthritis: A Randomised Clinical Trial. *Ann Rheum Dis* (2018) 77:898–904. doi: 10.1136/annrheumdis-2018-213222
22. Picchianti Diamanti A, Rosado MM, Scarsella M, Germano V, Giorda E, Cascioli S, et al. Abatacept (Cytotoxic T Lymphocyte Antigen 4-Immunoglobulin) Improves B Cell Function and Regulatory T Cell Inhibitory Capacity in Rheumatoid Arthritis Patients non-Responding to Anti-Tumour Necrosis Factor-α Agents. *Clin Exp Immunol* (2014) 177:630–40. doi: 10.1111/cei.12367
23. Moreland L, Bate G, Kirkpatrick P. Abatacept. *Nat Rev Drug Discovery* (2006) 5:185–6. doi: 10.1038/nrd1989
24. Hunter CA, Jones SA. IL-6 as a Keystone Cytokine in Health and Disease. *Nat Immunol* (2015) 16:448–57. doi: 10.1038/ni.3153
25. Ma CS, Deenick EK, Batten M, Tangye SG. The Origins, Function, and Regulation of T Follicular Helper Cells. *J Exp Med* (2012) 209:1241–53. doi: 10.1084/jem.20120994
26. Longhi MP, Wright K, Lauder SN, Nowell MA, Jones GW, Godkin AJ, et al. Interleukin-6 is Crucial for Recall of Influenza-Specific Memory CD4 T Cells. *PloS Pathog* (2008) 4:e1000006. doi: 10.1371/journal.ppat.1000006
27. Neidleman J, Luo X, McGregor M, Xie G, Murray M, Greene WC, et al. mRNA Vaccine-Induced T Cells Respond Identically to SARS-CoV-2 Variants of Concern But Differ in Longevity and Homing Properties Depending on Prior Infection Status. *bioRxiv* (2021) 2021.05.12.443888. doi: 10.1101/2021.05.12.443888
28. Sahin U, Muik A, Vogler I, Derhovanessian E, Kranz LM, Vormehr M, et al. BNT162b2 Vaccine Induces Neutralizing Antibodies and Poly-Specific T Cells in Humans. *Nature* (2021) 595:572–7. doi: 10.1038/s41586-021-03653-6
29. Barros-Martins J, Hammerschmidt SI, Cossmann A, Odak I, Stankov MV, Morillas Ramos G, et al. Immune Responses Against SARS-CoV-2 Variants After Heterologous and Homologous ChAdOx1 Ncov-19/BNT162b2 Vaccination. *Nat Med* (2021). doi: 10.1038/s41591-021-01449-9
30. Taborska P, Lastovicka J, Stakheev D, Strizova Z, Bartunkova J, Smrz D. SARS-CoV-2 Spike Glycoprotein-Reactive T Cells can be Readily Expanded From COVID-19 Vaccinated Donors. *Immun Inflammation Dis* (2021). doi: 10.1002/iid3.496
31. Goletti D, Petrone L, Manissero D, Bertoletti A, Rao S, Ndunda N, et al. The Potential Clinical Utility of Measuring Severe Acute Respiratory Syndrome Coronavirus 2-Specific T-Cell Responses. *Clin Microbiol Infect* (2021) S1198-743X(21)00378-5. doi: 10.1016/j.cmi.2021.07.005.
32. Cassaniti I, Percivalle E, Bergami F, Piralla A, Comolli G, Bruno R, et al. SARS-CoV-2 Specific T-Cell Immunity in COVID-19 Convalescent Patients and Unexposed Controls Measured by *Ex Vivo* ELISpot Assay. *Clin Microbiol Infect* (2021) 27:1029–34. doi: 10.1016/j.cmi.2021.03.010.
33. Murugesan K, Jagannathan P, Pham TD, Pandey S, Bonilla HF, Jacobson K, et al. Interferon-Gamma Release Assay for Accurate Detection of SARS-CoV-2 T Cell Response. *Clin Infect Dis* (2020) ciaa1537. doi: 10.1093/cid/ciaa1537
34. Petrone L, Petruccioli E, Vanini V, Cuzzi G, Najafi Fard S, Alonzi T, et al. A Whole Blood Test to Measure SARS-CoV-2-Specific Response in COVID-19 Patients. *Clin Microbiol Infect* (2021) 27:286.e7–286.e13. doi: 10.1016/j.cmi.2020.09.051.
35. Petrone L, Petruccioli E, Alonzi T, Vanini V, Cuzzi G, Najafi Fard S, et al. *In-Vitro* Evaluation of the Immunomodulatory Effects of Baricitinib: Implication for COVID-19 Therapy. *J Infect* (2021) 82:58–66. doi: 10.1016/j.jinf.2021.02.023
36. Petrone L, Petruccioli E, Vanini V, Cuzzi G, Gualano G, Vittozzi P, et al. Coinfection of Tuberculosis and COVID-19 Limits the Ability to *In Vitro* Respond to SARS-CoV-2. *Int J Infect Dis* (2021) S1201-9712(21)00176-4. doi: 10.1016/j.ijid.2021.02.090.
37. Petruccioli E, Najafi Fard S, Navarra A, Petrone L, Vanini V, Cuzzi G, et al. Exploratory Analysis to Identify the Best Antigen and the Best Immune Biomarkers to Study SARS-CoV-2 Infection. *J Transl Med* (2021) 19(1):272. doi: 10.1186/s12967-021-02938-8
38. Goletti D, Petrone L, Manissero D, Bertoletti A, Rao S, Ndunda N, et al. (2021), S1198–743X(21)00378-5. doi: 10.1016/j.cmi.2021.07.005
39. Nemes E, Rozot V, Geldenhuys H, Bilek N, Mabwe S, Abrahams D, et al. Optimization and Interpretation of Serial QuantiFERON Testing to Measure Acquisition of Mycobacterium Tuberculosis Infection. *Am J Respir Crit Care Med* (2017) 196:638–48. doi: 10.1164/rccm.201704-0817OC
40. Goletti D, Navarra A, Petruccioli E, Cimaglia C, Compagno M, Cuzzi G, et al. Latent Tuberculosis Infection Screening in Persons Newly-Diagnosed With HIV Infection in Italy: A Multicentre Study Promoted by the Italian Society of Infectious and Tropical Diseases. *Int J Infect Dis* (2020) 92:62–8. doi: 10.1016/j.ijid.2019.12.031.
41. Chiacchio T, Petruccioli E, Vanini V, Cuzzi G, Massafra U, Baldi G, et al. Characterization of QuantiFERON-TB-Plus Results in Latent Tuberculosis Infected Patients With or Without Immune-Mediated Inflammatory Diseases. *J Infect* (2019) 79:15–23. doi: 10.1016/j.jinf.2019.04.010.
42. Petruccioli E, Chiacchio T, Navarra A, Vanini V, Cuzzi G, Cimaglia C, et al. Effect of HIV-Infection on QuantiFERON-Plus Accuracy in Patients With Active Tuberculosis and Latent Infection. *J Infect* (2020) 80:536–46. doi: 10.1016/j.jinf.2020.02.009.

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CASE REPORT
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# Case Report: ANCA-Associated Vasculitis Presenting With Rhabdomyolysis and Pauci-Immune Crescentic Glomerulonephritis After Pfizer-BioNTech COVID-19 mRNA Vaccination

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As the coronavirus disease 2019 (COVID-19) pandemic is ongoing and new variants of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) are emerging, there is an urgent need for COVID-19 vaccines to control disease outbreaks by herd immunity. Surveillance of rare safety issues related to these vaccines is progressing, since more granular data emerge with regard to adverse events of COVID-19 vaccines during post-marketing surveillance. Interestingly, four cases of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) presenting with pauci-immune crescentic glomerulonephritis (GN) after COVID-19 mRNA vaccination have already been reported. We here expand our current knowledge of this rare but important association and report a case of AAV presenting with massive rhabdomyolysis and pauci-immune crescentic GN after Pfizer-BioNTech COVID-19 mRNA vaccination. As huge vaccination programs are ongoing worldwide, post-marketing surveillance systems must continue to assess vaccine safety important for the detection of any events associated with COVID-19 vaccination. This is especially relevant in complex diseases where diagnosis is often challenging, as in our patient with AAV presenting with massive rhabdomyolysis and pauci-immune crescentic GN.

**Keywords: coronavirus disease 2019 (COVID-19), vaccination, anti-neutrophil cytoplasmic antibody (ANCA), ANCA-associated vasculitis (AAV), rhabdomyolysis, acute kidney injury (AKI), pauci-immune crescentic glomerulonephritis (GN)**

## INTRODUCTION

As the coronavirus disease 2019 (COVID-19) pandemic is ongoing and new variants of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) are emerging, there is an urgent need for COVID-19 vaccines to control disease outbreaks by herd immunity (1). The use of novel vaccines containing a nucleoside-modified messenger ribonucleic acid (mRNA) or a viral deoxyribonucleic acid (DNA) vector that encodes the viral spike (S) glycoprotein of SARS-CoV-2 has already been approved. Large clinical trials have shown that these COVID-19 vaccines are safe and effective. Common adverse events include mild to moderate reactions at the injection site, fever, fatigue, body aches, and headache (2). Surveillance of rare safety issues related to these vaccines is progressing, since more granular data emerge with regard to adverse events of COVID-19 vaccines during post-marketing surveillance (3). Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis hallmarked by the presence of antibodies against autoantigens in cytoplasmic granules of neutrophils (4). AAV presents as granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (5). Generally, renal manifestations in AAV are estimated at 80% among all cases mainly manifesting as ANCA-associated glomerulonephritis (ANCA GN), and the overall prevalence does not seem to differ substantially between MPO-ANCA and PR3-ANCA AAV (6). Interestingly, five cases of renal AAV presenting with pauci-immune crescentic ANCA GN after COVID-19 mRNA vaccination have already been reported (7–10). We here expand our current knowledge of this rare but important association and report a case of AAV presenting with massive rhabdomyolysis and pauci-immune crescentic GN after Pfizer-BioNTech COVID-19 mRNA vaccination.

## CASE REPORT

A 79-year-old Caucasian female with a past medical history of hypertension, degenerative disc disease, and no documented history of COVID-19 received two doses of Pfizer-BioNTech COVID-19 mRNA vaccination. Two weeks thereafter, the patient presented to our emergency department with weakness and upper thigh pain. Vital parameters were stable, and physical examination was unremarkable. The patient had no allergies and denied illicit drug use. External routine laboratory assessments obtained 1 week prior to admission were normal for serum creatinine of 0.71 mg/dl (reference range: 0.5–0.95), estimated glomerular filtration rate (eGFR) of 84.4 ml/min/1.73 $m^2$, and urinalysis with the absence of hematuria or proteinuria. Repeat reverse transcription polymerase chain reaction (RT-PCR) testing for SARS-CoV-2 RNA from nasopharyngeal swabs was negative. Laboratory assessments at admission showed massive rhabdomyolysis with creatinine kinase (CK) levels of 14,243 U/L (reference range: 29–168), myoglobinemia of >12,000 µg/L (reference range: ≤106, **Figure 1A**), and acute kidney injury (AKI) with serum creatinine levels of 1.38 mg/dl (reference range: 0.7–1.2, **Figure 1B**) and an estimated glomerular filtration rate (eGFR) of 33.5 ml/min/1.73 $m^2$. Urinary analysis revealed leukocyturia, hematuria (no dysmorphic erythrocytes), few renal tubular epithelial cells, and nephrotic range proteinuria of >18,000 mg/g creatinine and albuminuria of <5,000 mg/g creatinine (reference range: <30 mg/g, **Figure 1C**). The patient received intravenous crystalloids with decreasing CK levels and myoglobinemia (**Figure 1A**). However, progressive deterioration of kidney function with worsening of serum creatinine levels up to 6.57 mg/dl (reference range: 0.7–1.2 mg/dl, **Figure 1B**) and an eGFR of <15 ml/min/1.73 $m^2$ occurred. ANCA immunofluorescence (IF) was positive at 1:1,000 (reference range: <1:10) with elevated MPO-ANCA levels >134 IU/ml (reference range: <3.5 IU/ml), while myositis antibodies, complement levels, and other serologic parameters were all tested negative (**Table 1**). Because of leukocytosis, a white blood differential was conducted revealing prominent peripheral blood eosinophilia (**Table 1**).

Based on suspected MPO-positive AAV, the patient received a steroid pulse with intravenous methylprednisolone for 3 days (250 mg per day) and oral prednisone 1 mg/kg daily thereafter (60 mg per day, **Figure 1D**). A kidney biopsy confirmed severe acute tubular injury with pauci-immune crescentic GN and interstital nephritis: cellular crescents in 1/15 (6.7%) glomeruli, global glomerular sclerosis in 2/15 (13.3%), mild (5%) interstitial fibrosis and tubular atrophy (IF/TA), interstitial inflammation (25%) with prominent eosinophilic infiltration, and severe acute tubular injury with myoglobin casts (**Figure 2** and **Table 2**). According to histopathological scoring, focal class ANCA GN and intermediate risk ANCA renal risk score (ARRS) were present (**Table 2**) (11, 12). Based on the diagnosis of AAV presenting with massive rhabdomyolysis and pauci-immune crescentic GN, intravenous cyclophosphamide (CYC) was initiated at 10 mg/kg (per CYCLOPS trial dosing, **Figure 1D**) (13). Thereafter, kidney function normalized without requirement of dialysis and proteinuria decreased to 1,603 mg/g creatinine and albuminuria to 351 mg/g creatinine (reference range: <30 mg/g, **Figures 1B, C**). Repeat serological testing confirmed that ANCA IF turned negative. Thereafter, oral prednisone was tapered down (currently 50 mg per day), and we do not plan to repeat administration of intravenous cyclophosphamide because rhabdomyolysis ceased and kidney function normalized.

## DISCUSSION

To our knowledge, we here present the first case of AAV presenting with massive rhabdomyolysis and pauci-immune crescentic GN after Pfizer-BioNTech COVID-19 mRNA vaccination. With millions of people being vaccinated for COVID-19, rare reports of adverse events are emerging. In this case, the temporal association between Pfizer-BioNTech

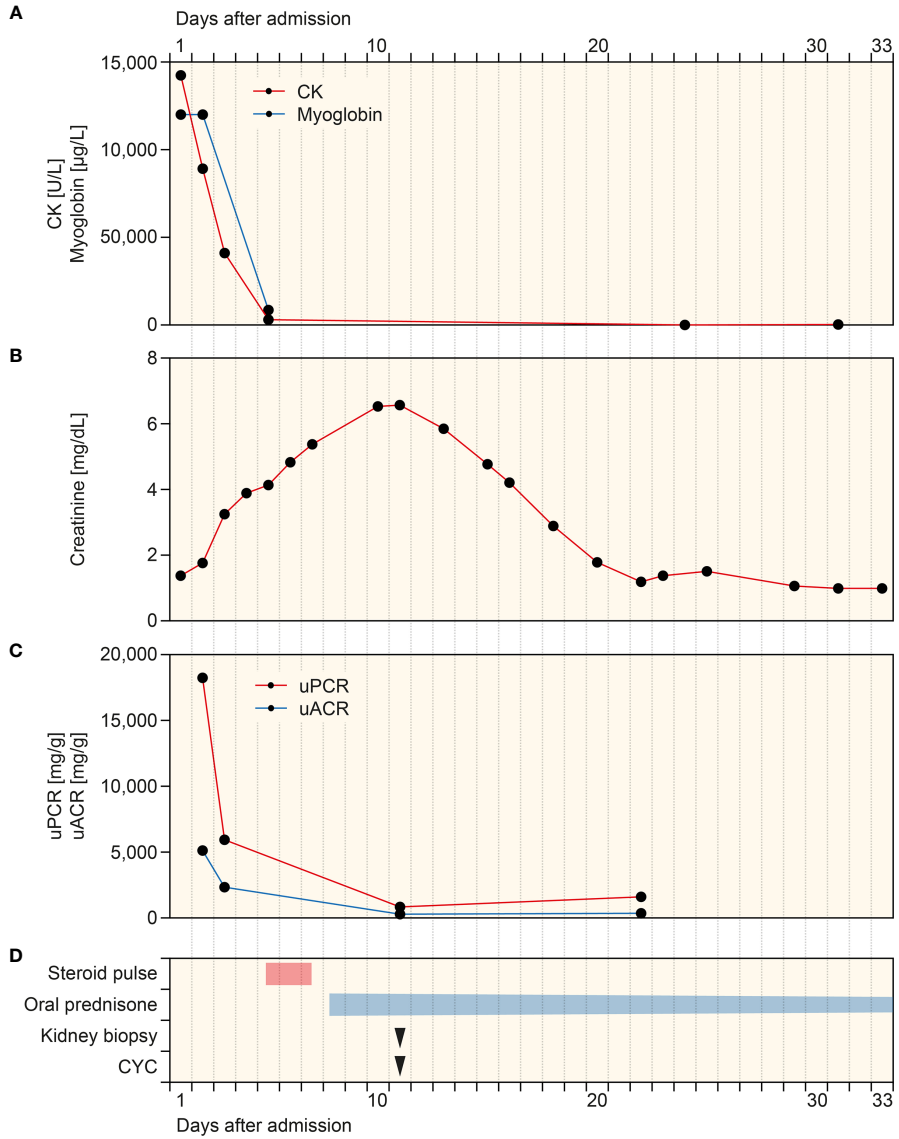


**FIGURE 1** | Timeline of the case after admission. **(A–C)** Time course of CK, myoglobin, plasma creatinine, and levels of uPCR and uACR. **(D)** Time of treatment regimens and kidney biopsy. CK, creatinine kinase; CYC, cyclophosphamide; uACR, urinary albumin-to-creatinine ratio; uPCR, urinary protein-to-creatinine ratio.

**TABLE 1** | Serologic parameters after admission.

| | Value | Reference range |
|---|---|---|
| Serologic parameters | | |
| HIV Ag/Ab—titer | Neg | Neg |
| HBsAg—titer | Neg | Neg |
| Anti-HCV—titer | Neg | Neg |
| Rheumatoid factor—IU/ml | <10 | <15.9 |
| Complement C3c—g/L | 0.97 | 0.82–1.93 |
| Complement C4—g/L | 0.19 | 0.15–0.57 |
| ANCA IF | 1:1,000 | <1:10 |
| PR3-ANCA—IU/ml | <0.2 | <2 |
| MPO-ANCA—IU/ml | >134 | <3.5 |
| ENA screen | <0.1 | <0.7 |
| Anti-DFS70—U/ml | <0.6 | <7 |
| Anti-ds-DNA—IU/ml | 4.4 | <15 |
| Histones—U/ml | 7.7 | <20 |
| ANA IF | 1:320 | <1:100 |
| RO52—blot | Neg | Neg |
| PM-Scl-100—blot | Neg | Neg |
| PM-Scl-75—blot | Neg | Neg |
| Ku—blot | Neg | Neg |
| SRP—blot | Neg | Neg |
| PL-7—blot | Neg | Neg |
| PL-12—blot | Neg | Neg |
| EJ—blot | Neg | Neg |
| OJ—blot | Neg | Neg |
| JO1—blot | Neg | Neg |
| Mi alpha—blot | Neg | Neg |
| Mi-2 beta—blot | Neg | Neg |
| TIF1 gamma—blot | Neg | Neg |
| MDA-5—blot | Neg | Neg |
| NXP2—blot | Neg | Neg |
| SAE1—blot | Neg | Neg |
| White blood differential | | |
| Leukocytes—1,000/µl | 22.9 | 4–11 |
| Lymphocytes—% | 4.7 | 20–45 |
| Monocytes—% | 4.5 | 3–13 |
| Eosinophils—% | 23.3 | ≤8 |
| Basophils—% | 0.2 | ≤2 |
| Neutrophils—% | 67.3 | 40-76 |

*ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibody; ds-DNA, double stranded-DNA; DSF70, dense-fine-speckled 70; ENA, EJ, glycine; ENA, extractable nuclear antigen; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; JO1, histidyl tRNA synthetase; MDA-5, melanoma differentiation-associated protein-5; MPO, myeloperoxidase; Neg, negative; NXP2, nuclear matrix protein 2; OJ, isoleucine; PM-Scl, PL-7, threonine; PL-12, alanine, polymyositis-scleroderma; PR3, proteinase 3; SAE1, small ubiquitin-like modifier activating enzyme; SRP, signal recognition particle; TIF1, transcriptional intermediary factor 1.*

COVID-19 mRNA vaccination and AAV presenting with rhabdomyolysis and pauci-immune crescentic GN suggests a neutrophilic immune response to mRNA as a potential trigger. This patient initially presented with upper thigh pain due to massive rhabdomyolysis after Pfizer-BioNTech COVID-19 mRNA vaccination. Rhabdomyolysis has been described in the context of COVID-19, and a direct viral tropism to myocytes has been suggested (14, 15). However, detection of SARS-CoV-2 infection in skeletal muscle cells has not been established yet (16). Rhabdomyolysis secondary to vaccination has previously been reported, mostly in the context of influenza vaccination (17, 18). In association with COVID-19 mRNA vaccination, the onset of fatigue, myalgias, and arthralgias following mRNA vaccination has been reported in a considerable subset of patients (19). Additionally, there is evidence that COVID-19 mRNA vaccination can directly induce myositis at the injection site, as previously observed in the deltoid muscle (20). In addition to rhabdomyolysis, we observed pauci-immune crescentic GN accompanied by MPO-ANCA autoantibodies after COVID-19 mRNA vaccination. To date, five cases of pauci-immune crescentic ANCA GN after the second dose of COVID-19 mRNA vaccination in all cases have been reported (7–10). In our case, kidney biopsy showed myoglobin casts due to massive rhabdomyolysis and pauci-immune crescentic GN, and it is likely that both contributed to deterioration of kidney function. It has already been reported that the first COVID-19 mRNA vaccination primes the innate immune system to mount a more potent response after the second booster immunization (21). It is possible that the enhanced immune response especially observed after the second dose of COVID-19 mRNA vaccination could be responsible for triggering the observed MPO-ANCA autoantibodies. Causal links between immune system activation by viral infections and AAV have been suggested due to onset of AAV predominantly during the winter (22, 23). Toll-like receptors (TLRs) are expressed on leukocytes and play crucial roles in the recognition of viral antigens, facilitating immune system activation and inflammation. Predominant TLR-2 and TLR-9 activation can stimulate autoimmunity in AAV, previously been described in the context of MPO-ANCA autoantibodies (24). Interestingly, TLR-2 activation in immunodominant cytotoxic T lymphocytes in response to SARS-CoV-2 S glycoprotein (as also produced by COVID-19 vaccines) has already been described (25). With regard to vaccination, there is some discussion about the relationship between vaccination and AAV recurrence in patients with pre-existing autoimmune disease after influenza vaccination as very rare but significant side effects (26). The temporal relationship could be explained theoretically, including molecular mimicry, polyclonal activation, or transient systemic proinflammatory cytokine responses that potentially provoke autoimmune diseases in genetically predisposed individuals (27). Interestingly, increased production of ANCA autoantibodies has already been described in response to viral mRNA-based influenza and rabies vaccines (27). Moreover, AAV and autoimmune reactions have been reported in the context of COVID-19, implicating a direct reaction to viral RNA (28–30). Therefore, the occurrence of AAV in the context of COVID-19 mRNA as compared with non-mRNA vaccines would be of great relevance. Huge vaccination programs are ongoing worldwide, and post-marketing surveillance systems must continue to assess vaccine safety important for the detection of any events associated with COVID-19 vaccination. This is especially relevant in complex diseases where diagnosis is often challenging, as in our patient with AAV presenting with massive rhabdomyolysis and pauci-immune crescentic GN. The limitation of this case report is only a temporal relationship between COVID-19 mRNA vaccination and onset of AAV. However, AAV onset in association with COVID-19

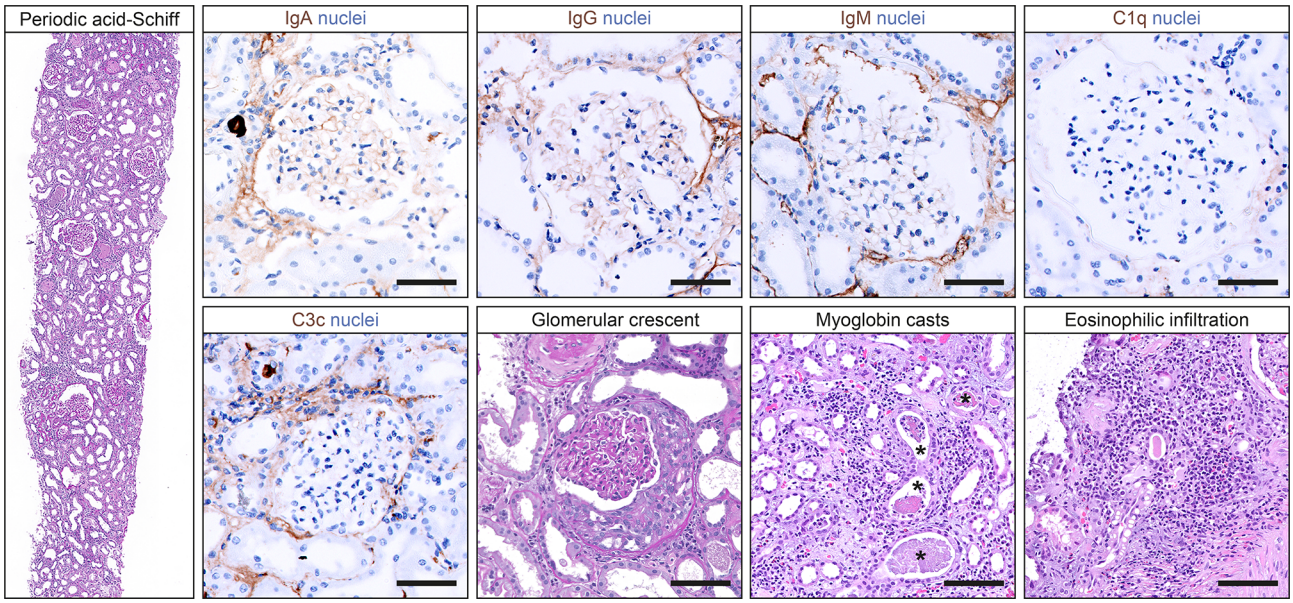


**FIGURE 2** | Histopathological findings in a kidney biopsy confirming pauci-immune crescentic GN. Representative photomicrographs of the kidney biopsy including staining for IgA (scale bar: 50 μm), IgG (scale bar: 50 μm), IgM (scale bar: 50 μm), C1q (scale bar: 50 μm), and C3c (scale bar: 50 μm); periodic acid-Schiff staining showing a glomerulus with crescent formation (scale bar: 50 μm); and hematoxylin/eosin staining with myoglobin casts (asterisks, scale bar: 100 μm) and tubulointerstitial inflammation with prominent eosinophilic infiltration (scale bar: 100 μm). C1q, complement component 1q; C3c, complement factor 3 conversion product; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; GN, glomerulonephritis.

**TABLE 2** | Histopathological findings in the kidney biopsy.

| | Value |
|---|---|
| Lesions | |
| Total glomeruli—no. | 15 |
| Cellular crescents—no. (%) | 1 (6.7) |
| Fibrocellular crescents—no. (%) | 0 (0) |
| Global glomerular sclerosis—no. (%) | 2 (13.3) |
| IF/TA—% | 5 |
| Interstitial inflammation—% | 25 |
| Scoring | |
| Berden class | Focal |
| ARRS | Intermediate risk |

*ARRS, ANCA renal risk score; IF/TA, interstitial fibrosis/tubular atrophy; no., number.*

mRNA vaccination has independently been observed before and requires further investigation with regard to the mechanisms linking autoimmunity to COVID-19 vaccines (7–9). Fortunately, treatment of AAV is possible and caution in such cases is warranted with regard to early testing if clinical symptoms are compatible with AAV in principle.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

Ethical 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.

## AUTHOR CONTRIBUTIONS

BT was directly involved in the treatment of the patient, conceived the case report, collected and analyzed the data, and wrote the manuscript. SH evaluated kidney biopsy findings and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES


1. Garcia-Montero C, Fraile-Martinez O, Bravo C, Torres-Carranza D, Sanchez-Trujillo L, Gómez-Lahoz AM, et al. An Updated Review of SARS-CoV-2 Vaccines and the Importance of Effective Vaccination Programs in Pandemic Times. *Vaccines (Basel)* (2021) 9(5):433. doi: 10.3390/vaccines9050433
2. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med* (2020) 383(27):2603–15. doi: 10.1056/NEJMoa2034577
3. Pollard AJ, Bijker EM. A Guide to Vaccinology: From Basic Principles to New Developments. *Nat Rev Immunol* (2021) 21(2):83–100. doi: 10.1038/s41577-020-00479-7
4. Jennette JC, Nachman PH. ANCA Glomerulonephritis and Vasculitis. *Clin J Am Soc Nephrol* (2017) 12(10):1680–91. doi: 10.2215/CJN.02500317
5. Falk RJ, Jennette JC. Anti-Neutrophil Cytoplasmic Autoantibodies With Specificity for Myeloperoxidase in Patients With Systemic Vasculitis and Idiopathic Necrotizing and Crescentic Glomerulonephritis. *N Engl J Med* (1988) 318(25):1651–7. doi: 10.1056/NEJM198806233182504
6. Franssen C, Gans R, Kallenberg C, Hageluken C, Hoorntje S. Disease Spectrum of Patients With Antineutrophil Cytoplasmic Autoantibodies of Defined Specificity: Distinct Differences Between Patients With Anti-Proteinase 3 and Anti-Myeloperoxidase Autoantibodies. *J Intern Med* (1998) 244(3):209–16. doi: 10.1046/j.1365-2796.1998.00357.x
7. Sekar A, Campbell R, Tabbara J, Rastogi P. ANCA Glomerulonephritis After the Moderna COVID-19 Vaccination. *Kidney Int* (2021) 100(2):473–4. doi: 10.1016/j.kint.2021.05.017
8. Anderegg MA, Liu M, Saganas C, Montani M, Vogt B, Huynh-Do U, et al. De Novo Vasculitis After mRNA-1273 (Moderna) Vaccination. *Kidney Int* (2021) 100(2):474–6. doi: 10.1016/j.kint.2021.05.016
9. Shakoor MT, Birkenbach MP, Lynch M. ANCA-Associated Vasculitis Following Pfizer-BioNTech COVID-19 Vaccine. *Am J Kidney Dis* (2021) 78 (4):611–3. doi: 10.1053/j.ajkd.2021.06.016
10. Dube GK, Benvenuto LJ, Batal I. ANCA-Associated Glomerulonephritis Following the Pfizer-BioNTech COVID-19 Vaccine. *Kidney Int Rep* (2021). doi: 10.1016/j.ekir.2021.08.012
11. Berden AE, Ferrario F, Hagen EC, Jayne DR, Jennette JC, Joh K, et al. Histopathologic Classification of ANCA-Associated Glomerulonephritis. *J Am Soc Nephrol* (2010) 21(10):1628–36. doi: 10.1681/ASN.2010050477
12. Brix SR, Noriega M, Tennstedt P, Vettorazzi E, Busch M, Nitschke M, et al. Development and Validation of a Renal Risk Score in ANCA-Associated Glomerulonephritis. *Kidney Int* (2018) 94(6):1177–88. doi: 10.1016/j.kint.2018.07.020
13. de Groot K, Harper L, Jayne DRW, Suarez LFF, Gregorini G, Gross WL, et al. Pulse Versus Daily Oral Cyclophosphamide for Induction of Remission in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis: A Randomized Trial. *Ann Intern Med* (2009) 150(10):670–80. doi: 10.7326/0003-4819-150-10-200905190-00004
14. Mack M, Nichols L, Guerrero DM. Rhabdomyolysis Secondary to COVID-19 Vaccination. *Cureus* (2021) 13(5):e15004. doi: 10.7759/cureus.15004
15. Haroun MW, Dieiev V, Kang J, Barbi M, Nia SFM, Gabr M, et al. Rhabdomyolysis in COVID-19 Patients: A Retrospective Observational Study. *Cureus* (2021) 13(1):e12552. doi: 10.7759/cureus.12552
16. Ding Y, He L, Zhang Q, Huang Z, Che X, Hou J, et al. Organ Distribution of Severe Acute Respiratory Syndrome (SARS) Associated Coronavirus (SARS-CoV) in SARS Patients: Implications for Pathogenesis and Virus Transmission Pathways. *J Pathol* (2004) 203(2):622–30. doi: 10.1002/path.1560
17. Callado RB, Carneiro TG, Parahyba CC, Lima Nde A, da Silva Junior GB, Daher Ede F. Rhabdomyolysis Secondary to Influenza A H1N1 Vaccine Resulting in Acute Kidney Injury. *Travel Med Infect Dis* (2013) 11(2):130–3. doi: 10.1016/j.tmaid.2012.11.004
18. Rajaratnam N, Govil S, Patel R, Ahmed M, Elias S. Rhabdomyolysis After Recombinant Zoster Vaccination: A Rare Adverse Reaction. *J Community Hosp Intern Med Perspect* (2021) 11(1):145–6. doi: 10.1080/20009666.2020.1841878
19. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med* (2021) 384 (5):403–16. doi: 10.1056/NEJMoa2035389
20. Theodorou DJ, Theodorou SJ, Axiotis A, Gianniki M, Tsifetaki N. COVID-19 Vaccine-Related Myositis. *QJM* (2021) hcab043. doi: 10.1093/qjmed/hcab043
21. Arunachalam PS, Scott MKD, Hagan T, Li C, Feng Y, Wimmers F, et al. Systems Vaccinology of the BNT162b2 mRNA Vaccine in Humans. *Nature* (2021) 596(7872):410–6. doi: 10.1038/s41586-021-03791-x
22. Konstantinov KN, Emil SN, Barry M, Kellie S, Tzamaloukas AH. Glomerular Disease in Patients With Infectious Processes Developing Antineutrophil Cytoplasmic Antibodies. *ISRN Nephrol* (2013) 2013:324315. doi: 10.5402/2013/324315
23. Tidman M, Olander R, Svalander C, Danielsson D. Patients Hospitalized Because of Small Vessel Vasculitides With Renal Involvement in the Period 1975-95: Organ Involvement, Anti-Neutrophil Cytoplasmic Antibodies Patterns, Seasonal Attack Rates and Fluctuation of Annual Frequencies. *J Intern Med* (1998) 244(2):133–41. doi: 10.1046/j.1365-2796.1998.00324.x
24. Summers SA, Steinmetz OM, Gan PY, Ooi JD, Odobasic D, Kitching AR, et al. Toll-Like Receptor 2 Induces Th17 Myeloperoxidase Autoimmunity While Toll-Like Receptor 9 Drives Th1 Autoimmunity in Murine Vasculitis. *Arthritis Rheumatol* (2011) 63(4):1124–35. doi: 10.1002/art.30208
25. Kumar N, Admane N, Kumari A, Sood D, Grover S, Prajapati VK, et al. Cytotoxic T-Lymphocyte Elicited Vaccine Against SARS-CoV-2 Employing Immunoinformatics Framework. *Sci Rep* (2021) 11(1):7653. doi: 10.1038/s41598-021-86986-6
26. Jeffs LS, Peh CA, Jose MD, Lange K, Hurtado PR. Randomized Trial Investigating the Safety and Efficacy of Influenza Vaccination in Patients With Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. *Nephrol (Carlton)* (2015) 20(5):343–51. doi: 10.1111/nep.12416
27. Jeffs LS, Nitschke J, Tervaert JW, Peh CA, Hurtado PR. Viral RNA in the Influenza Vaccine may Have Contributed to the Development of ANCA-Associated Vasculitis in a Patient Following Immunisation. *Clin Rheumatol* (2016) 35(4):943–51. doi: 10.1007/s10067-015-3073-0
28. Uppal NN, Kello N, Shah HH, Khanin Y, De Oleo IR, Epstein E, et al. De Novo ANCA-Associated Vasculitis With Glomerulonephritis in COVID-19. *Kidney Int Rep* (2020) 5(11):2079–83. doi: 10.1016/j.ekir.2020.08.012
29. Izci Duran T, Turkmen E, Dilek M, Sayarlioglu H, Arik N. ANCA-Associated Vasculitis After COVID-19. *Rheumatol Int* (2021) 41(8):1523–9. doi: 10.1007/s00296-021-04914-3
30. Vlachoyiannopoulos PG, Magira E, Alexopoulos H, Jahaj E, Theophilopoulou K, Kotanidou A, et al. Autoantibodies Related to Systemic Autoimmune Rheumatic Diseases in Severely Ill Patients With COVID-19. *Ann Rheum Dis* (2020) 79(12):1661–3. doi: 10.1136/annrheumdis-2020-218009


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REVIEW
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# Severe Acute Respiratory Syndrome Coronavirus-2 Infection and Autoimmunity 1 Year Later: The Era of Vaccines

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Impressive efforts have been made by researchers worldwide in the development of target vaccines against the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and in improving the management of immunomodulating agents. Currently, different vaccine formulations, such as viral vector, mRNA, and protein-based, almost all directed toward the spike protein that includes the domain for receptor binding, have been approved. Although data are not conclusive, patients affected by autoimmune rheumatic diseases (ARDs) seem to have a slightly higher disease prevalence, risk of hospitalization, and death from coronavirus disease-2019 (COVID-19) than the general population. Therefore, ARD patients, under immunosuppressive agents, have been included among the priority target groups for vaccine administration. However, specific cautions are needed to optimize vaccine safety and effectiveness in these patients, such as modification in some of the ongoing immunosuppressive therapies and the preferential use of mRNA other than vector-based vaccines. Immunomodulating agents can be a therapeutic opportunity for the management of COVID-19 patients; however, their clinical impact depends on how they are handled. To place in therapy immunomodulating agents in the correct window of opportunity throughout the identification of surrogate markers of disease progression and host immune response is mandatory to optimize patient's outcome.

**Keywords: SARS-CoV-2, COVID-19, infections, vaccines, autoimmune rheumatic disease (ARD)**

## INTRODUCTION

During the first wave of the coronavirus disease-2019 (COVID-19) pandemic, we discussed the complex relationship between autoimmunity and the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (1). At that moment, in the absence of specific antiviral treatments or available vaccines, we focused on the immunologic rationale and the initial scientific evidences for

the effectiveness of immunomodulatory treatments, commonly adopted in autoimmune rheumatic diseases (ARDs), in the management of the aberrant hyper-inflammatory response induced by COVID-19. We concluded that anti-cytokine therapy, in particular the anti-IL-6 tocilizumab (TCZ) and the Janus kinase (JAK) inhibitor baricitinib, seemed to be the most promising drugs provided that their use was tailored to both timing of symptom onset and COVID-19 severity.

More than 1 year later, we are still facing a COVID-19 pandemic. However, combined adoption of universal containment measures and effective vaccine campaigns had a successful impact in terms of reduction of SARS-CoV-2 infections and COVID-19 hospitalizations. In addition, the use of immunosuppressive agents in the clinical management of COVID-19 patients has been clarified by consolidated data (2–4). First immunosuppressive agents, such as chloroquine and hydroxychloroquine (HCQ), have been discharged, whereas others have been better tailored to COVID-19 severity, timing of symptom onset, and association with other therapies. A considerable effort has been put forward to identify innovative vaccine platforms and to produce neutralizing monoclonal antibodies (mAbs) against SARS-CoV-2. In December 2020, the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved the first mRNA vaccine (BNT162b2) against SARS-CoV-2, followed by other vaccines using mRNA platforms or inactivated viral vectors. Conversely, protein-based vaccines and conventional vaccines based on live attenuated or inactivated whole virus were left behind with still ongoing clinical trials or under approval by regulatory agencies.

The likely choice of use of different SARS-CoV-2 vaccines raised several questions and special concerns for its application in patients with ARD. First, ARD patients are part of vulnerable categories. Considering their intrinsic dysregulated immune system and the clinical impact of immunosuppressive treatments, a vaccine priority should be deserved. Second, safety and effectiveness of vaccination can be influenced by intrinsic vaccine formulations (mRNA *vs.* viral vectors), by disease-specific factors (ongoing immunosuppressive treatment) or by host factors (disease activity).

The aim of this review is to identify in the selected category of ARD patients the main controversial issues in terms of safety, efficacy, and effectiveness of SARS-CoV-2 vaccines and how to place vaccination in the context of the therapy with immunosuppressive agents, as well as to clinical parameters of disease severity and host immune response.

## CORONAVIRUS DISEASE-19 CLINICAL SETTING

To better understand the characteristics of the COVID-19 patients enrolled in randomized controlled trials (RCTs) evaluating the effectiveness and safety of immunosuppressive agents is fundamental to remind the generally accepted classification of disease severity (5).

Mild Infection (Approximately 80% of Cases): Individuals have signs and symptoms of COVID-19 (e.g., fever, cough, headache, muscle pain, nausea, diarrhea, and loss of taste and smell) with no evidence of viral pneumonia or hypoxia; they are usually outpatients.

Moderate Infection: Individuals show evidence of pneumonia during clinical assessment or imaging and have saturation of oxygen ($SpO_2$) ≥92%–94% on room air at sea level; they are usually inpatients with no need of supplemental oxygen.

Severe Infection: Individuals have signs of severe pneumonia plus $SpO_2$ <92%–94% on room air at sea level and/or a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen ($PaO_2/FiO_2$) <300 mmHg, and/or respiratory rate >30 breaths/min; they are usually inpatients requiring high flow supplemental oxygen or mechanical ventilation.

Critical Infection (Approximately 5% of Cases): Individuals have acute respiratory distress syndrome (ARDS), with 100 mmHg < $PaO_2/FiO_2a$ ≤ 300 mmHg, and acute life-threatening organ dysfunction and can experience cytokine storm syndrome (CSS) caused by a dysregulated host response; they are usually inpatients admitted in intensive care units requiring non-invasive (NIV) or invasive mechanical ventilation (IMV).

## IMMUNOMODULATING ANTI-RHEUMATIC AGENTS USED IN SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 PATIENTS

### Hydroxychloroquine

HCQ is an oral drug frequently adopted in the treatment of systemic autoimmune diseases, with immunomodulatory effects and antiviral properties. The initial great enthusiasm for the potential benefits of this agent, sustained by some encouraging results from case series and retrospective studies (6), was subsequently denied by a deeper analysis of the data.

The FDA, EMA, and National Institutes of Health (NIH) have recommended against the use chloroquine or HCQ for COVID-19 patients (5).

### Colchicine

Colchicine is a well-known alkaloid agent currently approved in Italy for the treatment of gout and acute/recurrent pericarditis, but it is frequently used with effectiveness in the management of different autoinflammatory conditions (7, 8). It acts on several anti-inflammatory pathways, but the most recognized is the inhibition of microtubule polymerization, which can lead to impaired neutrophil migration, phagocytosis, and release of superoxide anion (9, 10). In addition, this agent limits neutrophil adhesion by both altering neutrophil L-selectin expression and E-selectin distribution in endothelial cells and inhibits Nod-Like Receptor Protein 3 (NLRP3) inflammasome known to be implicated in hyper-inflammatory syndrome (10, 11). These data provided the rationale for a possible role of this agent in the prevention of

hyperinflammation and coagulation activation pathognomonic of COVID-19. Among RCTs, the GRECCO study showed that colchicine significantly improved time to clinical deterioration as compared with that in controls in hospitalized patients with moderate/severe COVID-19 not receiving ventilation support (12). In contrast, the RECOVERY trial closed the recruitment to the colchicine arm because there was no significant difference in the primary endpoint of 28-day mortality with respect to standard of care (SOC) in hospitalized COVID-19 patients (13). More recently, the largest RCT on colchicine in non-hospitalized COVID-19 patients (COLCORONA) did not meet the primary endpoint but demonstrated that in patients with PCR-confirmed COVID-19, colchicine led to a significant reduction in the composite rate of death or hospitalization (14).

In conclusion, there is no evidence for the efficacy of colchicine in hospitalized patients, and the drug is not included in the NIH recommendations. Its early use in outpatient settings to prevent hospitalization appears an attractive option to be confirmed.

## INTERLEUKIN INHIBITORS

mAbs directed toward different inflammatory cytokines have been adopted in SARS-CoV-2 infection to reduce the aberrant immune response in COVID-19 patients with contrasting results. Most of available data are present for the humanized and human mAbs, recognizing the soluble and membrane-bound forms of IL-6 receptor, TCZ, and sarilumab, and also for anakinra, the non-glycosylated recombinant human IL-1Ra that blocks the binding of both IL-1α and IL-1β to its native receptor. IL-6 and IL-1 indeed play a central role in CSS (15–18) and are involved in several immunological activities such as the differentiation of naïve CD4-positive T cells into Th17 cells, increase acute-phase proteins and signaling, homing of immune cells to the site of primary infection, epithelial cell activation, and release of several other inflammatory cytokines (19, 20).

### Tocilizumab

TCZ was the first biologic agent to be used in severe COVID-19 patients also in view of the fact that it was the only approved biologic agent for the treatment of cytokine release syndrome (21, 22).

The first results from RCTs were unsatisfactory; however, the efforts in defining the right timing/segment of COVID-19 population combined with the concomitant use of a more effective SOC has made the difference in reaching the therapeutic targets.

Indeed, in all the first RCTs conducted, the COVACTA, the CORIMUNO-19 (23, 24), the Boston Area COVID-19 Consortium (BACC) Bay Tocilizumab (25), and the RCT by Salvarani et al. (2), TCZ did not show a significant improvement in clinical status or mortality reduction. Of note, these studies shared in common a very low use of glucocorticoid (GC) as SOC. Furthermore, in the COVACTA trial, more than two-thirds of participants were under mechanical ventilation.

The EMPACTA (Evaluating Minority Patients with Actemra, tocilizumab) was the first trial including a relevant percentage of patients receiving dexamethasone (DEX) and remdesivir (26). TCZ reached the composite primary outcome of progression to NIV/IMV, but without improving the survival rate.

More recently, the REMAP-CAP and RECOVERY, the two largest RCTs on TCZ, showed a significant efficacy of TCZ in mortality reduction in a well-defined COVID-19 population and in combination with DEX (27). Indeed, the REMAP-CAP enrolled severe/critical COVID-19 patients within 24 h after starting NIV or IMV, and most of the patients received concomitant GC. TCZ reduced both hospital mortality and time to hospital discharge. The RECOVERY trial enrolled severe COVID-19 hospitalized patients with hypoxemia, with or without mechanical ventilation, and C-reactive protein (CRP) level ≥75 mg/L. Patients on TCZ had a 4% mortality reduction through day 28, as well as the median time of hospitalization; the mortality benefit was observed in participants who were receiving concomitant GC. Considering all the above studies, NIH and WHO recommendations have recently stated that TCZ should be administered in combination with DEX in all severe/critical COVID-19 patients recently hospitalized (28).

### Sarilumab

Some small prospective studies explored COVID-19 patients receiving sarilumab with contrasting results on clinical outcomes, mortality, and time of hospitalization.

A phase 3 trial assessing 400 mg of sarilumab plus SOC *vs.* SOC alone, in critical COVID-19 patients (requiring IMV), was stopped because it did not meet its primary and secondary endpoints (29). In contrast with these results, the REMAP-CAP study reported median adjusted ORs for hospital survival at day 28 of 2.01 for i.v. sarilumab plus SOC *vs.* SOC alone (30). In July 2021, WHO recommends use of sarilumab with the same TCZ indications (5).

### Anakinra

A recent systematic review and meta-analysis reported data of seven studies on a total of 346 patients (31). These studies were different for design (four prospective and three retrospective), route and dose of anakinra administration, and population setting (mainly severe/critical COVID-19 patients); thus, results are hard to compare (32–38).

By now, only one RCT has been published on the use of anakinra in COVID-19 patients with disappointing results. It was a multicenter, open-label, Bayesian trial nested within the CORIMUNO-19 cohort, in patients with moderate-to-severe COVID-19, neither receiving mechanical ventilation nor admitted at ICU, and with a CRP >25 mg/L. Patients received either SOC plus i.v. anakinra at 400 mg/day for 3 days, 200 mg on day 4, and 100 mg on day 5, or SOC alone (39). The trial showed no significant difference between the two groups in terms of 4-day improvement, 14-day ventilation requirement, and 28-day mortality. The mean disease duration was 10 days before randomization, and the selected narrow segment target population (patients with a WHO score of 5 points and requiring oxygen, without mechanical ventilation) limits any conclusions.

Recently, the SAVE-MORE RCT was conducted in over 600 COVID-19 inpatients with elevated soluble urokinase plasminogen activator receptor (suPAR), a plasma biomarker that reflects immune activation. The comparative 11-point WHO Contracting & Procurement Services (CPS), at day 28, demonstrated significant improvement in patients receiving anakinra plus SOC *versus* SOC (40).

By now, the use of anakinra is not indicated by the NIH and WHO recommendations considering paucity of data; however, in July 2021, EMA started evaluating the use of anakinra in adult COVID-19 patients at increased risk of severe respiratory failure.

## JAK-STAT INHIBITORS

JAK inhibitors are a class of orally administered targeted synthetic immunosuppressants that act by inhibiting the activity of one or more of the JAK members (JAK1, JAK2, JAK3, and TYK2), thereby interfering with the JAK-STAT signaling pathway and inhibiting the signaling of several inflammatory cytokines involved in autoimmunity diseases (41). Baricitinib reversibly inhibits JAK1/2-dependent cytokines (IL-6 and IFN-γ) and to a lesser extent JAK1/TYK2-dependent cytokines (IL-10 and IFN-α) (42).

Baricitinib has also shown to inhibit a regulator of endocytosis, the AP2-associated protein kinase 1 (AAK1), at therapeutic dosage for rheumatoid arthritis (RA); therefore, it can interrupt virus entry through receptor-mediated endocytosis (43). In addition, it has low interaction with the CYP drug-metabolizing enzymes, thus being safe in combination with antiviral drugs (44).

By now, the only RCT available is the Adaptive COVID-19 Treatment Trial 2 (ACTT-2) involving hospitalized patients with COVID-19 randomized to receive 4 mg/day of baricitinib plus remdesivir or remdesivir alone (45, 46).

Patients receiving baricitinib showed a significantly lower median time to recovery, with 30% higher odds of improvement in clinical status at day 15, and a similar incidence of serious adverse events (SAEs). The reduction in the time of hospitalization and the odds of improvement in clinical status were more clinically meaningful in the subgroup of patients with a baseline severity ordinal score of 6 (receiving high-flow oxygen or NIV) (47). The results of the CoV barrier in hospitalized COVID-19 patients who received once-daily baricitinib 4 mg plus SOC or SOC have been just released (unpublished preprint data). Treatment with baricitinib significantly reduced mortality in particular in subjects on high-flow oxygen/NIV, with a good safety profile (48).

The FDA currently approved the use of baricitinib in addition to remdesivir, in patients with moderate-to-severe COVID-19 requiring high-flow oxygen or NIV.

## GLUCOCORTICOIDS

Most of the data come from the RECOVERY trial, the largest and first trial to be published. It demonstrated that the use of 6 mg of DEX daily up to 10 days plus SOC resulted in a significant decrease in 28-day mortality among patients who required mechanical ventilation or supplemental oxygen with respect to SOC (49). A systematic review and meta-analysis published in September 2020 selected six further RCTs evaluating the efficacy and safety of GCs in COVID-19 patients (50): the Efficacy of Dexamethasone Treatment for Patients With ARDS Caused by COVID-19 (DEXA-COVID 19) (51) and the COVID-19 Dexamethasone (CoDEX) trial, evaluated DEX in severe/critical COVID-19 patients under mechanical ventilation (52); the Randomized Embedded Multifactorial Adaptive Platform for Community-acquired Pneumonia (REMAP-CAP) (53), CAPECOVID (54), and COVIDSTEROID trial (55), evaluated hydrocortisone (HCT) in patients admitted to ICU or to an intermediate care unit who were receiving supplemental oxygen; finally, the Steroids-SARI trials was the only trial evaluating methylprednisolone (MTP) and enrolled patients admitted to ICU (56). The extent of concomitant SOC varied substantially among the trials. Overall death occurred in 32.7% of patients in GC group *vs.* 41.5% of SOC. The summary ORs for the association with all-cause mortality were 0.64 for DEX, 0.69 for HCT, and 0.91 for MTP. SAEs were reported by 18% of patients randomized to GC *vs.* 23% of patients in the control group (50).

In light of these data, DEX, alone or in combination with remdesivir, has become the SOC in severe and critical COVID-19 requiring supplemental oxygen with or without mechanical ventilation; alternatively, MTP or HCT can be adopted (52). It should be taken into account that GCs are not exempt from known severe side effects; in particular, high-dose GC can inhibit immune response, reduce pathogen clearance, and increase viral replication; thus, their use in mild/moderate patients not requiring oxygen supplementation and in the first stage of disease is contraindicated; moreover, low-dose (up to 6 mg of DEX or equivalent) and short-term GCs (up to 6 days) are recommended.

## ANTI-SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 MONOCLONAL ANTIBODIES

Before the COVID-19 pandemic, little attention had been paid to the use of mAbs, specific for pathogen epitopes, as therapy to treat infections. Despite, there are more than 100 mAbs that had been licensed as therapeutics for cancer, inflammation, and autoimmunity. The reasons behind the low interest to develop mAbs against infectious agents are mostly because of their high cost, which does not make them suitable for a broader use.

The high pressure generated from the SARS-CoV-2 pandemic induced first-line clinicians to explore the use of antibodies on the management of hospitalized patients. The initial approach was to transfuse plasma from previously infected convalescent individuals to hospitalized patients. Plasma samples were selected based on the titers of SARS-CoV-2-neutralizing antibodies, meaning that only individuals presenting high titers for the S1 subunit of SARS-CoV-2 spike protein, which contains the receptor-binding domain (RBD), were picked as donors. Such antibodies are able to prevent the entry

of SARS-CoV-2 into cells by blocking the binding of the viral spike (S) protein to its cellular main receptor, angiotensin-converting enzyme 2 (ACE2) (57), or S-mediated membrane fusion. As plasma is obtained by apheresis, in addition to the SARS-CoV-2-neutralizing antibodies, each convalescent plasma (CP) preparation also contains the entire repertoire of antibody specificities, including natural antibodies, as well as proteins potentially working as immunomodulators (anti-inflammatory cytokines, clotting and anti-thrombotic factors, defensins, complement, and many others) (58). Actually, it is known that natural antibodies present in intravenous immunoglobulin (IVIg) preparations may sequester BAFF and reduce B-cell survival/proliferation or bind Fas and induce apoptosis, whereas exposure of dendritic cells to IVIg compromises dendritic cell (DC) maturation and downregulates costimulatory molecules such as CD86, CD80, and CD40 complement. IVIg also modulates the balance between CD4 and CD8 T cells and reduces the proliferation of Th17 cells as well as the secretion of inflammatory cytokines while promoting proliferation and survival of Treg cells (58). All the described immunomodulatory features of IVIgs are likely to be also present in the plasma of convalescent individuals; thus, CP therapy may benefit patients with COVID-19.

Because composition of CP is variable and patients receiving CP are under multiple concurrent therapies, it is not surprising that positive immunomodulatory effects of CP, on patients with COVID-19, diverge. In spite of that, plasma therapy was the first treatment authorized by the FDA for emergency use (40) based on trials demonstrating moderate benefit of plasma therapy, in particular when used promptly upon hospitalization, during the initial stages of disease, or in contexts in which patients are unable to produce their own antibodies against the coronavirus, such as in immunodeficient or in B-cell-depleted patients (40).

It is worthy to note that different studies reported low antibody responses in asymptomatic subjects and positive correlations between disease severity and intensity of the antibody response, with the highest Abs levels being observed in hospitalized patients. Moreover, B-cell receptor (BCR) sequence analysis revealed that SARS-CoV-2-neutralizing antibodies have few mutations as compared with their respective germline sequences (59, 60), suggesting poor germinal center reactions and thus a reduced capacity to induce long-lived plasma cells (61). These observations are extremely important for vaccine design and for the screening of neutralizing antibodies.

The generation of sub-neutralizing (or non-neutralizing) antibodies, in some infections, is responsible for antibody-dependent enhancement (ADE) of disease meaning that antibodies facilitate viral entry into host cells and enhance viral infections (62). ADE has been documented to occur through two distinct mechanisms in viral infections: by enhanced antibody-mediated virus uptake across Fc gamma receptor IIa (FcγRIIa)-expressing phagocytic cells leading to increased viral infection and replication or by excessive antibody Fc-mediated effector functions or immune complex formation, causing enhanced inflammation and immunopathology. All the viruses causing ADE have the ability to replicate in macrophages and/or alter their function. Although macrophages do not seem to be a major target for SARS-CoV-2, and the expression of ACE2 on different monocyte and macrophage populations is highly variable, previous data regarding SARS-CoV suggest that FcγRs can facilitate uptake of the virus into macrophages and B cells (63). While correlations between antibody titers and infection severity have been reported, the remaining question is whether the high antibody titers promote disease or whether severe infections elicit higher antibody titers (64).

In addition, sustaining a role for ADE in SARS-CoV *in vitro* studies showed that macrophages treated with serum from patients with SARS had exaggerated inflammatory cytokine profiles (65). It is worth noting that studies performed in 2014 by Wang and colleagues, during the first SARS infection, strongly suggested that SARS-CoV ADE is primarily mediated by anti-spike antibodies rather than antibodies against nucleocapsid proteins (66). This contributes to raise the concern for the use of CP without having done rigorous scientific studies as the ones done for other experimental treatments.

The ADE issue is important and has to be kept in mind during vaccine design and mAb production. The latter has been immediately activated by several academic laboratories (67) and pharmaceutical companies resulting in the synthesis of mAbs targeting SARS-CoV-2 spike protein RBD able to neutralize the virus at concentrations below 10 ng/ml (48). One of the first to be used was the LY-CoV555 (also known as LY3819253), developed by Eli Lilly after its discovery by researchers at AbCellera and at the Vaccine Research Center of the National Institute of Allergy and Infectious Diseases. This and other mAbs entered clinical testing within the first 6 months of the pandemic and showed to accelerate viral clearance and reduce hospitalization by 75% when administered early after infection (68). More recently, various groups are exploiting the possibility of combining mAbs (69), binding to different portions of the RBD, aiming at increasing the potency of protection but also preventing viral escape of immune responses due to viral mutations (70, 71).

In particular, two RCTs showed a significant reduction in hospitalizations and deaths among mild-to-moderate COVID-19 outpatient subjects receiving bamlanivimab plus etesevimab and casirivimab plus imdevimab compared with placebo. However, in both trials, the percentage of patients with hospitalization or death was also low in the placebo group (2.1% *vs.* 7.0% and 1% *vs.* 3.2%) (69, 72). Finally, the ACTIV-3 trial demonstrated that bamlanivimab plus remdesivir was not effective in hospitalized patients (73). Currently, the use of these mAbs is limited to outpatients with confirmed diagnosis of mild/moderate COVID-19 and specific risk factors for disease progression and hospitalization, within 10 days from the onset of symptoms. Nevertheless, UK RECOVERY investigators announced in June 2021 that high doses of casirivimab plus imdevimab reduced risk of death by 20% in patients hospitalized with COVID-19 who had not mounted their own immune response (74).

## SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 VACCINES

To date, several vaccines against SARS-CoV-2 have been approved by both the FDA and EMA and are currently being widely used in

high-income countries. Several, of more than 70, vaccine candidates have reached the final stages for vaccine safety and protection efficacy in RCTs, but no direct comparative data between different vaccines or different platforms are available, and assays to titer viral neutralizing antibodies in the sera of vaccine recipients are not fully standardized. Theoretically a good vaccine, for any pathogenic agent, is a vaccine capable of reducing infection, disease, and transmission. The spike protein is required for SARS-CoV-2 receptor recognition, viral entry, and cell fusion and consequent infection. S protein, including its RBD, was shown to induce robust antibody response including generation of neutralizing antibodies, rendering it the target of almost all the approved and under-development vaccines against SARS-CoV-2. With the last decade technological achievements, vaccines can be produced on various platforms (75): viral vector, RNA, DNA, and protein-based in addition to the "traditional" inactivated and attenuated virus vaccines. These different types of vaccine induce immune responses through different mechanisms. Vaccines using adenoviral vector as antigen delivery are known to induce both cellular and humoral immunity already after a single immunization, with two immunizations raising a durable and long-lasting immune response (76, 77). Russia was the first country to approve a vaccine against COVID-19 named "Sputnik V," formerly known as the "Gam-COVID-Vac." This vaccine is based on a heterologous adenovirus strategy using two recombinant serotypically distinct adenovirus, namely, Adenovirus26 and Adenovirus5, both vectors including DNA coding for the spike protein. However, the world scientific community has raised doubts about the pivotal trial to prove its safety and efficacy, as its approval was obtained before public data disclosure, which only recently has been unleashed (78, 79). Published data showed that from 21 days after the first dose, the vaccine efficacy was 91.6% according to the number of confirmed COVID-19 cases.

Similar adenoviral vaccines are already used in large-scale vaccination programs all over the world. One of them is based on chimpanzee adenovirus called ChAdOx1 developed by the University of Oxford and AstraZeneca (80, 81), which showed a particularly high efficacy of 90.0% by inoculating two doses with a low first dose of vaccine (82). The other is an Adenovirus26 vector-based produced by Johnson & Johnson (83–85). Both vaccines are able to elicit potent and protective immune response, the latter even with a single dose (86). mRNA, FDA-approved vaccines first launched into the market were Pfizer/BioNTech and Moderna, which produced a viral modified mRNA encoding a more stable spike protein in a pre-fusion conformation form (87, 88). Because it is important for the immune system to respond to the virus before it enters into the cell, both Pfizer/BioNTech and Moderna vaccines deliver spike-specific mRNA into host cells through lipid nanoparticles (89).

The spike protein is synthesized translating the information encoded in the mRNA. The expression of SARS-CoV-2 spike protein elicits high titers of neutralizing antibodies and robust antigen-specific $CD8^+$ and Th1-type $CD4^+$ T-cell responses (88, 90). The efficacy rate in preventing COVID-19 infections for the Moderna (mRNA-1273) and Pfizer/BioNTech (BNT162b2) vaccines was 94.1% and 95.0%, respectively, with negligible side effects (91). The rapid emergence of SARS-CoV-2 variants, in particular the so-called UK and South African (SA) variants, carrying multiple mutations in the spike protein, raised the question whether mRNA vaccine-elicited antibodies were able to neutralize such variants. Neutralization geometric mean titers against N501Y from the United Kingdom and SA, 69/70-deletion/N501Y/D614G from the United Kingdom, and E484K/N501Y/D614G from SA variants of sera from subjects immunized with the BNT162b2 were 0.8- to 1.5-fold those against the parental virus (92). Noteworthy, these differences are much lower than those relative to the hemagglutination inhibition titers taken in account to consider a strain change in influenza vaccines (93). The virus-based vaccine candidates use weakened or inactivated virus processed by conventional technology by passing the virus through animal or human cells leading to mutations or by chemical substances (most commonly formaldehyde and heat) that make it less virulent.

Currently, two inactivated vaccines against SARS-CoV-2 were approved by at least one country. The first, Covaxin or BBV152, is a whole-virion inactivated SARS-CoV-2 vaccine (3 or 6 μg) formulated with a toll-like receptor 7/8 agonist molecule (IMDG) adsorbed to alum. It has been developed in India; and during the phase 1 trial, Covaxin was able to induce high neutralizing antibody responses that remained high in all participants at 3 months after the second vaccination. The phase 2 trial showed better reactogenicity and safety with enhanced humoral and cell-mediated immune responses compared with the phase 1 trial (94). The second vaccine has been developed in China by Sinopharm BBIBPCorV (95), which gives 79% protection (96).

Other whole virus-based vaccines with an inactivated virus, such as CoronaVac (97) and adsorbed vaccine COVID-19 by Sinovac (98), have just passed phase 3 clinical trials, and the sole live-attenuated COVI-VAC (developed in Russia, Codagenix Inc.) is in phase 1.

# SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 VACCINE IN RHEUMATIC AUTOIMMUNE DISEASES

## Rheumatic Autoimmune Diseases and Infections

Patients with ARD have an increased risk of infections, due to the immune-dysregulatory impact of the disease itself and the use of immunosuppressive agents.

Infections mainly involve bone and joints, skin, soft tissues, and respiratory tract; they are characterized by a more severe clinical outcome than in the general population and may induce disease flares, representing a frequent cause of death (99, 100).

In RA patients, the risk for serious infections is reported to be about double with respect to healthy subjects (101). Regarding common infections such as influenza, the reported incidence risk ratio (IRR) in RA patients *versus* general population is 1.2 (95% CI: 1.1–1.4), with a 2.75-fold increase in influenza-related complications (102). For *Streptococcus pneumoniae*, the reported IRR is 4.4 (95% CI: 3.8–5.2) in patients with RA and 4.3 (95% CI: 3.8–4.7) in patients with systemic lupus erythematosus (SLE), with an also increased risk of invasive pneumococcal disease (102). Beyond

common risk factors (e.g., older age and comorbidities), the known specific risk factors for developing infections in ARD are mainly the concomitant immunosuppressive therapy, a high disease activity, and the presence of organ involvement (e.g., lungs, kidney, and leukopenia) (103–105). Regarding immunosuppressive treatments, it has been reported that the use of conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) such as MTX and HCQ did not significantly affect the rate of influenza or its complications, whereas treatment with biological DMARDs (bDMARDs) are at a moderate higher risk than csDMARDs, with no significant differences among them. GC use over 7.5 mg/day in particular in combination with anti-TNF-α appears to be the most pro-infective treatment (104–108).

## Prevalence and Outcome of Severe Acute Respiratory Syndrome Coronavirus-2 Infection in Rheumatic Autoimmune Diseases

In our previous review, we hypothesized that the impairment of immune response caused by the ongoing therapy in ARD could be a double-edged sword in the context of COVID-19 pandemia.

On the one hand, indeed, immunosuppressive therapy could increase the risk of COVID-19, but on the other hand, the cytokine release could be restrained by the effect of the immunomodulating agents, thus potentially reducing COVID-19 symptoms and severity, and the risk of the aberrant inflammatory response observed in some critical infected patients.

More than 1 year later, interpretation of data on whether or not patients with ARD are at higher risk of COVID-19 prevalence and complications are still not straightforward due to the heterogenicity of autoimmune diseases analyzed, study design, ongoing therapeutic regimen, and disease activity.

A systematic review and meta-analysis published in September 2020 showed a COVID-19 prevalence of 0.009 (95% CI: 0.005–0.014) in patients with ARD (0.034 in the studies evaluating only SLE, Sjogren's syndrome, and systemic sclerosis), by analyzing 33 observational studies with a total of 54,679 subjects (109). Regarding COVID-19 severity, the overall rates of hospitalization and death assessed by 34 studies in 1,449 patients with ARD were 0.54 (95% CI: 0.46–0.63) and 0.113 (95% CI: 0.098–0.13), respectively (0.33 and 0.069 in the studies evaluating only SLE, Sjogren's syndrome, and systemic sclerosis). The six case–control studies demonstrated that the risk of COVID-19 in ARD was significantly higher than that in controls (OR: 1.59, 95% CI: 1.04 to 4.58, p = 0.008), whereas there were no significant differences in clinical outcomes, such as hospitalization and death.

Since then, several data have been published on a large population such as those from the COVID-19 Global Rheumatology Alliance (GRA) and from nationwide cohort studies and registries (110–113). Furthermore, another systematic review and meta-analysis evaluating 1,138 patients with various rheumatic diseases from 31 studies (16 of which were different from those included by Akiyama et al.) have recently reported that ARD represents a risk factor for poor outcomes in patients with COVID-19.

In particular, the rates of hospitalization and fatality among COVID-19-infected patients with ARD were 0.58 (95% CI: 0.48–0.67) and 0.07 (0.13 among hospitalized patients) (95% CI: 0.03–0.11), respectively (114).

In conclusion, although data are partly contrasting and hard to compare, ARD seem to have a similar or slightly higher prevalence, risk of hospitalization, and death from COVID-19 than the general population, as already stated by the last European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) recommendations (115, 116). As for the general population, older age, male gender, and comorbidities such as hypertension, diabetes, lung disease, and obesity have been described as independent risk factors associated with COVID-19 death and worse outcome. Whereas among disease-specific risk factors, the use of prednisolone-equivalent dosage >5/10 mg/day, some csDMARDs (e.g., azathioprine, cyclophosphamide (CyC), and mycophenolate), and rituximab (RTX) and having an active disease were the most frequently reported factors associated with poor prognosis. Although there is no clear evidence of worse outcome due to specific factors related to the disease itself other than GC use, also in SLE patients (117), the presence of kidney involvement should be taken into account considering that it is a known risk factor for severe disease and mortality in COVID-19 patients (118). On the other hand, MTX and bDMARD monotherapy (other than RTX) were reported to have a protective rather than negative effect on COVID-19 outcome. Data on targeted synthetic (ts) DMARDs are still contrasting and on small sample size (109, 110, 119).

## Efficacy and Safety of Common Vaccines in Rheumatic Autoimmune Diseases

The particular relevance of vaccinations in ARD patients is indisputable, considering that vaccines can lead to a lower prevalence of infections and related complications such as hospitalization and death. In the last years, the efforts of several groups gave important contributions in this field, generally overcoming prejudices that limited this good clinical practice in patients with ARD, such as the fear of an impaired efficacy (as a result of immunosuppression) and decreased safety (linked to a possible exacerbation of the underlying autoimmune disease).

Among the vaccine-preventable respiratory diseases, influenza and pneumococcal infections have been the more extensively studied in ARD patients. Last, EULAR recommendations stated that both these vaccines should be strongly considered for the majority of patients with ARD (120).

Indeed, as reported by a systematic review and meta-analysis, most studies evaluating immunogenicity (expressed as seroconversion rate; ≥4-fold increase in hemagglutinin titer) of inactivated influenza vaccine showed that autoimmune patients (mainly RA and SLE) reached protective antibody levels, although reduced with respect to healthy controls (121, 122). In particular, for the H1N1 strain, the pooled mean proportion of 11 studies on 883 RA patients was 50.5% *vs.* 67% of healthy controls (p = 0.05), whereas for H3N2 strain, the pooled mean proportion of eight studies on 333 patients was 54.7% *vs.* 50.2% (ns). The percentage of adverse events was similar to that of controls, and the disease activity was generally stable. The humoral immunogenicity of the 23-valent pneumococcal

polysaccharide vaccine (PPSV23) and to a lesser extent for the 13-valent conjugate vaccine (PCV13) has been demonstrated for both RA and SLE patients with no significant safety concerns independently of the type of vaccine (120, 122).

## Efficacy and Safety of Severe Acute Respiratory Syndrome Coronavirus-2 Vaccine in Rheumatic Autoimmune Diseases

In Italy, as well as in other countries, autoimmune patients under immunosuppressive therapy have been included among priority target groups for vaccine administration (123–125). While waiting for evidence-based data, at least three questions are currently a challenge for ARD (**Figure 1**):

- Is there a preferred vaccine for ARD patients?
- What is the effect of ongoing immunosuppressive therapy?
- Can disease activity influence or be influenced by SARS-CoV-2 vaccine?

## Influence of Vaccine Formulation

According to the previously cited ACR recommendations, there is no preference for one COVID-19 vaccine over another in ARD patients. Indeed, ARD patients were mainly excluded from clinical trials, so there were inadequate data to draw any conclusions on the efficacy and safety of COVID-19 vaccination in these patients. Nevertheless, different observational data are currently becoming available for mRNA vaccine in this patient category (**Table 1**), whereas we still have no data for viral vector vaccines; furthermore, some immunologic insights are tipping the scale in favor of mRNA-based vaccine. Indeed, is important to consider that after vector-based vaccines, you can respond not only to the spike but also to the adenovirus developing anti-vector antibodies and a lower spike-specific response, thus requiring frequent booster doses with a consequent increased risk of autoimmune flares. On the other hand, mRNA vaccines by reducing the toll-like receptor-7 stimulation could potentially attenuate the risk of disease flares in ARD patients (131).

After small pivotal trials of the mRNA-based vaccine BNT162b2 in few ARD patients (127–129, 132), a larger study, in the Israel population, showed that the majority of the RA patients respond to the vaccine with a seropositivity rate of 86%, although with a lower humoral response than the control group. Overall, BNT162b2 showed a good safety and no significant disease flares (126).

Some immunosuppressive treatments, such as RTX, abatacept, mycophenolate mofetil (MMF), and methotrexate (MTX), showed negative impact on vaccine immunogenicity; however, data are partly contrasting and need to be evaluated by additional real-life studies (**Table 1**). In the attempt to provide useful insights, we

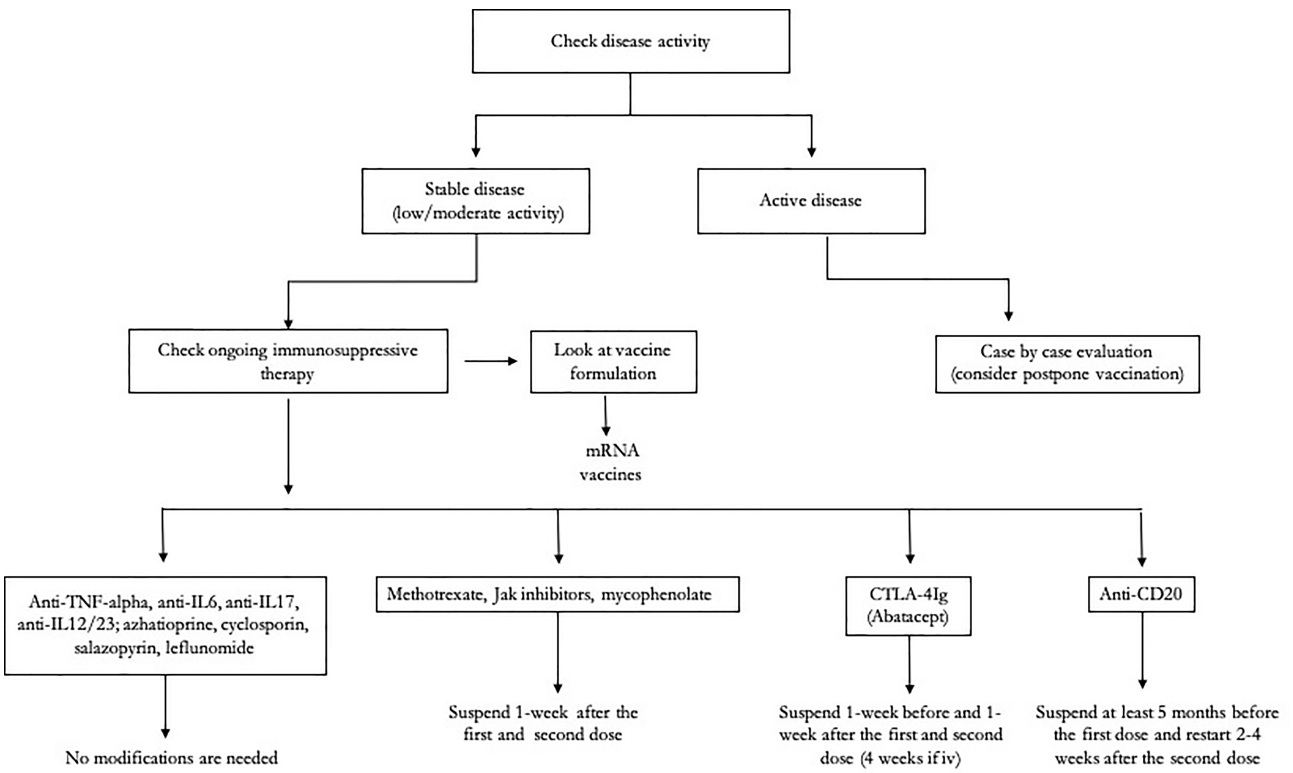


**FIGURE 1** | Clinical algorithm for the management of patients with autoimmune rheumatic diseases (ARDs) undergoing severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) vaccination.

**TABLE 1** | Core relevant data from studies evaluating efficacy and safety of SARS-CoV-2 vaccine in ARD patients.

| | ARD Total number Subtype number | Type of vaccine | Age Mean, years (range) | Timing of assessment (weeks) | Seropositivity rate %(serum anti-S1 IgG titer, mean) | | | | | | | T-cell responses | Side effects/ disease relapses |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Controls | Patients(therapy) | | | | | | | |
| | | | | | | Tot | MTX | TNFi | IL6i | JAKi | CTLA4i | CD20i | |
| Furer et al. (126) | **Tot: 686**<br>RA 263<br>PsA/SpA 233<br>SLE 101<br>Vasculitis 66<br>Other 23 | BNT162b2 | 59 (19-88) | 2–6 weeks from 2nd dose | 100 (218.6) | 86* (132.9) | 92 | 98 | 100 | 90 | 71 | 41 | NA | Similar to HCs/ stable in most pts |
| Geisen et al. (127) | **Tot: 26**<br>RA 8<br>PsA/SpA 5<br>SLE 2<br>Other 11 | BNT162b2 | 50.5 (24–89) | 1 week from 2nd dose | 100 (2,685) | 100 (2,053**) | NR | NR | NR | NR | NR | NR | NA | Similar to HCs/ none |
| Haberman et al. (128) | **Tot: 51**<br>RA 22<br>PsA/PsO 24<br>Other 5 | BNT162b2 | 56 (22–79) | 1 week from 2nd dose | 96 (104.3) | 82 (80.9) | 72# (46.9) | NR | NR | NR | NR | NR | ^Reduced in pts receiving MTX | NA |
| Simon et al. (129) | **Tot: 84**<br>RA 24<br>SpA 27<br>SLE 16<br>Other 16 | BNT162b2 | 53 (NA) | At least 10 days from 2nd dose | 100 (9.36) | 94$ (6.46) | NR | NR | NR | NR | NR | NR | NA | Similar to HCs/NA |
| Picchianti Diamanti et al. (130) | **Tot: 35 RA** | BNT 162b2 | 59 (55-65) | 2–6 weeks from 2nd dose | 167 (2351) | 97 (785§) | 100 (1526) | 100 (1239) | 100 (492§) | NR | 92 (465§) | NA | Reduced in pts receiving TNFi, IL6i and CTLA4i | Similar to HCsNone |

*NA, not assessed; NR, not reported; ARD, autoimmune rheumatic disease; RA, rheumatoid arthritis; PsA, psoriatic arthritis; SpA, spondyloarthritis; SLE, systemic lupus erythematosus; MTX, methotrexate; TNFi, tumor necrosis factor inhibitors; JAKi, Janus kinase inhibitors; CD20i, CD20 inhibitors.*

**p < 0.0001 (BAU/ml, cutoff = 15).*

***p = 0.037 (BAU/ml, cutoff = above 2,000 but not specifically reported).*

*#p = 0.023 (median; cutoff = not specifically reported).*

*$p = 0.003 (optical density, cutoff = 5.7 nm).*

*^Evaluated by high-parameter spectral flow cytometry.*

have recently assessed the induction of specific humoral and T-cell response after mRNA BNT162b2 SARS-COV-2 vaccination in RA patients who underwent a strategy of temporary modification of immunosuppressive treatment according to ACR indications. The vaccine was safe and no disease relapses were observed. The vaccine induced an antibody-specific response in almost all patients, although the titer was significantly reduced in those under abatacept or IL-6-inhibitor compared to HCs. Spike-specific T-cell response was positive in 69% of RA patients vs. 100% of HCs with significantly lower levels in those under a biological therapy.Our data suggest that holding treatment with MTX and abatacept at the first and second vaccine dose can be a succesfull strategy in clinically stable patients (130).

In particular, the timing of vaccination related to the immunosuppressive therapy should be better defined, which could play a crucial role on the size of the response.

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## Influence of Immunosuppressive Agents

Whether to modify the ongoing immunosuppressive treatment in ARD patients who are to receive SARS-CoV-2 vaccinations can be challenging, because the balance of the risk of disease relapse with that of a decreased immunogenicity has to be weighed in. Until now, recommendations for SARS-CoV-2 vaccine in ARD have been formulated by the ACR (130, 133). The authors stated that ARD patients should be prioritized for vaccination and have no specific contraindications. However, expected response to COVID-19 vaccination for many patients on systemic immunomodulatory therapies is likely to be blunted in its magnitude and duration. The authors reported that most of the csDMARDs such as HCQ, ciclosporin, salazopyrin, azathioprine, leflunomide, oral CyC, and GC <20 mg/day do not require modifications in therapeutic regimen or vaccination timing. On the other hand, MTX, mycophenolate, JAK inhibitors, and i.v. CyC should be held 1 week after each vaccine dose, at least for patients with well-controlled disease. Among bDMARDs, anti-TNF-α, anti-IL-6R, anti-IL-1, anti-IL-17, anti-IL-12/23, and anti-BLYS do not require specific

modifications on therapy frequency/dosage or vaccination timing, except for abatacept, which should be stopped 1 week prior to and 1 week after the first dose only (4 weeks for i.v. route) (**Figure 1**).

The anti-CD20 RTX deserves special clinical attention. Indeed, vaccine timing should be modified so that the first dose is initiated 4 weeks prior to next scheduled RTX cycle; moreover, after vaccination, RTX should be delayed 2–4 weeks after the second vaccine dose, if disease activity allows. These recommendations were previously released by the ACR Board of Directors in February 2021 and then published in June. Considering the almost absent available scientific evidence, they were based on data from common vaccinations. Indeed, regarding seasonal and pandemic influenza, and pneumococcal inactivated vaccines, the use of TNF-α-blocking agents, anti-IL-6, and csDMARDs (other than MTX), in the majority of studies did not induce a reduction of immunogenicity (120, 134–139). Fewer studies with small sample size evaluated the effect of abatacept and JAK inhibitors with contrasting data; however, most of them reported a mild reduction in humoral immunogenicity (140–143). By now, studies on SARS-CoV-2 mRNA vaccine in ARD patients have generally confirmed the neutral impact of cytokine inhibitors and most of the csDMARDs on vaccine immunogenicity, as well as a significant impairment caused by abatacept (126). Data on the impact of MTX in vaccine immunogenicity are more controversial. Indeed, no negative effect of MTX was found for influenza vaccination, in most studies including one meta-analysis in patients with RA (144–148). Only two studies by Park et al. in patients with RA showed that temporary discontinuation of MTX significantly improves immunogenicity to seasonal influenza vaccine (149, 150). In particular, suspension of MTX for 2 weeks after vaccination led to an 11%–16% (depending on the strain) higher seroprotection rate compared with that in RA patients who continued MTX. Two other studies reported a reduced pneumococcal humoral immunogenicity in patients receiving MTX (131, 151, 152). In line with these data, the 2019 EULAR recommendations for vaccination in ARD patients stated that it is not recommended to stop MTX before or after influenza and pneumococcal vaccines (120). These concerns have not been resolved by recently published data on COVID-19 mRNA vaccines in ARD patients who report contrasting results on the effect of MTX, ranging from a relevant to mild reduction in humoral response with respect to controls (**Table 1**). Regarding RTX, this agent has been associated with hampered antibody responses following influenza and pneumococcal vaccination in multiple studies, and the EULAR recommendations report that they should be scheduled at least 6 months after the administration and 4 weeks before the next course of anti-CD20 therapy (119). In these patients, B-cell depletion is due to different mechanisms such as antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and apoptosis (153), whereas, it did not significantly affect T cell-mediated response in RA patients receiving influenza vaccine (154). Data from the 2003 SARS outbreak and the current SARS-CoV-2 pandemic showed the role of protective memory T-cell responses in recovered and asymptomatic individuals, with the emergence of memory T cells also in the absence of an antibody response (155–157). Indeed, asymptomatic people who have cleared SARS-CoV-2 can have a detectable specific T-cell response, without mounting an antibody response (157–160), observation confirmed by the recovery of people with B-cell immunodeficiency, or receiving B-cell-depleting therapy (161, 162). Furthermore, considering that most of the SARS-CoV-2 vaccines need a second dose after 3–4 weeks, this prolonged interruption of RTX could lead to an increased risk of disease relapse.

Current data confirm the severe reduction of humoral response to mRNA SARS-CoV-2 vaccine in patients under RTX, however accompanied by a substantial maintenance of the adaptive cellular immunity (163).

In view of the above, the immunologic response following SARS-CoV-2 vaccine in ARD patients receiving MTX and RTX deserves to be better investigated by additional real-life studies.

## Severe Acute Respiratory Syndrome Coronavirus-2 Vaccination and Disease Activity

Autoimmune diseases, infections, and vaccines are also joined with disease activity. A high disease activity is a poor prognostic factor for COVID-19 severity, and infections even by SARS-CoV-2 can induce disease flares in ARD. Vaccines could work in a similar manner, thus potentially inducing clinical relapses in ARD and having a reduced immunogenicity in active patients. Indeed, high disease activity can reflect an ongoing active inflammation against self, which could deplete the immune resources and partly deviate them from the signals delivered by vaccines (164); moreover, these patients are generally under a stronger immunosuppressive therapy that can further decrease immunogenicity. Based on these assumptions, few studies on common vaccination included patients with active disease. In one study, patients with juvenile SLE and high activity reduced seroconversion rates to influenza A H1N1 vaccination (165). Conversely, in a study of 340 RA patients, high disease activity levels did not reduce immunogenicity (166). Data are reassuring with regard to disease reactivation after common vaccines in ARD patients; almost all the studies, indeed, reported that vaccination did not influence activity of the underlying ARD such as RA and SLE (122, 167–174).

Concerns about the possible risk of disease relapse in these patient populations, related to the use of new vaccine technologies, have been reduced by recently published manuscripts on BNT162b2 vaccine in ARD patients who showed a good safety and no significant disease flares. Moreover, a cross-sectional study based on a web-based survey on 696 SLE participants reported that COVID-19 vaccination (both mRNA and adenoviral platforms) appears well tolerated with only a minimal risk of flare (3%) (175).

Anyway, considering the paucity of data on active patients, the EULAR recommendations report that vaccination in patients with ARD should be preferably administered during quiescent disease (120). In apparent contrast, the ACR recommendations stated that COVID-19 vaccination should occur as soon as possible for patients for whom it is being recommended, irrespective of disease activity and severity (130).

In conclusion, considering the severity of the SARS-CoV-2 infection, the current pandemic emergency, and the evidence from common vaccinations, the chance of protect also active patients seem to weight more in the balance than the possible risk of a reduced immunogenicity and safety concerns.

## CONCLUSIONS

Eighteen months after SARS-CoV-2 identification, we are still facing the COVID-19 pandemic despite all public health measures put in place. Nevertheless, the spread of the virus is being strongly reduced by vaccines based on various platforms currently adopted in most countries. Available data suggest that ARD patients have a similar or slightly higher prevalence of SARS-CoV-2 infections, risk of hospitalization, and death from COVID-19 than have the general population. They have been included among priority groups for vaccination, but special measures should be adopted to improve vaccine safety and effectiveness, throughout modification in ongoing immunosuppressive regimen and preferential use of mRNA-based vaccines as demonstrated by emerging data on mRNA vaccination efficacy in ARD patients.

Immunomodulating agents are an important therapeutic opportunity for the management of COVID-19 patients. To place therapy immunomodulating agents in the correct window of opportunity throughout the identification of surrogate markers of disease progression and host immune response is mandatory to optimize patient's outcome.

## AUTHOR CONTRIBUTIONS

AP and BL conceived the manuscript. AP, MR, and CP wrote the manuscript. EN and GS critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES

1. Picchianti Diamanti A, Rosado MM, Pioli C, Sesti G, Lagana B. Cytokine Release Syndrome in COVID-19 Patients, A New Scenario for an Old Concern: The Fragile Balance Between Infections and Autoimmunity. *Int J Mol Sci* (2020) 21 (9):3330. doi: 10.3390/ijms21093330
2. Alunno A, Najm A, Mariette X, De Marco G, Emmel J, Mason L, et al. Immunomodulatory Therapies for SARS-CoV-2 Infection: A Systematic Literature Review to Inform EULAR Points to Consider. *Ann Rheum Dis* (2021) 80(6):803–15. doi: 10.1136/annrheumdis-2020-219725
3. Atzeni F, Masala IF, Rodriguez-Carrio J, Rios-Garces R, Gerratana E, La Corte L, et al. The Rheumatology Drugs for COVID-19 Management: Which and When? *J Clin Med* (2021) 10(4):783. doi: 10.3390/jcm10040783
4. Cantini F, Goletti D, Petrone L, Najafi Fard S, Niccoli L, Foti R. Immune Therapy, or Antiviral Therapy, or Both for COVID-19: A Systematic Review. *Drugs* (2020) 80(18):1929–46. doi: 10.1007/s40265-020-01421-w
5. 3/15/2021, h.w.c.t.n.g.o. .
6. Gao J, Tian Z, Yang X. Breakthrough: Chloroquine Phosphate has Shown Apparent Efficacy in Treatment of COVID-19 Associated Pneumonia in Clinical Studies. *Biosci Trends* (2020) 14(1):72–3. doi: 10.5582/bst.2020.01047
7. https://farmaci.agenziafarmaco.gov.it/.
8. https://www.accessdata.fda.gov/.
9. Leung YY, Yao Hui LL, Kraus VB. Colchicine–Update on Mechanisms of Action and Therapeutic Uses. *Semin Arthritis Rheum* (2015) 45(3):341–50. doi: 10.1016/j.semarthrit.2015.06.013
10. Slobodnick A, Shah B, Krasnokutsky S, Pillinger MH. Update on Colchicine, 2017. *Rheumatol (Oxford)* (2018) 57(suppl_1):i4–i11. doi: 10.1093/rheumatology/kex453
11. Brunetti L, Diawara O, Tsai A, Firestein BL, Nahass RG, Poiani G, et al. Colchicine to Weather the Cytokine Storm in Hospitalized Patients With COVID-19. *J Clin Med* (2020) 9(9):2961. doi: 10.3390/jcm9092961
12. Deftereos SG, Giannopoulos G, Vrachatis DA, Siasos GD, Giotaki SG, Gargalianos P, et al. Effect of Colchicine *vs* Standard Care on Cardiac and Inflammatory Biomarkers and Clinical Outcomes in Patients Hospitalized With Coronavirus Disease 2019: The GRECCO-19 Randomized Clinical Trial. *JAMA Netw Open* (2020) 3(6):e2013136. doi: 10.1001/jamanetworkopen.2020.13136
13. https://www.recoverytrial.net/news/recovery-trial-closes-recruitment-to-colchicine-treatment-for-patients-hospitalised-with-covid-19.
14. Tardif J-C, Bouabdallaoui N, L'Allier PL, Gaudet D, Shah B, Pillinger MH, et al. Efficacy of Colchicine in Non-Hospitalized Patients With COVID-19. *medRxiv* (2021) 9(8):924–32. doi: 10.1016/S2213-2600(21)00222-8
15. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, et al. Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. *Clin Infect Dis* (2020) 71(15):762–8. doi: 10.1093/cid/ciaa248
16. Behrens EM, Koretzky GA. Review: Cytokine Storm Syndrome: Looking Toward the Precision Medicine Era. *Arthritis Rheumatol* (2017) 69(6):1135–43. doi: 10.1002/art.40071
17. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the Eye of the Cytokine Storm. *Microbiol Mol Biol Rev* (2012) 76(1):16–32. doi: 10.1128/MMBR.05015-11
18. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and Immunological Features of Severe and Moderate Coronavirus Disease 2019. *J Clin Invest* (2020) 130(5):2620–9. doi: 10.1172/JCI137244
19. Picchianti Diamanti A, Markovic M, Argento G, Giovagnoli S, Ricci A, Lagana B, et al. Therapeutic Management of Patients With Rheumatoid Arthritis and Associated Interstitial Lung Disease: Case Report and Literature Review. *Ther Adv Respir Dis* (2017) 11(1):64–72. doi: 10.1177/1753465816668780
20. Choy E, Rose-John S. Interleukin-6 as a Multifunctional Regulator: Inflammation, Immune Response, and Fibrosis. *J Scleroderma Related Disord* (2017) 2(2):S1–5. doi: 10.5301/jsrd.5000265
21. https://www.ema.europa.eu/en/documents/smop/chmp-post-authorisation-summary-positive-opinion-roactemra_en-3.pdf.
22. https://www.fda.gov/NewsEvents/. U.S.F.d.a.P.a.-F.a.b.f.g.t.t.t.U.S.
23. Zhang W, Zhao Y, Zhang F, Wang Q, Li T, Liu Z, et al. The Use of Anti-Inflammatory Drugs in the Treatment of People With Severe Coronavirus Disease 2019 (COVID-19): The Perspectives of Clinical Immunologists From China. *Clin Immunol* (2020) 214:108393. doi: 10.1016/j.clim.2020.108393
24. Xu X, Han M, Li T, Sun W, Wang D, Fu B, et al. Effective Treatment of Severe COVID-19 Patients With Tocilizumab. *Proc Natl Acad Sci USA* (2020) 117(20):10970–5. doi: 10.1073/pnas.2005615117
25. Rosas IO, Brau N, Waters M, Go RC, Hunter BD, Bhagani S, et al. Tocilizumab in Hospitalized Patients With Severe Covid-19 Pneumonia. *N Engl J Med* (2021) 384(16):1503–16. doi: 10.1056/NEJMoa2028700

26. Hermine O, Mariette X, Tharaux PL, Resche-Rigon M, Porcher R, Ravaud P, et al. Effect of Tocilizumab *vs* Usual Care in Adults Hospitalized With COVID-19 and Moderate or Severe Pneumonia: A Randomized Clinical Trial. *JAMA Intern Med* (2021) 181(1):32–40. doi: 10.1001/jamainternmed.2020.6820
27. Salvarani C, Dolci G, Massari M, Merlo DF, Cavuto S, Savoldi L, et al. Effect of Tocilizumab *vs* Standard Care on Clinical Worsening in Patients Hospitalized With COVID-19 Pneumonia: A Randomized Clinical Trial. *JAMA Intern Med* (2021) 181(1):24–31. doi: 10.1001/jamainternmed.2020.6615
28. https://www.covid19treatmentguidelines.nih.gov/.
29. (2020). h.w.s.c.e.m.-r.p.-r.
30. Investigators R-C, Gordon AC, Mouncey PR, Al-Beidh F, Rowan KM, Nichol AD, et al. Interleukin-6 Receptor Antagonists in Critically Ill Patients With Covid-19. *N Engl J Med* (2021) 384:1491–502. doi: 10.1056/NEJMoa2100433
31. Khan FA, Stewart I, Fabbri L, Moss S, Robinson K, Smyth AR, et al. Systematic Review and Meta-Analysis of Anakinra, Sarilumab, Siltuximab and Tocilizumab for COVID-19. *Thorax* (2021) 76(9):907–19. doi: 10.1136/thoraxjnl-2020-215266
32. Cauchois R, Koubi M, Delarbre D, Manet C, Carvelli J, Blasco VB, et al. Early IL-1 Receptor Blockade in Severe Inflammatory Respiratory Failure Complicating COVID-19. *Proc Natl Acad Sci USA* (2020) 117(32):18951–3. doi: 10.1073/pnas.2009017117
33. Cavalli G, De Luca G, Campochiaro C, Della-Torre E, Ripa M, Canetti D, et al. Interleukin-1 Blockade With High-Dose Anakinra in Patients With COVID-19, Acute Respiratory Distress Syndrome, and Hyperinflammation: A Retrospective Cohort Study. *Lancet Rheumatol* (2020) 2(6):e325–31. doi: 10.1016/S2665-9913(20)30127-2
34. Balkhair A, Al-Zakwani I, Al Busaidi M, Al-Khirbash A, Al Mubaihsi S, BaTaher H, et al. Anakinra in Hospitalized Patients With Severe COVID-19 Pneumonia Requiring Oxygen Therapy: Results of a Prospective, Open-Label, Interventional Study. *Int J Infect Dis* (2021) 103:288–96. doi: 10.1016/j.ijid.2020.11.149
35. Narain S, Stefanov DG, Chau AS, Weber AG, Marder G, Kaplan B, et al. Comparative Survival Analysis of Immunomodulatory Therapy for Coronavirus Disease 2019 Cytokine Storm. *Chest* (2021) 159(3):933–48. doi: 10.1016/j.chest.2020.09.275
36. Kyriazopoulou E, Panagopoulos P, Metallidis S, Dalekos GN, Poulakou G, Gatselis N, et al. An Open Label Trial of Anakinra to Prevent Respiratory Failure in COVID-19. *Elife* (2021) 10:1:21. doi: 10.7554/eLife.66125
37. Huet T, Beaussier H, Voisin O, Jouveshomme S, Dauriat G, Lazareth I, et al. Anakinra for Severe Forms of COVID-19: A Cohort Study. *Lancet Rheumatol* (2020) 2(7):e393–400. doi: 10.1016/S2665-9913(20)30164-8
38. Kooistra EJ, Waalders NJB, Grondman I, Janssen NAF, de Nooijer AH, Netea MG, et al. Anakinra Treatment in Critically Ill COVID-19 Patients: A Prospective Cohort Study. *Crit Care* (2020) 24(1):688. doi: 10.1186/s13054-020-03364-w
39. group, C.-C. Effect of Anakinra *Versus* Usual Care in Adults in Hospital With COVID-19 and Mild-to-Moderate Pneumonia (CORIMUNO-ANA-1): A Randomised Controlled Trial. *Lancet Respir Med* (2021) 9(3):295–304. doi: 10.1016/S2213-2600(20)30556-7
40. Liu STH, Lin HM, Baine I, Wajnberg A, Gumprecht JP, Rahman F, et al. Convalescent Plasma Treatment of Severe COVID-19: A Propensity Score-Matched Control Study. *Nat Med* (2020) 26(11):1708–13. doi: 10.1038/s41591-020-1088-9
41. Kontzias A, Kotlyar A, Laurence A, Changelian P, O'Shea JJ. Jakinibs: A New Class of Kinase Inhibitors in Cancer and Autoimmune Disease. *Curr Opin Pharmacol* (2012) 12(4):464–70. doi: 10.1016/j.coph.2012.06.008
42. McInnes IB, Byers NL, Higgs RE, Lee J, Macias WL, Na S, et al. Comparison of Baricitinib, Upadacitinib, and Tofacitinib Mediated Regulation of Cytokine Signaling in Human Leukocyte Subpopulations. *Arthritis Res Ther* (2019) 21(1):183. doi: 10.1186/s13075-019-1964-1
43. Richardson P, Griffin I, Tucker C, Smith D, Oechsle O, Phelan A, et al. Baricitinib as Potential Treatment for 2019-Ncov Acute Respiratory Disease. *Lancet* (2020) 395(10223):e30–1. doi: 10.1016/S0140-6736(20)30304-4
44. A TV, Haikarainen T, Raivola J, Silvennoinen O. Selective JAKinibs: Prospects in Inflammatory and Autoimmune Diseases. *BioDrugs* (2019) 33(1):15–32. doi: 10.1007/s40259-019-00333-w
45. Cantini F, Niccoli L, Nannini C, Matarrese D, Natale MED, Lotti P, et al. Beneficial Impact of Baricitinib in COVID-19 Moderate Pneumonia; Multicentre Study. *J Infect* (2020) 81(4):647–79. doi: 10.1016/j.jinf.2020.06.052
46. Cantini F, Niccoli L, Matarrese D, Nicastri E, Stobbione P, Goletti D. Baricitinib Therapy in COVID-19: A Pilot Study on Safety and Clinical Impact. *J Infect* (2020) 81(2):318–56. doi: 10.1016/j.jinf.2020.04.017
47. Goletti D, Cantini F. Baricitinib Therapy in Covid-19 Pneumonia - An Unmet Need Fulfilled. *N Engl J Med* (2021) 384(9):867–9. doi: 10.1056/NEJMe2034982
48. Andreano E, Nicastri E, Paciello I, Pileri P, Manganaro N, Piccini G, et al. Extremely Potent Human Monoclonal Antibodies From COVID-19 Convalescent Patients. *Cell* (2021) 184(7):1821–1835.e16. doi: 10.1016/j.cell.2021.02.035
49. Group RC, Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, et al. Dexamethasone in Hospitalized Patients With Covid-19. *N Engl J Med* (2021) 384(8):693–704.
50. Group, T.W.R.E.A.f.C.-T.W. Association Between Administration of Systemic Corticosteroids and Mortality Among Critically Ill Patients With COVID-19: A Meta-Analysis. *JAMA* (2020) 324(13):1330–41. doi: 10.1001/jama.2020.17023
51. Villar J, Anon JM, Ferrando C, Aguilar G, Munoz T, Ferreres J, et al. Efficacy of Dexamethasone Treatment for Patients With the Acute Respiratory Distress Syndrome Caused by COVID-19: Study Protocol for a Randomized Controlled Superiority Trial. *Trials* (2020) 21(1):717. doi: 10.1186/s13063-020-04643-1
52. Tomazini BM, Maia IS, Cavalcanti AB, Berwanger O, Rosa RG, Veiga VC, et al. Effect of Dexamethasone on Days Alive and Ventilator-Free in Patients With Moderate or Severe Acute Respiratory Distress Syndrome and COVID-19: The CoDEX Randomized Clinical Trial. *JAMA* (2020) 324 (13):1307–16. doi: 10.1001/jama.2020.17021
53. Angus DC, Berry S, Lewis RJ, Al-Beidh F, Arabi Y, van Bentum-Puijk W, et al. The REMAP-CAP (Randomized Embedded Multifactorial Adaptive Platform for Community-Acquired Pneumonia) Study. Rationale and Design. *Ann Am Thorac Soc* (2020) 17(7):879–91. doi: 10.1513/AnnalsATS.202003-192SD
54. Dequin PF, Heming N, Meziani F, Plantefeve G, Voiriot G, Badie J, et al. Effect of Hydrocortisone on 21-Day Mortality or Respiratory Support Among Critically Ill Patients With COVID-19: A Randomized Clinical Trial. *JAMA* (2020) 324(13):1298–306. doi: 10.1001/jama.2020.16761
55. Petersen MW, Meyhoff TS, Helleberg M, Kjaer MN, Granholm A, Hjortsø CJS, et al. Low-Dose Hydrocortisone in Patients With COVID-19 and Severe Hypoxia (COVID STEROID) Trial-Protocol and Statistical Analysis Plan. *Acta Anaesthesiol Scand* (2020) 64(9):1365–75. doi: 10.1111/aas.13673
56. https://covid-19.cochrane.org/studies/crs-13107160.
57. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* (2020) 181(2):271–280 e8. doi: 10.1016/j.cell.2020.02.052
58. Rojas M, Rodriguez Y, Monsalve DM, Acosta-Ampudia Y, Camacho B, Gallo JE, et al. Convalescent Plasma in Covid-19: Possible Mechanisms of Action. *Autoimmun Rev* (2020) 19(7):102554. doi: 10.1016/j.autrev.2020.102554
59. Seydoux E, Homad LJ, MacCamy AJ, Parks KR, Hurlburt NK, Jennewein MF, et al. Analysis of a SARS-CoV-2-Infected Individual Reveals Development of Potent Neutralizing Antibodies With Limited Somatic Mutation. *Immunity* (2020) 53(1):98–105 e5. doi: 10.1016/j.immuni.2020.06.001
60. Cao Y, Su B, Guo X, Sun W, Deng Y, Bao L, et al. Potent Neutralizing Antibodies Against SARS-CoV-2 Identified by High-Throughput Single-Cell Sequencing of Convalescent Patients' B Cells. *Cell* (2020) 182(1):73–84 e16. doi: 10.1016/j.cell.2020.05.025
61. Kaneko N, Kuo HH, Boucau J, Farmer JR, Allard-Chamard H, Mahajan VS, et al. Loss of Bcl-6-Expressing T Follicular Helper Cells and Germinal Centers in COVID-19. *Cell* (2020) 183(1):143–157.e13. doi: 10.1016/j.cell.2020.08.025
62. Wan Y, Shang J, Sun S, Tai W, Chen J, Geng Q, et al. Molecular Mechanism for Antibody-Dependent Enhancement of Coronavirus Entry. *J Virol* (2020) 94(5):1:15. doi: 10.1128/JVI.02015-19

63. Merad M, Martin JC. Pathological Inflammation in Patients With COVID-19: A Key Role for Monocytes and Macrophages. *Nat Rev Immunol* (2020) 20(6):355–62. doi: 10.1038/s41577-020-0331-4
64. Lee N, Chan PK, Ip M, Wong E, Ho J, Ho C, et al. Anti-SARS-CoV IgG Response in Relation to Disease Severity of Severe Acute Respiratory Syndrome. *J Clin Virol* (2006) 35(2):179–84. doi: 10.1016/j.jcv.2005.07.005
65. Liu L, Wei Q, Lin Q, Fang J, Wang H, Kwok H, et al. Anti-Spike IgG Causes Severe Acute Lung Injury by Skewing Macrophage Responses During Acute SARS-CoV Infection. *JCI Insight* (2019) 4(4):1–20. doi: 10.1172/jci.insight.123158
66. Wang SF, Tseng SP, Yen CH, Yang JY, Tsao CH, Shen CW, et al. Antibody-Dependent SARS Coronavirus Infection is Mediated by Antibodies Against Spike Proteins. *Biochem Biophys Res Commun* (2014) 451(2):208–14. doi: 10.1016/j.bbrc.2014.07.090
67. Chen Y, Zuiani A, Fischinger S, Mullur J, Atyeo C, Travers M, et al. Quick COVID-19 Healers Sustain Anti-SARS-CoV-2 Antibody Production. *Cell* (2020) 183(6):1496–1507 e16. doi: 10.1016/j.cell.2020.10.051
68. Chen P, Nirula A, Heller B, Gottlieb RL, Boscia J, Morris J, et al. SARS-CoV-2 Neutralizing Antibody LY-CoV555 in Outpatients With Covid-19. *N Engl J Med* (2021) 384(3):229–37. doi: 10.1056/NEJMoa2029849
69. Gottlieb RL, Nirula A, Chen P, Boscia J, Heller B, Morris J, et al. Effect of Bamlanivimab as Monotherapy or in Combination With Etesevimab on Viral Load in Patients With Mild to Moderate COVID-19: A Randomized Clinical Trial. *JAMA* (2021) 325(7):632–44. doi: 10.1001/jama.2021.0202
70. Yao H, Sun Y, Deng YQ, Wang N, Tan Y, Zhang NN, et al. Rational Development of a Human Antibody Cocktail That Deploys Multiple Functions to Confer Pan-SARS-CoVs Protection. *Cell Res* (2021) 31(1):25–36. doi: 10.1038/s41422-020-00444-y
71. Wang N, Sun Y, Feng R, Wang Y, Guo Y, Zhang L, et al. Structure-Based Development of Human Antibody Cocktails Against SARS-CoV-2. *Cell Res* (2021) 31(1):101–3. doi: 10.1038/s41422-020-00446-w
72. https://newsroom.regeneron.com/news-releases/news-release-details/phase-3-treatment-trial-recently-infected-asymptomatic-patients/.
73. ACTIV-3/TICO LY-CoV555 Study Group, Lundgren JD, Grund B, Barkauskas EC, Holland TL, Gottlieb LR, et alA Neutralizing Monoclonal Antibody for Hospitalized Patients With Covid-19. *N Engl J Med* (2020) 384(10):905–14. doi: 10.1056/NEJMoa2033130
74. Group RC, Horby PW, Mafham M, Peto L, Campbell M, Pessoa-Amorim G, et al. Casirivimab and Imdevimab in Patients Admitted to Hospital With COVID-19 (RECOVERY): A Randomised, Controlled, Open-Label, Platform Trial. *medRxiv* (2021) 2021.06.15.21258542.
75. van Riel D, de Wit E. Next-Generation Vaccine Platforms for COVID-19. *Nat Mater* (2020) 19(8):810–2. doi: 10.1038/s41563-020-0746-0
76. Antrobus RD, Coughlan L, Berthoud TK, Dicks MD, Hill AV, Lambe T, et al. Clinical Assessment of a Novel Recombinant Simian Adenovirus ChAdOx1 as a Vectored Vaccine Expressing Conserved Influenza A Antigens. *Mol Ther* (2014) 22(3):668–74. doi: 10.1038/mt.2013.284
77. Tatsis N, Ertl HC. Adenoviruses as Vaccine Vectors. *Mol Ther* (2004) 10(4):616–29. doi: 10.1016/j.ymthe.2004.07.013
78. Jones I, Roy P. Sputnik V COVID-19 Vaccine Candidate Appears Safe and Effective. *Lancet* (2021) 397(10275):642–3. doi: 10.1016/S0140-6736(21)00191-4
79. Logunov DY, Dolzhikova IV, Shcheblyakov DV, Tukhvatulin AI, Zubkova OV, Dzharullaeva AS, et al. Safety and Efficacy of an Rad26 and Rad5 Vector-Based Heterologous Prime-Boost COVID-19 Vaccine: An Interim Analysis of a Randomised Controlled Phase 3 Trial in Russia. *Lancet* (2021) 397(10275):671–81. doi: 10.1016/S0140-6736(21)00234-8
80. Folegatti PM, Ewer KJ, Aley PK, Angus B, Becker S, Belij-Rammerstorfer S, et al. Safety and Immunogenicity of the ChAdOx1 Ncov-19 Vaccine Against SARS-CoV-2: A Preliminary Report of a Phase 1/2, Single-Blind, Randomised Controlled Trial. *Lancet* (2020) 396(10249):467–78. doi: 10.1016/S0140-6736(20)31604-4
81. Ramasamy MN, Minassian AM, Ewer KJ, Flaxman AL, Folegatti PM, Owens DR, et al. Safety and Immunogenicity of ChAdOx1 Ncov-19 Vaccine Administered in a Prime-Boost Regimen in Young and Old Adults (COV002): A Single-Blind, Randomised, Controlled, Phase 2/3 Trial. *Lancet* (2021) 396(10267):1979–93. doi: 10.1016/S0140-6736(20)32466-1
82. Voysey M, Clemens SAC, Madhi SA, Weckx LY, Folegatti PM, Aley PK, et al. Safety and Efficacy of the ChAdOx1 Ncov-19 Vaccine (AZD1222) Against SARS-CoV-2: An Interim Analysis of Four Randomised Controlled Trials in Brazil, South Africa, and the UK. *Lancet* (2021) 397(10269):99–111. doi: 10.1016/S0140-6736(20)32661-1
83. Sadoff J, Le Gars M, Shukarev G, Heerwegh D, Truyers C, de Groot AM, et al. Interim Results of a Phase 1-2a Trial of Ad26.COV2.S Covid-19 Vaccine. *N Engl J Med* (2021)384(19):1824–35. doi: 10.1056/NEJMoa2034201
84. Bos R, Rutten L, van der Lubbe JEM, Bakkers MJG, Hardenberg G, Wegmann F, et al. Ad26 Vector-Based COVID-19 Vaccine Encoding a Prefusion-Stabilized SARS-CoV-2 Spike Immunogen Induces Potent Humoral and Cellular Immune Responses. *NPJ Vaccines* (2020) 5:91. doi: 10.1038/s41541-020-00243-x
85. Stephenson KE, Le Gars M, Sadoff J, de Groot AM, Heerwegh D, Truyers C, et al. Immunogenicity of the Ad26.COV2.S Vaccine for COVID-19. *JAMA* (2021) 325(15):1535–44.
86. Yahalom-Ronen Y, Tamir H, Melamed S, Politi B, Shifman O, Achdout H, et al. A Single Dose of Recombinant VSV-ΔG-Scs Protection Against SARS-CoV-2 Challenge. *Nat Commun* (2020) 11(1):6402. doi: 10.1001/jama.2021.3645
87. Walsh EE, Frenck RWJr., Falsey AR, Kitchin N, Absalon J, Gurtman A, et al. Safety and Immunogenicity of Two RNA-Based Covid-19 Vaccine Candidates. *N Engl J Med* (2020) 383(25):2439–50. doi: 10.1056/NEJMoa2027906
88. Jackson LA, Anderson EJ, Rouphael NG, Roberts PC, Makhene M, Coler RN, et al. An mRNA Vaccine Against SARS-CoV-2 - Preliminary Report. *N Engl J Med* (2020) 383(20):1920–31. doi: 10.1056/NEJMoa2022483
89. Englezou PC, Sapet C, Demoulins T, Milona P, Ebensen T, Schulze K, et al. Self-Amplifying Replicon RNA Delivery to Dendritic Cells by Cationic Lipids. *Mol Ther Nucleic Acids* (2018) 12:118–34. doi: 10.1016/j.omtn.2018.04.019
90. Sahin U, Muik A, Derhovanessian E, Vogler I, Kranz LM, Vormehr M, et al. Concurrent Human Antibody and TH1 Type T-Cell Responses Elicited by a COVID-19 RNA Vaccine. *medRxiv* (2020), 2020.07.17.20140533.
91. Mulligan MJ, Lyke KE, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Phase I/II Study of COVID-19 RNA Vaccine BNT162b1 in Adults. *Nature* (2020) 586(7830):589–93. doi: 10.1038/s41586-020-2639-4
92. Xie X, Liu Y, Liu J, Zhang X, Zou J, Fontes-Garfias CR, et al. Neutralization of SARS-CoV-2 Spike 69/70 Deletion, E484K and N501Y Variants by BNT162b2 Vaccine-Elicited Sera. *Nat Med* (2021) 27(4):620–1. doi: 10.1038/s41591-021-01270-4
93. Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus ADME, et al. Mapping the Antigenic and Genetic Evolution of Influenza Virus. *Science* (2004) 305(5682):371–6. doi: 10.1126/science.1097211
94. Ella R, Reddy S, Jogdand H, Sarangi V, Ganneru B, Prasad S, et al. Safety and Immunogenicity of an Inactivated SARS-CoV-2 Vaccine, BBV152: Interim Results From a Double-Blind, Randomised, Multicentre, Phase 2 Trial, and 3-Month Follow-Up of a Double-Blind, Randomised Phase 1 Trial. *Lancet Infect Dis* (2021) 21(7):950–61. doi: 10.1016/S1473-3099(21)00070-0
95. Wang H, Zhang Y, Huang B, Deng W, Quan Y, Wang W, et al. Development of an Inactivated Vaccine Candidate, BBIBP-CorV, With Potent Protection Against SARS-CoV-2. *Cell* (2020) 182(3):713–21.e9. doi: 10.1016/j.cell.2020.06.008
96. Xia S, Zhang Y, Wang Y, Wang H, Yang Y, Gao GF, et al. Safety and Immunogenicity of an Inactivated SARS-CoV-2 Vaccine, BBIBP-CorV: A Randomised, Double-Blind, Placebo-Controlled, Phase 1/2 Trial. *Lancet Infect Dis* (2021) 21(1):39–51. doi: 10.1016/S1473-3099(20)30831-8
97. Zhang Y, Zeng G, Pan H, Li C, Hu Y, Chu K, et al. Safety, Tolerability, and Immunogenicity of an Inactivated SARS-CoV-2 Vaccine in Healthy Adults Aged 18-59 Years: A Randomised, Double-Blind, Placebo-Controlled, Phase 1/2 Clinical Trial. *Lancet Infect Dis* (2021) 21(2):181–92. doi: 10.1016/S1473-3099(20)30843-4
98. Palacios R, Patiño EG, de Oliveira Piorelli R, Conde M, Batista AP, Zeng G, et al. Double-Blind, Randomized, Placebo-Controlled Phase III Clinical Trial to Evaluate the Efficacy and Safety of Treating Healthcare Professionals With the Adsorbed COVID-19 (Inactivated) Vaccine Manufactured by Sinovac - PROFISCOV: A Structured Summary of a Study Protocol for a Randomised Controlled Trial. *Trials* (2020) 21(1):853. doi: 10.1186/s13063-020-04775-4

99. Gluck T, Muller-Ladner U. Vaccination in Patients With Chronic Rheumatic or Autoimmune Diseases. *Clin Infect Dis* (2008) 46(9):1459–65. doi: 10.1086/587063
100. Holvast B, Huckriede A, Kallenberg CG, Bijl M. Influenza Vaccination in Systemic Lupus Erythematosus: Safe and Protective? *Autoimmun Rev* (2007) 6(5):300–5. doi: 10.1016/j.autrev.2006.09.012
101. Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of Infection in Patients With Rheumatoid Arthritis Compared With Controls: A Population-Based Study. *Arthritis Rheum* (2002) 46(9):2287–93. doi: 10.1002/art.10524
102. Furer V, Rondaan C, Heijstek M, van Assen S, Bijl M, Agmon-Levin N, et al. Incidence and Prevalence of Vaccine Preventable Infections in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases (AIIRD): A Systemic Literature Review Informing the 2019 Update of the EULAR Recommendations for Vaccination in Adult Patients With AIIRD. *RMD Open* (2019) 5(2):e001041. doi: 10.1136/annrheumdis-2019-eular.7706
103. Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Predictors of Infection in Rheumatoid Arthritis. *Arthritis Rheum* (2002) 46(9):2294–300. doi: 10.1002/art.10529
104. Ramiro S, Sepriano A, Chatzidionysiou K, Nam JL, Smolen JS, van der Heijde D, et al. Safety of Synthetic and Biological DMARDs: A Systematic Literature Review Informing the 2016 Update of the EULAR Recommendations for Management of Rheumatoid Arthritis. *Ann Rheum Dis* (2017) 76(6):1101–36. doi: 10.1136/annrheumdis-2016-210708
105. Hou C, Jin O, Zhang X. Clinical Characteristics and Risk Factors of Infections in Patients With Systemic Lupus Erythematosus. *Clin Rheumatol* (2018) 37(10):2699–705. doi: 10.1007/s10067-018-4198-8
106. Germano V, Cattaruzza MS, Osborn J, Tarantino A, Di Rosa R, Salemi S, et al. Infection Risk in Rheumatoid Arthritis and Spondyloarthropathy Patients Under Treatment With DMARDs, Corticosteroids and TNF-Alpha Antagonists. *J Transl Med* (2014) 12:77. doi: 10.1186/1479-5876-12-77
107. Dixon WG, Suissa S, Hudson M. The Association Between Systemic Glucocorticoid Therapy and the Risk of Infection in Patients With Rheumatoid Arthritis: Systematic Review and Meta-Analyses. *Arthritis Res Ther* (2011) 13(4):R139. doi: 10.1186/ar3453
108. Atzeni F, Sarzi-Puttini P, Botsios C, Carletto A, Cipriani P, Favalli EG, et al. Long-Term Anti-TNF Therapy and the Risk of Serious Infections in a Cohort of Patients With Rheumatoid Arthritis: Comparison of Adalimumab, Etanercept and Infliximab in the GISEA Registry. *Autoimmun Rev* (2012) 12(2):225–9. doi: 10.1016/j.autrev.2012.06.008
109. Akiyama S, Hamdeh S, Micic D, Sakuraba A. Prevalence and Clinical Outcomes of COVID-19 in Patients With Autoimmune Diseases: A Systematic Review and Meta-Analysis. *Ann Rheum Dis* (2020) 384–91. doi: 10.1136/annrheumdis-2020-219394
110. Strangfeld A, Schäfer M, Gianfrancesco MA, Lawson-Tovey S, Liew JW, Ljung L, et al. Factors Associated With COVID-19-Related Death in People With Rheumatic Diseases: Results From the COVID-19 Global Rheumatology Alliance Physician-Reported Registry. *Ann Rheumatic Dis* (2021) 80(7):930–42. doi: 10.1136/annrheumdis-2020-219498.
111. Attauabi M, Seidelin JB, Felding OK, Wewer MD, Vinther Arp LK, Sarikaya MZ, et al. Coronavirus Disease 2019, Immune-Mediated Inflammatory Diseases and Immunosuppressive Therapies - A Danish Population-Based Cohort Study. *J Autoimmun* (2021) 118:102613. doi: 10.1016/j.jaut.2021.102613
112. Bower H, Frisell T, Di Giuseppe D, Delcoigne B, Ahlenius GM, Baecklund E, et al. Impact of the COVID-19 Pandemic on Morbidity and Mortality in Patients With Inflammatory Joint Diseases and in the General Population: A Nationwide Swedish Cohort Study. *Ann Rheum Dis* (2021). doi: 10.1136/annrheumdis-2021-219845
113. Hasseli R, Mueller-Ladner U, Hoyer BF, Krause A, Lorenz HM, Pfeil A, et al. Older Age, Comorbidity, Glucocorticoid Use and Disease Activity are Risk Factors for COVID-19 Hospitalisation in Patients With Inflammatory Rheumatic and Musculoskeletal Diseases. *RMD Open* (2021) 7(1):1:8. doi: 10.1136/rmdopen-2020-001464
114. Xu C, Yi Z, Cai R, Chen R, Thong BY-H, Mu R. Clinical Outcomes of COVID-19 in Patients With Rheumatic Diseases: A Systematic Review and Meta-Analysis of Global Data. *Autoimmun Rev* (2021) 20(4):102778. doi: 10.1016/j.autrev.2021.102778
115. Landewe RB, Machado PM, Kroon F, Bijlsma HW, Burmester GR, Carmona L, et al. EULAR Provisional Recommendations for the Management of Rheumatic and Musculoskeletal Diseases in the Context of SARS-CoV-2. *Ann Rheum Dis* (2020) 79(7):851–8. doi: 10.1136/annrheumdis-2020-217877
116. Mikuls TR, Johnson SR, Fraenkel L, Arasaratnam RJ, Baden LR, Bermas BL, et al. American College of Rheumatology Guidance for the Management of Rheumatic Disease in Adult Patients During the COVID-19 Pandemic: Version 2. *Arthritis Rheumatol* (2020) 72(9):e1–e12.
117. Fernandez-Ruiz R, Paredes JL, Niewold TB. COVID-19 in Patients With Systemic Lupus Erythematosus: Lessons Learned From the Inflammatory Disease. *Transl Res* (2021) 232:13–36. doi: 10.1016/j.trsl.2020.12.007
118. Bruchfeld A. The COVID-19 Pandemic: Consequences for Nephrology. *Nat Rev Nephrol* (2021) 17(2):81–2. doi: 10.1038/s41581-020-00381-4
119. Hyrich KL, Machado PM. Rheumatic Disease and COVID-19: Epidemiology and Outcomes. *Nat Rev Rheumatol* (2021) 17(2):71–2. doi: 10.1038/s41584-020-00562-2
120. Furer V, Rondaan C, Heijstek MW, Agmon-Levin N, van Assen S, Bijl M, et al. 2019 Update of EULAR Recommendations for Vaccination in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases. *Ann Rheum Dis* (2020) 79(1):39–52. doi: 10.1136/annrheumdis-2019-215882
121. Huang Y, Wang H, Tam WWS. Is Rheumatoid Arthritis Associated With Reduced Immunogenicity of the Influenza Vaccination? A Systematic Review and Meta-Analysis. *Curr Med Res Opin* (2017) 33(10):1901–8. doi: 10.1080/03007995.2017.1329140
122. Rondaan C, et al. Efficacy, Immunogenicity and Safety of Vaccination in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases: A Systematic Literature Review for the 2019 Update of EULAR Recommendations. *RMD Open* (2019) 5(2):e001035–e001035. doi: 10.1136/rmdopen-2019-001035
123. http://www.salute.gov.it/imgs/C_17_pubblicazioni_2986_allegato.pdf.
124. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/951928/uk-covid-19-vaccines-delivery-plan-final.pdf.
125. https://www.ecdc.europa.eu/sites/default/files/documents/Overview-of-EU_EEA-UK-vaccination-deployment-plans.pdf.
126. Furer V, Rondaan C, Agmon-Levin N, van Assen S, Bijl M, Kapetanovic MC, et al. Immunogenicity and Safety of the BNT162b2 mRNA COVID-19 Vaccine in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases and in the General Population: A Multicentre Study. *Ann Rheum Dis* (2021) 7(1):e001594. doi: 10.1136/annrheumdis-2021-220647
127. Geisen UM, Berner DK, Tran F, Sümbül M, Vullriede L, Ciripoi M, et al. Immunogenicity and Safety of Anti-SARS-CoV-2 mRNA Vaccines in Patients With Chronic Inflammatory Conditions and Immunosuppressive Therapy in a Monocentric Cohort. *Ann Rheumatic Dis* (2021), annrheumdis–2021-220272. doi: 10.1136/annrheumdis-2021-220272
128. Haberman RH, Herati R, Simon D, Samanovic M, Blank RB, Tuen M, et al. Methotrexate Hampers Immunogenicity to BNT162b2 mRNA COVID-19 Vaccine in Immune-Mediated Inflammatory Disease. *Ann Rheum Dis* (2021). doi: 10.1101/2021.05.11.21256917
129. Simon D, Tascilar K, Fagni F, Krönke G, Kleyer A, Meder C, et al. SARS-CoV-2 Vaccination Responses in Untreated, Conventionally Treated and Anticytokine-Treated Patients With Immune-Mediated Inflammatory Diseases. *Ann Rheumatic Dis* (2021) annrheumdis–2021-220461. doi: 10.1136/annrheumdis-2021-220461
130. Picchianti Diamanti A, Aiello A, Laganà B, Agrati C, Castilletti C, Meschi S, et al. Immunosuppressive Therapies Differently Modulate Humoral and T Cell Specifc Response to COVID-19 mRNA Vaccine in Rheumatoid Arthritis Patients. *Front Immunol*. doi: 10.3389/fimmu.2021.740249
131. Coulson E, Saravanan V, Hamilton J, So KL, Morgan L, Heycock C, et al. Pneumococcal Antibody Levels After Pneumovax in Patients With Rheumatoid Arthritis on Methotrexate. *Ann Rheum Dis* (2011) 70 (7):1289–91. doi: 10.1136/ard.2010.144451
132. Furer V, Rondaan C, Agmon-Levin N, van Assen S, Bijl M, Crnkic Kapetanovic M, et al. Point of View on the Vaccination Against COVID-19 in Patients With Autoimmune Inflammatory Rheumatic Diseases. *RMD Open* (2021) 7(1):1:10.. doi: 10.1136/rmdopen-2021-001594
133. Curtis JR, Johnson SR, Anthony DD, Arasaratnam RJ, Baden LR, Bass AR, et al. American College of Rheumatology Guidance for COVID-19

Vaccination in Patients With Rheumatic and Musculoskeletal Diseases: Version 2. *Arthritis Rheumatol* (2021) 73(8):e30–45. doi: 10.1002/art.41877
134. Salemi S, Picchianti-Diamanti A, Germano V, Donatelli I, Di Martino A, Facchini M, et al. Influenza Vaccine Administration in Rheumatoid Arthritis Patients Under Treatment With TNFalpha Blockers: Safety and Immunogenicity. *Clin Immunol* (2010) 134(2):113–20. doi: 10.1016/j.clim.2009.09.014
135. Polachek A, Korobko U, Mader-Balakirski N, Arad U, Levartovsky D, Kaufman I, et al. Immunogenicity and Safety of Vaccination Against Seasonal 2012 Influenza Virus Among Patients With Psoriatic Arthritis and Psoriasis. *Clin Exp Rheumatol* (2015) 33(2):181–6.
136. Crowe SR, Merrill JT, Vista ES, Dedeke AB, Thompson DM, Stewart S, et al. Influenza Vaccination Responses in Human Systemic Lupus Erythematosus: Impact of Clinical and Demographic Features. *Arthritis Rheum* (2011) 63 (8):2396–406. doi: 10.1002/art.30388
137. Kapetanovic MC, Saxne T, Nilsson JA, Geborek P. Influenza Vaccination as Model for Testing Immune Modulation Induced by Anti-TNF and Methotrexate Therapy in Rheumatoid Arthritis Patients. *Rheumatol (Oxford)* (2007) 46(4):608–11. doi: 10.1093/rheumatology/kel366
138. Kubota T, Nii T, Nanki T, Kohsaka H, Harigai M, Komano Y, et al. Anti-Tumor Necrosis Factor Therapy Does Not Diminish the Immune Response to Influenza Vaccine in Japanese Patients With Rheumatoid Arthritis. *Mod Rheumatol* (2007) 17(6):531–3. doi: 10.3109/s10165-007-0632-5
139. Fomin I, Caspi D, Levy V, Varsano N, Shalev Y, Paran D, et al. Vaccination Against Influenza in Rheumatoid Arthritis: The Effect of Disease Modifying Drugs, Including TNF Alpha Blockers. *Ann Rheum Dis* (2006) 65(2):191–4. doi: 10.1136/ard.2005.036434
140. Crnkic Kapetanovic M, Saxne T, Jönsson G, Truedsson L, Geborek P. Rituximab and Abatacept But Not Tocilizumab Impair Antibody Response to Pneumococcal Conjugate Vaccine in Patients With Rheumatoid Arthritis. *Arthritis Res Ther* (2013) 15(5):R171. doi: 10.1186/ar4358
141. Ribeiro AC, Laurindo IM, Guedes LK, Saad CG, Moraes JC, Silva CA, et al. Abatacept and Reduced Immune Response to Pandemic 2009 Influenza A/H1N1 Vaccination in Patients With Rheumatoid Arthritis. *Arthritis Care Res (Hoboken)* (2013) 65(3):476–80. doi: 10.1002/acr.21838
142. Migita K, Akeda Y, Akazawa M, Tohma S, Hirano F, Ideguchi H, et al. Effect of Abatacept on the Immunogenicity of 23-Valent Pneumococcal Polysaccharide Vaccination (PPSV23) in Rheumatoid Arthritis Patients. *Arthritis Res Ther* (2015) 17:357. doi: 10.1186/s13075-015-0863-3
143. Winthrop KL, Silverfield J, Racewicz A, Neal J, Lee EB, Hrycaj P, et al. The Effect of Tofacitinib on Pneumococcal and Influenza Vaccine Responses in Rheumatoid Arthritis. *Ann Rheum Dis* (2016) 75(4):687–95. doi: 10.1136/annrheumdis-2014-207191
144. Jain VK, Bhashini N, Balajee LK, Sistla S, Parija SC, Negi VS. Effect of Disease-Modifying Antirheumatic Drug Therapy on Immune Response to Trivalent Influenza Vaccine in Rheumatoid Arthritis. *Indian J Med Res* (2017) 145(4):464–70. doi: 10.4103/ijmr.IJMR_920_15
145. Kobie JJ, Zheng B, Bryk P, Barnes M, Ritchlin CT, Tabechian DA, et al. Decreased Influenza-Specific B Cell Responses in Rheumatoid Arthritis Patients Treated With Anti-Tumor Necrosis Factor. *Arthritis Res Ther* (2011) 13(6):R209. doi: 10.1186/ar3542
146. Kapetanovic MC, Kristensen LE, Saxne T, Aktas T, Morner A, Geborek P. Impact of Anti-Rheumatic Treatment on Immunogenicity of Pandemic H1N1 Influenza Vaccine in Patients With Arthritis. *Arthritis Res Ther* (2014) 16(1):R2. doi: 10.1186/ar4427
147. Kogure T, Harada N, Tatsumi T, Fujinaga H. Investigation of Clinical Characteristics as Predictive Factors for the Humoral Immune Response to the Influenza Vaccine in Patients With Rheumatoid Arthritis. *Clin Rheumatol* (2014) 33(3):323–8. doi: 10.1007/s10067-013-2483-0
148. Hua C, Barnetche T, Combe B, Morel J. Effect of Methotrexate, Anti-Tumor Necrosis Factor Alpha, and Rituximab on the Immune Response to Influenza and Pneumococcal Vaccines in Patients With Rheumatoid Arthritis: A Systematic Review and Meta-Analysis. *Arthritis Care Res (Hoboken)* (2014) 66(7):1016–26. doi: 10.1002/acr.22246
149. Park JK, Lee MA, Lee EY, Song YW, Choi Y, Winthrop KL, et al. Effect of Methotrexate Discontinuation on Efficacy of Seasonal Influenza Vaccination in Patients With Rheumatoid Arthritis: A Randomised Clinical Trial. *Ann Rheum Dis* (2017) 76(9):1559–65. doi: 10.1136/annrheumdis-2017-211128
150. Park JK, Lee YJ, Shin K, Ha YJ, Lee EY, Song YW, et al. Impact of Temporary Methotrexate Discontinuation for 2 Weeks on Immunogenicity of Seasonal Influenza Vaccination in Patients With Rheumatoid Arthritis: A Randomised Clinical Trial. *Ann Rheum Dis* (2018) 77(6):898–904. doi: 10.1136/annrheumdis-2018-213222
151. Kapetanovic MC, Nagel J, Nordstrom I, Saxne T, Geborek P, Rudin A. Methotrexate Reduces Vaccine-Specific Immunoglobulin Levels But Not Numbers of Circulating Antibody-Producing B Cells in Rheumatoid Arthritis After Vaccination With a Conjugate Pneumococcal Vaccine. *Vaccine* (2017) 35(6):903–8. doi: 10.1016/j.vaccine.2016.12.068
152. Migita K, Akeda Y, Akazawa M, Tohma S, Hirano F, Ideguchi H, et al. Pneumococcal Polysaccharide Vaccination in Rheumatoid Arthritis Patients Receiving Tacrolimus. *Arthritis Res Ther* (2015) 17:149. doi: 10.1186/s13075-015-0662-x
153. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc Receptors Modulate *In Vivo* Cytotoxicity Against Tumor Targets. *Nat Med* (2000) 6 (4):443–6. doi: 10.1038/74704
154. Arad U, Tzadok S, Amir S, Mandelboim M, Mendelson E, Wigler I, et al. The Cellular Immune Response to Influenza Vaccination is Preserved in Rheumatoid Arthritis Patients Treated With Rituximab. *Vaccine* (2011) 29 (8):1643–8. doi: 10.1016/j.vaccine.2010.12.072
155. Cox RJ, Brokstad KA. Not Just Antibodies: B Cells and T Cells Mediate Immunity to COVID-19. *Nat Rev Immunol* (2020) 20(10):581–2. doi: 10.1038/s41577-020-00436-4
156. Le Bert N, Tan AT, Kunasegaran K, Tham CYL, Hafezi M, Chia A, et al. SARS-CoV-2-Specific T Cell Immunity in Cases of COVID-19 and SARS, and Uninfected Controls. *Nature* (2020) 584(7821):457–62. doi: 10.1038/s41586-020-2550-z
157. Sekine T, Perez-Potti A, Rivera-Ballesteros O, Stralin K, Gorin JB, Olsson A, et al. Robust T Cell Immunity in Convalescent Individuals With Asymptomatic or Mild COVID-19. *Cell* (2020) 183(1):158–168 e14. doi: 10.1016/j.cell.2020.08.017
158. Baker D, Roberts CAK, Pryce G, Kang AS, Marta M, Reyes S, et al. COVID-19 Vaccine-Readiness for Anti-CD20-Depleting Therapy in Autoimmune Diseases. *Clin Exp Immunol* (2020) 202(2):149–61. doi: 10.1111/cei.13495
159. Robbiani DF, Gaebler C, Muecksch F, Lorenzi JCC, Wang Z, Cho A, et al. Convergent Antibody Responses to SARS-CoV-2 in Convalescent Individuals. *Nature* (2020) 584(7821):437–42. doi: 10.1038/s41586-020-2456-9
160. Gallais F, Velay A, Nazon C, Wendling MJ, Partisani M, Sibilia J, et al. Intrafamilial Exposure to SARS-CoV-2 Associated With Cellular Immune Response Without Seroconversion, France. *Emerg Infect Dis* (2021) 27 (1):113–21. doi: 10.3201/eid2701.203611
161. Montero-Escribano P, Matias-Guiu J, Gomez-Iglesias P, Porta-Etessam J, Pytel V, Matias-Guiu JA. Anti-CD20 and COVID-19 in Multiple Sclerosis and Related Disorders: A Case Series of 60 Patients From Madrid, Spain. *Mult Scler Relat Disord* (2020) 42:102185. doi: 10.1016/j.msard.2020.102185
162. Louapre C, Maillart E, Roux T, Pourcher V, Bussone G, Lubetzki C, et al. Patients With MS Treated With Immunosuppressive Agents: Across the COVID-19 Spectrum. *Rev Neurol (Paris)* (2020) 176(6):523–5. doi: 10.1016/j.neurol.2020.04.009
163. Bonelli MM, Mrak D, Perkmann T, Haslacher H, Aletaha D. SARS-CoV-2 Vaccination in Rituximab-Treated Patients: Evidence for Impaired Humoral But Inducible Cellular Immune Response. *Ann Rheum Dis* (2021). doi: 10.1136/annrheumdis-2021-220997
164. Kanterman J, Sade-Feldman M, Baniyash M. New Insights Into Chronic Inflammation-Induced Immunosuppression. *Semin Cancer Biol* (2012) 22 (4):307–18. doi: 10.1016/j.semcancer.2012.02.008
165. Campos LM, Silva CA, Aikawa NE, Jesus AA, Moraes JC, Miraglia J, et al. High Disease Activity: An Independent Factor for Reduced Immunogenicity of the Pandemic Influenza a Vaccine in Patients With Juvenile Systemic Lupus Erythematosus. *Arthritis Care Res (Hoboken)* (2013) 65(7):1121–7. doi: 10.1002/acr.21948
166. Ribeiro AC, Guedes LK, Moraes JC, Saad CG, Aikawa NE, Calich AL, et al. Reduced Seroprotection After Pandemic H1N1 Influenza Adjuvant-Free Vaccination in Patients With Rheumatoid Arthritis: Implications for Clinical Practice. *Ann Rheum Dis* (2011) 70(12):2144–7. doi: 10.1136/ard.2011.152983
167. Borba EF, Saad CGS, Pasoto SG, Calich ALG, Aikawa NE, Ribeiro ACM, et al. Influenza A/H1N1 Vaccination of Patients With SLE: Can Antimalarial

Drugs Restore Diminished Response Under Immunosuppressive Therapy? *Rheumatology* (2012) 51(6):1061–9. doi: 10.1093/rheumatology/ker427
168. Saad CGS, Borba EF, Aikawa NE, Silva CA, Pereira RMR, Calich AL, et al. Immunogenicity and Safety of the 2009 non-Adjuvanted Influenza A/H1N1 Vaccine in a Large Cohort of Autoimmune Rheumatic Diseases. *Ann Rheumatic Dis* (2011) 70(6):1068–73. doi: 10.1136/ard.2011.150250
169. Elkayam O, Amir S, Mendelson E, Schwaber M, Grotto I, Wollman J, et al. Efficacy and Safety of Vaccination Against Pandemic 2009 Influenza A (H1N1) Virus Among Patients With Rheumatic Diseases. *Arthritis Care Res* (2011) 63(7):1062–7. doi: 10.1002/acr.20465
170. Adler S, Krivine A, Weix J, Rozenberg F, Launay O, Huesler J, et al. Protective Effect of A/H1N1 Vaccination in Immune-Mediated Disease–a Prospectively Controlled Vaccination Study. *Rheumatol (Oxford)* (2012) 51 (4):695–700. doi: 10.1093/rheumatology/ker389
171. Milanetti F, Germano V, Nisini R, Donatelli I, Di Martino A, Facchini M, et al. Safety and Immunogenicity of Co-Administered MF59-Adjuvanted 2009 Pandemic and Plain 2009-10 Seasonal Influenza Vaccines in Rheumatoid Arthritis Patients on Biologicals. *Clin Exp Immunol* (2014) 177(1):287–94. doi: 10.1111/cei.12292
172. Migita K, Akeda Y, Akazawa M, Tohma S, Hirano F, Ideguchi H, et al. Opsonic and Antibody Responses to Pneumococcal Polysaccharide in Rheumatoid Arthritis Patients Receiving Golimumab Plus Methotrexate. *Med (Baltimore)* (2015) 94(52):e2184. doi: 10.1097/MD.0000000000002184
173. Mori S, Ueki Y, Akeda Y, Hirakata N, Oribe M, Shiohira Y, et al. Pneumococcal Polysaccharide Vaccination in Rheumatoid Arthritis Patients Receiving Tocilizumab Therapy. *Ann Rheum Dis* (2013) 72 (8):1362–6. doi: 10.1136/annrheumdis-2012-202658
174. Bingham CO3rd, Rizzo W, Kivitz A, Hassanali A, Upmanyu R, Klearman M. Humoral Immune Response to Vaccines in Patients With Rheumatoid Arthritis Treated With Tocilizumab: Results of a Randomised Controlled Trial (VISARA). *Ann Rheum Dis* (2015) 74(5):818–22. doi: 10.1136/annrheumdis-2013-204427
175. Felten R, Kawka L, Dubois M, Ugarte-Gil MF, Fuentes-Silva Y, Piga M, et al. Tolerance of COVID-19 Vaccination in Patients With Systemic Lupus Erythematosus: The International VACOLUP Study. *Lancet Rheumatol* (2021) 3(9):e613–5. doi: 10.1016/S2665-9913(21)00221-6

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# Identification of a Novel Serological Marker in Seronegative Rheumatoid Arthritis Using the Peptide Library Approach

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Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation mainly affecting the joints leading to cartilage and bone destruction. The definition of seropositive or seronegative RA is based on the presence or absence of rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPAs). Other autoantibodies have been identified in the last decade such as antibodies directed against carbamylated antigens, peptidyl-arginine deiminase type 4 and v-Raf murine sarcoma viral oncogene homologue B. In order to identify relevant autoantigens, we screened a random peptide library (RPL) with pooled IgGs obtained from 50 patients with seronegative RA. Patients' sera were then used in an ELISA test to identify the most frequently recognized peptide among those obtained by screening the RPL. Sera from age- and sex-matched healthy subjects were used as controls. We identified a specific peptide (RA-peptide) recognized by RA patients' sera, but not by healthy subjects or by patients with other immune-mediated diseases. The majority of sera from seronegative and seropositive RA patients (73.8% and 63.6% respectively) contained IgG antibodies directed against the RA-peptide. Interestingly, this peptide shares homology with some self-antigens, such as Protein-tyrosine kinase 2 beta, B cell scaffold protein, Liprin-alfa1 and Cytotoxic T lymphocyte protein 4. Affinity purified anti-RA-peptide antibodies were able to cross react with these autoantigens. In conclusion, we identified a peptide that is recognized by seropositive and, most importantly, by seronegative RA patients' sera, but not by healthy subjects, conferring to this epitope a high degree of specificity. This peptide shares also homology with other autoantigens which can be recognized by autoantibodies present in seronegative RA sera. These newly identified autoantibodies, although present also in a percentage of seropositive RA patients, may be considered as novel serum biomarkers for seronegative RA, which lacks the presence of RF and/or ACPAs.

**Keywords: peptide library approach, RA-peptide, seronegative rheumatoid arthritis, autoantibodies, autoantigen targets**

## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation and synovial hyperplasia that lead to progressive and destructive arthritis. It is estimated that RA has a prevalence of 1% of world population (1) with a higher frequency in women and elderly people (2, 3).

Early diagnosis and treatment are essential in order to prevent disease progression and irreversible joint damage (4).

The diagnosis of RA is based on clinical features, radiographic images and serological markers, such as rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPAs) (3). The presence of such autoantibodies in the serum of RA patients allows to distinguish seropositive RA from seronegative RA patients. It is estimated that one third of RA patients have no ACPAs.

These two forms of RA are characterized by different genetic and environmental risk factors, differences in preclinical and early phases, and different synovial fluid cytokine profile (2).

Seronegative RA patients have, indeed, a more active disease in the initial phase and have a slower response to treatment compared with seropositive RA patients (5). However, no differences in the treatment of the two subsets of the disease are provided by the current guidelines (6).

ACPAs are pathogenetic autoantibodies that are highly specific for RA (7). Anti-cyclic citrullinated peptide antibodies (anti-CCP2) are the most commonly ACPAs measured in patients' serum. These antibodies are useful for distinguishing RA from other rheumatic diseases and they appear to be highly predictive of future RA development in either healthy subjects or in patients with undifferentiated arthritis (8, 9).

The presence of ACPAs is also related to the development of bone erosion (10, 11). Citrullinated peptides may be potential autoantigens and may be recognized by T cells that are activated and either contribute to the pro-inflammatory environment that may cause joint damage or trigger B cells to produce autoantibodies (12). Both ACPAs and inflammatory cytokines can be found even 2-4 years before the clinical outset of the disease (13, 14).

The use of the combined detection of RF and ACPAs makes the diagnosis of seropositive RA more accurate (15). On the contrary, in seronegative RA patients there is not a specific marker currently available; therefore, much effort should be made to identify markers present in this form of the disease.

Indeed, other autoantibodies associated with RA have also been identified, such as anti-carbamylated (anti-CarP), anti-v raf murine sarcoma viral oncogene homologue B1 (anti-BRAF) and anti-human peptidyl-arginine deiminase type 4 (anti-PAD4) antibodies (16). Anti-CarP antibodies are present in serum and synovial fluid (SF) of both seronegative and seropositive RA patients and are correlated with more severe disease and radiographic progression (17). In patients with arthralgias, the presence of anti-CarP antibodies predicts the development of RA independently of anti-CCP2 antibodies (18–21). Moreover, anti-CarP antibodies can be found in the sera of healthy first-degree relatives (HFDRs) of RA patients and their prevalence is significantly higher than in normal healthy subjects (22).

Anti-BRAF antibodies are detected in RA patients' serum and are able to activate BRAF kinase, which is the first step in mitogen-activated protein kinase (MAP kinase) activation, leading to pro-inflammatory cytokine production and joint inflammation (23).

Anti-PAD4 antibodies are present in 36-42% of RA patients and appear to be specific markers for RA, as well as associated with more severe disease (24).

Recently, Colasanti et al., combining both proteomic and immunological approaches, were able to identify Hcy-A1AT (Homocysteinylated alpha 1 antitrypsin) as a new antigenic target in seronegative RA (25).

However, while there is an effective combination of serological markers in the diagnosis of seropositive RA, a specific marker is not currently available for the diagnosis of seronegative arthritis. Also, for this reason, diagnosis of seronegative RA is more difficult and may be delayed.

The aim of this work was to identify possible markers of seronegative RA. To this aim, we have used a random peptide library approach, that we have previously successfully applied to identify novel autoantigen targets in other autoimmune diseases (26–28).

## MATERIAL AND METHODS

### Study Population

In this study, 50 patients affected by seronegative RA (13 males and 37 females, mean age: 65.7 ± 10.83 years) and 25 by seropositive RA (7 males and 18 females, mean age: 55.88 ± 12.15 years) were enrolled by the Rheumatology Unit of the Perugia University Hospital. A cohort of 30 seronegative RA (8 males and 22 females, mean age: 62.57 ± 18.26) and 30 seropositive RA (5 males and 25 females, mean age: 58.9 ± 11.45 years) was enrolled by the Unit of Autoimmune Diseases at the University Hospital of Verona, as validation group.

The diagnosis of RA was performed according to the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (3). All the subjects were screened for both RF and ACPAs, which were absent in seronegative RA patients.

**Abbreviations:** Abs, antibodies; ACPAs, anti-citrullinated peptide antibodies; AUC, area under the curve; BANK-1, B cell scaffold protein with ANKyrin repeats; BSA, bovine serum albumin; CI, confidence interval; CRP, C reactive protein; CTLA-4, cytotoxic T lymphocyte protein 4; DAS 28, disease activity score 28; DAUDI, Burkitt lymphoma cell; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; HD, healthy donors; HFLS, normal human fibroblast-like synoviocytes; HFLS-RA, human fibroblast-like synoviocytes: rheumatoid arthritis; HL-60, human promyelocytic leukemia cell; IgG, immunoglobulin G; IP, immunoprecipitation; LIPRIN-1/LIP-1, liprin-alpha 1; mAbs, monoclonal antibodies; NHDF, normal human dermal fibroblast; O.D., optical density; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PsA, psoriatic arthritis; PYK2/FADK2, protein-tyrosine kinase 2 beta/focal adhesion kinase; RA, rheumatoid arthritis; RF, rheumatoid factor; ROC, receiver operating characteristic; RPLs, random peptide libraries; SA, spondyloarthritis; S.D., standard deviation; SF, synovial fluid; SSc, systemic sclerosis; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SJ, swollen joint count; TBS-T, tris-buffered saline containing 0.1% Tween 20; TJ, tender joint count; WB, western blot.

A further group of 30 patients affected by other immune-mediated diseases, such as systemic sclerosis (SSc) (29), spondyloarthritis (SA) (30) and psoriatic arthritis (PsA) (31) was also included in the study. Finally, 25 age- and sex-matched healthy donors (HD) were included as a control group.

Blood was collected and centrifuged at 3000xg for 10 min. All sera were aliquoted and frozen at -20°C until used.

Written informed consent was obtained from adult patients and healthy donors. The study was approved by the local Ethical Committee of the Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy (protocol number: 1538, version number 3) and by Comitato Universitario di Bioetica of Perugia, Italy (identification code: 2013-012 approved 4-4-2013).

## Peptide Library

The screening procedure of the FliTrx random dodecamer peptides library (FliTrx Panning Kit, Invitrogen, Carlsbad, CA, USA) has been described elsewhere by our group (26–28).

Briefly, a random peptides library was screened with pooled immunoglobulins (IgGs) obtained from the sera of 50 patients with seronegative RA, according to the manufacturer's instructions. To remove the bacteria expressing peptides not related to the disease, a 'pre-panning' step with pooled IgGs obtained from 25 healthy donors was carried out,thereby allowing to discard non-specific peptides without the loss of disease-related data. Then we proceed with five sequential cycles of bio-panning. The enriched library was grown, and single colonies were selected, expanded and incubated with tryptophan to induce the expression of the fusion peptides. Bacteria were then lysed in sample buffer and tested by western blot procedures with the pooled IgG fraction from seronegative RA to identify positive clones. DNA was then extracted from these clones and sequenced. Peptide sequence was deduced from DNA sequence. A set of 17 out of 33 peptides was synthesized and used in an ELISA assay to test individual sera. The remaining 16 peptides were excluded due to a low amount of plasmid DNA isolation or because the DNA sequence did not yield a consensus peptide (e.g. DNA sequence contained stop codons).

## Peptide Synthesis

All the five synthetic peptides, including RA-peptide (LAVLANLASRTL), BANK-1 peptide (DMILANLSIKKK), PYK2/FADK2 peptide (AKVLANLAHPPA), LIPRIN-1 peptide (ASVLANVAQAFE) and CTLA-4 peptide (HKAQL NLATRTW) were purchased from TIBMolbiol (Genoa, Italy).

## Enzyme-Linked Immunosorbent Assays (ELISA)

The ELISA test for antibody binding to the synthetic peptides has already been described elsewhere (26–28, 32), and was carried out with minor modifications (see **Supplementary Material and Methods**).

All serum samples were diluted 1:100 in diluting buffer.

The absorbance value (O.D.) for each sample was calculated subtracting the O.D. plates with serum and no peptide to the O.D. plates with serum and peptide. O.D. values higher than the mean plus 3 standard deviations (S.D.) of O.D. value measured in control group were considered as positive (cutoff 0.079), as previously reported (32). The experiment has been carried out in duplicate (see **Supplementary Material and Methods**).

## Binding of Affinity Purified Abs to the Peptides

Affinity purified peptide Abs were obtained from sera of 15 seronegative RA patients (see **Supplementary Material and Methods**).

Different dilutions ranging from 20 μg/mL to 1.25 μg/mL of affinity purified Abs were tested in a plate coated with RA-peptide. As internal negative control, affinity purified anti-irrelevant-peptide Abs were used at the same dilutions.

The same procedure was also used to assess the direct binding of the other purified Abs (anti-Pyk/Fadk2, anti-Bank-1, anti-Liprin-1, anti-CTLA-4 and anti-irrelevant peptide) to their specific peptides (using plates coated with Pyk/Fadk2, Bank-1, Liprin-1 and CTLA-4, respectively) and to test the ability of the purified Abs to cross-recognize the other peptides, at the same dilution conditions.

For inhibition test, we pre-incubated the purified anti-RA-peptide Abs (20 μg/mL) with increasing concentration of competitors (synthetic RA-peptide or irrelevant peptide: 12.5, 25, 50, 100, 200 μg/mL) for 1 hour at 37 °C. The mixtures were then transferred to the RA-peptide coated plate. The assay was performed as the direct binding test above described. Results were expressed as inhibition percentage.

## Cell Culture

Normal Human Fibroblast-Like Synoviocytes (HFLS), Human Fibroblast-Like Synoviocytes: Rheumatoid Arthritis (HFLS-RA) and human synoviocytes complete growth medium were purchased from Cell applications (San Diego, CA, USA). Normal human dermal fibroblast (NHDF) and their growth media were purchased from Promocell Bioscience Alive (Heidelberg, Germany). Cells were cultured in standard conditions at 37°C in 5%$CO_2$ and used between passages 3 and 6.

Human promyelocytic leukemia cell (HL-60) and Burkitt lymphoma cell (DAUDI) lines were purchased from ATCC (Manassas, VA, USA) and routinely maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2 mM stable glutamine, 100U/ml penicillin and 100U/ml streptomycin (Sigma) in a humidified atmosphere with 5% $CO_2$ at 37°C.

## Isolation of B and T Lymphocytes From Healthy Subjects

Normal B or T lymphocytes were isolated from PBMCs after blood separation on Ficoll HyPaque Plus (GE Healthcare, Little Chalfont, UK) and purification by negative selection with EasySep™ Human B Cell isolation Kit or EasySep™ Human T

Cell isolation Kit (Stemcell Technologies, Vancouver, BC, Canada). Purity of B or T lymphocyte preparations were evaluated by flow cytometry with anti-CD19 mAb and with anti-CD3 mAb (BD Biosciences, San Jose, CA, USA).

## Immunoprecipitation

B, T and PBMCs or Synoviocytes (HFLS and HFLS-RA) were lysed in cold lysis buffer (0.5% Nonidet P-40, 10 mM TRIS [pH 7.4], 0.15 M sodium chloride, 5 mM magnesium chloride) and protease inhibitors (Sigma, Saint Louis, MO). Protein concentration of cell lysates was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). All cell lysates were immunoprecipitated with anti-RA specific peptide antibodies coupled to protein A magnetic beads using SureBeads™ Protein A Magnetic Beads (BioRad Laboratories, Inc, Hercules, CA, United States) following manufacturer's instructions for standard immunoprecipitation protocol. Purified target proteins (20 μg) were run in a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Amersham Bioscience, Piscataway, New Jersey, United States). Immunoblots were performed using the following primary antibodies: anti-RA specific peptide antibodies, mouse polyclonal antibodies directed against PYK2 (Novus Biologicals, Littleton, CO, USA), rabbit polyclonal antibodies directed against Liprin (Novus Biologicals Littleton, CO, USA) and rabbit polyclonal antibodies directed against Bank (Abgent, San Diego, CA, USA) followed by their specific peroxidase-linked secondary antibodies. Blots were then developed using chemiluminescent substrate (Amersham; GE Healthcare Life Sciences, Milan, Italy) and images were acquired by Image Quant Las 4000mini (GE Healthcare Life Sciences, Milan, Italy).

## Statistical Analysis

All calculations were performed using the IBM SPSS 23.0 (IBM Inc., Armonk, NY) or GraphPad Prism 5 (GraphPad Software, San Diego, CA) statistical packages. Continuous variables are shown as mean values ± standard deviations. With the aim to compare O.D. values of patients with seronegative RA with those of i) patients with seropositive RA, ii) patients with immune-mediated diseases, or iii) healthy donors, a two-tailed non-parametric Mann-Whitney test was used. Analyses of receiver-operating characteristic (ROC) were carried out and ROC curves were plotted with area under the curve (AUC) as an indicator of the diagnostic value. Within the group of seronegative RA patients, more homogeneous and thus allowing the use of parametric tests, continuous variables having a skewed distribution (e.g. O.D., ESR, CRP) were ln-transformed and the statistical analyses were performed on ln-transformed values. However, for sake of clarity, also their results have been reported as mean values ± standard deviations. Differences of continuous variables between sample's subgroups were analyzed by t-test, while correlations were assessed by Pearson's analysis. Categorical variables are reported as relative proportions and were analyzed by chi-square test, with chi-square for linear trend when appropriate. A P value < 0.05 was considered as statistically significant.

# RESULTS

## Clinical and Laboratory Features and Treatment of RA Patients

The main clinical features, biochemical characteristics and treatment of all seronegative RA patients included in the study are summarized in **Table 1**. **Supplementary Table 1** shows the data related to patients with seropositive RA, used as control. The RA patients were predominantly female and the gender percentage in the two seronegative RA groups were equally balanced.

## Use of Random Peptide Library and ELISA Test

A dodecamer random peptide library was used to identify possible relevant autoantigens in seronegative RA. An IgG pool derived from the first cohort of 50 patients with seronegative RA was used to screen the peptide library, while a pool of IgGs from 25 healthy donors was used for the pre-screening step (see material and methods). Finally, a set of 17 putative peptides was used to evaluate the binding of individual patient's sera

By using a direct ELISA test, we identified a peptide (RA-peptide: LAVLANLASRTL) that was recognized by individual serum IgG of 70% of patients with seronegative RA (35 out of 50; 9 men and 26 women) (**Figure 1A**). Such reactivity was not detected by the individual sera of 25 healthy subjects, using a O.D. cutoff of 0.079 (mean ± 3 s.d).

We also analyzed the sera of 25 seropositive RA patients and found that 64% of them recognized the RA-peptide (16 out of 25; 6 men and 10 women) (**Figure 1A**). Statistically significant differences were found in the binding to RA-peptide between the seronegative RA patients' and healthy donors' sera (absorbance mean ± s.d.: 0.1676 ± 0.1308 vs 0.02972 ± 0.01638, $p<0.0001$) or between the seropositive RA and healthy donors (absorbance mean ± s.d: 0.1532 ± 0.1264 vs 0.02972 ± 0.01638, $p<0.0001$), but not between seronegative and seropositive patients ($p>0.05$).

The potential diagnostic value of anti-RA-peptide antibodies in either seronegative or seropositive RA patients was assessed by a receiver operating characteristic (ROC) curve. The area below the ROC curve (AUC) was 0.9244 [95% confidence interval (CI) = 0.8658 to 0.9830, $p<0.0001$] between anti-peptide Abs of seronegative RA patients and healthy donors (**Figure 1B**) and 0.8840 [95% confidence interval (CI) =0.7797 to 0.9883, $p<0.0001$] between anti-peptide Abs of seropositive RA patients and healthy donors (**Supplementary Figure 1A**). No significant difference was found in ROC curve between the seronegative and seropositive RA ($p=0.5514$) (**Supplementary Figure 1B**).

In order to validate the binding specificity of RA-peptide, a second study sample of seronegative RA patients (n=30) was analyzed, as well as a group of patients with other immune-mediated diseases,like SA, PsA, and SSc (n=30).

In the validation analysis 83.3% of subjects with seronegative RA (25 out of 30; 7 men and 18 women) and 66.7% of patients with seropositive RA (20 out of 30; 5 men and 15 women) had serum IgG antibodies able to recognize the RA-peptide (**Figure 1C**).

**TABLE 1** | Clinical and laboratory features and treatment of seronegative rheumatoid arthritis (RA) patients from the Perugia (Pg) and Verona (Vr) cohorts.

| | RA (Pg) (n=50) | RA (Vr) (n=30) |
|---|---|---|
| *Clinical characteristics* | | |
| **Age (years)** | 65.7 ± 10.83 | 62.57 ± 18.26 |
| **Gender: female/male** | 37/13 | 22/8 |
| **Age at diagnosis (years)** | 57.32 ± 11.11 | 55.9 ± 17.45 |
| **Disease duration (years)** | 8.38 ± 5.51 | 6.67 ± 5.46 |
| **Erosion** | 5/44 | 6/19 |
| **presence/absence, n.** | (n= 49) | (n= 25) |
| **Tender joint** | 1.69 ± 2.52 | 1.6 ± 2.42 |
| **count (0-10)** | (n= 49) | (n= 25) |
| **Swollen joint** | 0.71 ± 1.84 | 1.56 ± 2.43 |
| **count (0-10)** | (n= 49) | (n= 25) |
| **DAS28-ESR** | 2.59 ± 1.10 | 2.76 ± 1.30 |
| | (n=50) | (n= 30) |
| *Therapy* | **RA** | **RA** |
| | **(n=50)** | **(n=30)** |
| **Methotrexate (%)** | 20 (40%) | 12 (40%) |
| **Hydroxychloroquine (%)** | 19 (38%) | 9 (30%) |
| **Leflunomide (%)** | 9 (18%) | 5 (16.7%) |
| **Biological/others (%)** | 2 (4%) | 2 (6.7%) |
| **n° treatment (%)** | 0 (0%) | 2 (6.7%) |
| **Prednisolon/prednisone (%)** | 16 (32%) | 14 (56%) |
| | (n=50) | (n=25) |
| *Inflammatory markers* | **RA** | **RA** |
| **ESR (mm/h)** | 24.66 ± 19.05 | 21.36 ± 14 |
| | (n=50) | (n=28) |
| **CRP (mg/dl)** | 0.70 ± 1.01 | 0.67 ± 0.92 |
| | (n= 50) | (n=28) |

*RA, rheumatoid arthritis; DAS 28, Disease activity score 28; ESR, Erythrocyte sedimentation rate; CRP, C reactive protein. Values are shown as mean ± SD and as percentage (%).*

Five out of 30 (16.7%) patients with SSc, 12 out of 30 (40%) with SA and 10 out of 30 (33.3%) with PsA showed a low reactivity to RA-peptide. Only a SA patient had a strong value of absorbance. There were differences in the absorbance between the seronegative RA and SSc patients (0.1710 ± 0.09651 vs 0.0543 ± 0.03228, p<0.0001) or SA patients (0.0788 ± 0.05045, p<0.0001) or PsA patients (0.05997 ± 0.03771, p<0.0001), but not between seronegative and seropositive patients (0.1710 ± 0.09651 vs 0.1380 ± 0.09002, p>0.05) (**Figure 1C**). Differences were also observed by comparing seropositive RA patients with SSc/SA/PsA patients (p=0.0001, p=0.0089, p=0.0004, respectively).

In the ROC curve analysis, AUC was 0.9044 [95% confidence interval (CI) = 0.8239 to 0.9849, p<0.0001] when anti-peptide Abs of seronegative RA and SSc subjects were compared (**Figure 1D**); 0.8206 [95% confidence interval (CI) = 0.7102 to 0.9309, p<0.0001] between RA and SA (**Figure 1E**); 0.8850 [95% confidence interval (CI) =0.7984 to 0.9716, p<0.0001] between RA and PsA (**Figure 1F**).

Similarly to the first cohort analysis, ROC curves between seropositive RA and seronegative RA were not significant (**Supplementary Figure 1C**), while the comparison of seropositive RA with SSc/SA/PsA showed significant differences in AUC (**Supplementary Figures 1D–F**).

Notably, many patients with SSc, SA and PsA showed a slight positivity of anti-RA-peptide Abs with a cutoff of 0.079. Therefore, on the basis of ROC curve analysis, we decided to increase the cutoff value to an arbitrary threshold of 0.090. Such threshold allows to preserve the sensibility of the biomarker (only 2 subjects became negative at testing with the new threshold in seropositive (n=1) and seronegative (n=1) RA cohorts), while the specificity was improved [3 out of 30 (10%) patients with SSc, 5 out of 30 (16.7%) with SA and 5 out of 30 (16.7%) with PsA showed a reactivity to RA-peptide]. Therefore, 14 out of 27 previously positive patients with immune-mediated disease became negative using the new O.D. threshold level.

Then, we evaluated the diagnostic power of our test by assessing the sensitivity, specificity, positive predictive power (PPV), negative predictive power (NPV) and accuracy, by using the two different cutoffs (0.079 or 0.090) and by comparing to healthy donors both seronegative and seropositive RA patients (**Tables 2A, B** respectively).

From the data obtained by this analysis, we consider the arbitrary cutoff as a valid limit. Therefore, by pooling both cohorts of seronegative RA patients, we observed that 59 out of 80 (73.8%) sera were characterized by the presence of IgG antibodies against the RA-peptide, which were present in 35 out of 55 (63.6%) seropositive RA patients.

## Associations of O.D. With Clinical and Laboratory Parameters and O.D. Quartile Distribution of RA Patients Taking or Not Corticosteroid Therapy

Within the group of seronegative RA patients there was no difference in O.D. values between females and males (0.182 ± 0.131 vs 0.131 ± 0.062, p=0.465) or between subjects with or without joint erosions (0.253 ± 0.183 vs 0.155 ± 0.099, p=0.151).

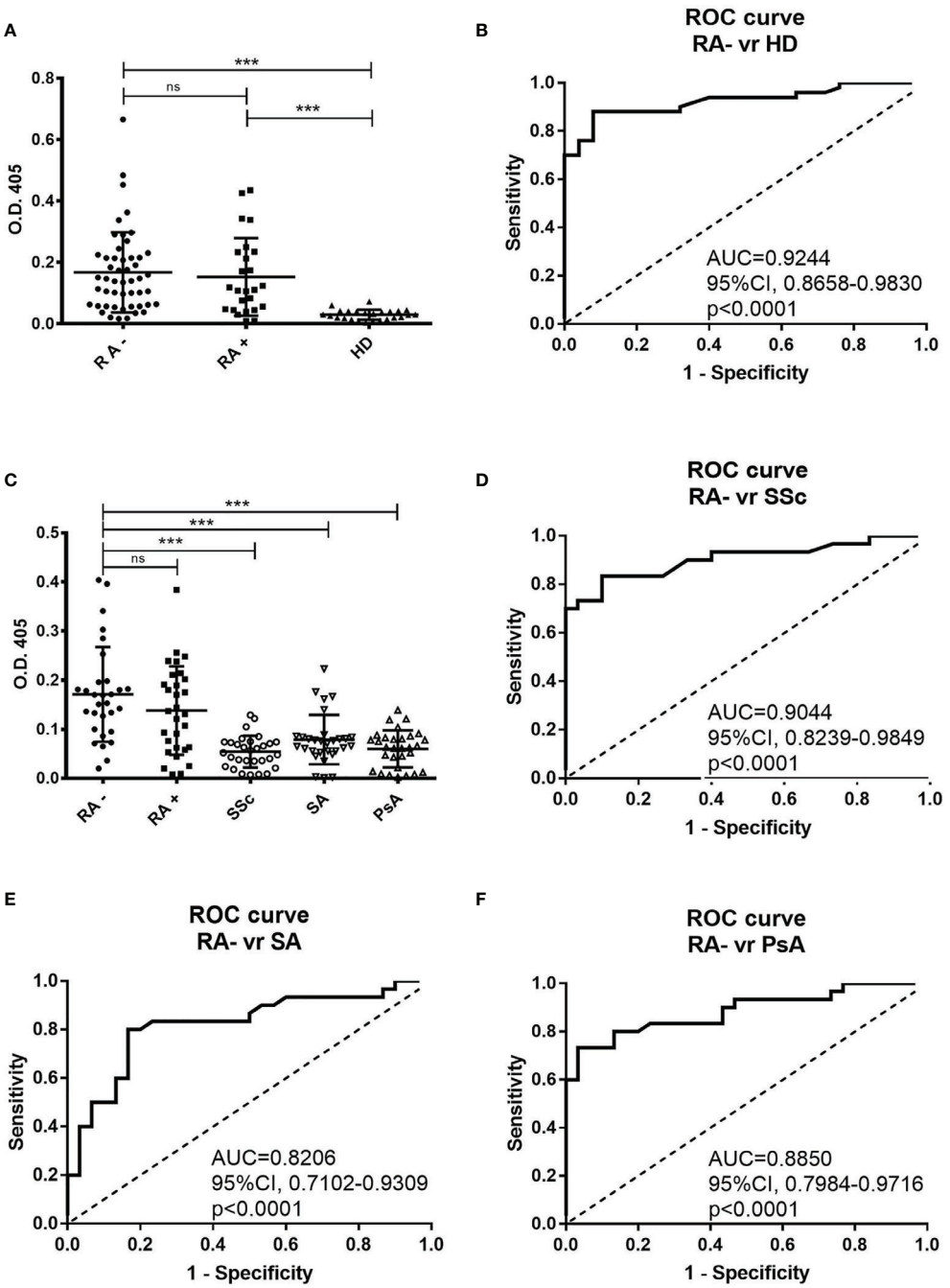


**FIGURE 1** | Direct binding of individual patients' and healthy donors' sera to RA-peptide obtained from peptide library screening. **(A)** RA-peptide is recognized by serum IgG antibodies of seronegative RA patients (●), by serum IgG of seropositive RA patients (■), but not by serum IgG of healthy donors (▲). The results are expressed as absorbance (O.D.) at 405 nm. Results shown are the mean values of three independent experiments. Bar line represents the mean absorbance value and standard deviation for each group. The differences among the different groups of patients and the healthy donors were determined by non-parametric Mann-Whitney test. Statistical significance is defined as following: ***P < 0,0001, ns, not significant. **(B)** Receiver operating characteristic (ROC) analysis of O.D. value of serum from seronegative RA patients and healthy donors. AUC= area under the curve. **(C)** Binding of RA-peptide by serum IgG antibodies of the validation cohort of seronegative RA (●), seropositive RA (■), and of patients with SSc (○), with SA (▽) and with PsA (△). The results are expressed as absorbance (O.D.) at 405 nm. Lines indicate the mean absorbance value and standard deviation for each group. The non-parametric Mann-Whitney test was used to compare the different groups. ***P < 0,0001, ns, not significant. **(D–F)** Receiver operating characteristic (ROC) analysis of O.D. value of serum from seronegative RA patients and SSc or SA or PsA patients, respectively. RA, Rheumatoid arthritis; SSc, Systemic sclerosis; SA, Spondyloarthritis; PsA, Psoriatic arthritis.

**TABLE 2** | Association of high level of anti-RA peptide Abs with diagnosis of RA.

| **A** | | **cut-off = 0.079** | | | | **cut-off = 0.090** | | |
|---|---|---|---|---|---|---|---|---|
| | | **Positive** | **Negative** | **total** | | **Positive** | **Negative** | **total** |
| **RA seronegative patients** (%) | | **60** (75%) | **20** (25%) | **80** | | **59** (73,8%) | **21** (26,3%) | **80** |
| **Healthy donors** (%) | | **0** (0%) | **25** (100%) | **25** | | **0** (0%) | **25** (100%) | **25** |
| | **total** | **60** | **45** | **105** | **total** | **59** | **46** | **105** |
| **sensitivity** | **75%** | 95% CI: 0.6406 to 0.8401 | | | **73.8%** | 95% CI: 0.6271 to 0.8296 | | |
| **specificity** | **100%** | 95% CI: 0.8628 to 1.000 | | | **100%** | 95% CI: 0.8628 to 1.000 | | |
| **PPV** | **100%** | 95% CI: 0.9404 to 1.000 | | | **100%** | 95% CI: 0.9394 to 1.000 | | |
| **NPV** | **55.6%** | 95% CI: 0.4000 to 0.7036 | | | **54.3%** | 95% CI: 0.3901 to 0.6910 | | |
| **accuracy** | **81%** | | | | **80%** | | | |
| **B** | | **cut-off = 0.079** | | | | **cut-off = 0.090** | | |
| | | **Positive** | **Negative** | **total** | | **Positive** | **Negative** | **total** |
| **RA seropositive patients** (%) | | **36** (65.5%) | **19** (34.5%) | **55** | | **35** (63.6%) | **20** (36.4%) | **55** |
| **Healthy donors** (%) | | **0** (0%) | **25** (100%) | **25** | | **0** (0%) | **25** (100%) | **25** |
| | **total** | **36** | **44** | **80** | **total** | **35** | **45** | **80** |
| **sensitivity** | **65.5%** | 95% CI: 0.5142 to 0.7776 | | | **63.6%** | 95% CI: 0.4956 to 0.7619 | | |
| **specificity** | **100%** | 95% CI: 0.8628 to 1.000 | | | **100%** | 95% CI: 0.8628 to 1.000 | | |
| **PPV** | **100%** | 95% CI: 0.9026 to 1.000 | | | **100%** | 95% CI: 0.9000 to 1.000 | | |
| **NPV** | **56.8%** | 95% CI: 0.4103 to 0.7165 | | | **55.6%** | 95% CI: 0.4000 to 0.7036 | | |
| **accuracy** | **76.3%** | | | | **75%** | | | |

***(A)*** *RA seronegative patients and healthy donors;* ***(B)*** *RA seropositive patients and healthy donors; diagnostic powers of the Elisa test. High levels are defined as above the threshold value of mean +/- 3 S.D. of healthy donors (HD), cut-off = 0.079; or above a hypothetical cutoff value with a more stringent threshold, cut-off = 0.090. Data are analyzed as categorical variable with GraphPad Prism 5. Fisher's exact test, two-sided; p value < 0.0001. Abs, antibodies; PPV, positive predictive value; NPV, negative predictive value.*

There was no correlation between O.D. levels and age at enrollment, age at diagnosis, disease duration, ESR, CRP, DAS28, tender and swollen joint counts (**Supplementary Table 2A**). There was no difference in O.D. values among patients receiving different disease modifying anti-rheumatic drugs (**Supplementary Table 2B**), while treatment with corticosteroids was associated with lower O.D. levels (0.132 ± 0.095 vs 0.193 ± 0.127, p=0.028 by t-test).

Finally, we stratified the whole study sample according the O.D. quartile distribution. Considering the potential influence of corticosteroid therapy, these analyses were performed in subgroups of subjects taking or not corticosteroids (**Figure 2**). As expected, healthy donors were strictly prevalent in the lowest quartile while seropositive RA (RA+) or seronegative RA (RA-) patients were more frequent in the higher quartiles in both the subgroups. A progressive increase of prevalence of RA+/RA- patients by increasing O.D. values, from the lowest to the highest quartile, was particularly evident among subjects not taking corticosteroids (**Figure 2A**), while the trend – although significant – appeared milder in those taking corticosteroids (**Figure 2B**).

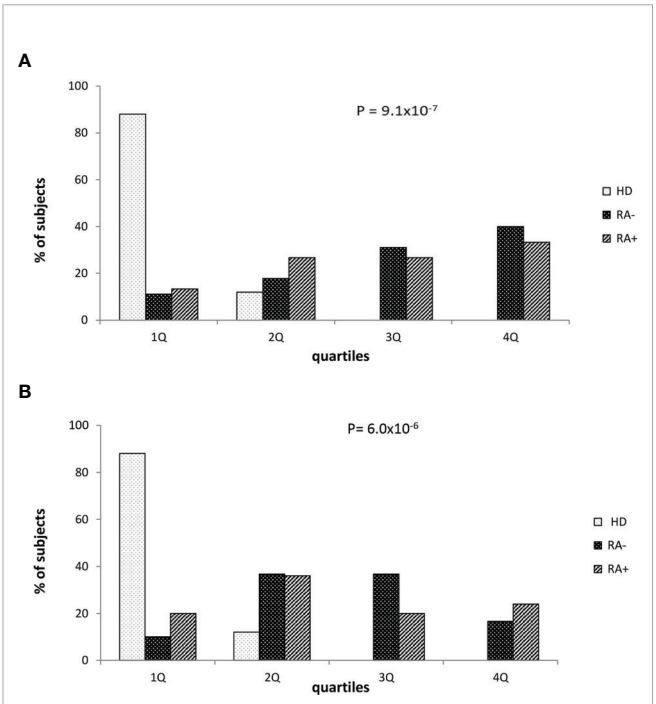


**FIGURE 2** | Healthy donors and patients with seronegative or seropositive arthritis according to the quartile distribution of absorbance levels in patients who were not **(A)** or were treated with corticosteroid **(B)**. RA seronegative (RA-) patients, RA seropositive (RA+) patients and healthy donors (HD). Quartile distribution was determined on the basis of the following threshold values: Q1 < 0.045; 0.045 ≤ Q2 ≤ 0.112 or Q2 0.045-0.112; 0.113≤ Q3 ≤ 0.197 or Q3 0.113-0.197; Q4 ≥ 0.198. Statistical p value was obtained by $\chi^2$ for linear trend.

## Sequence Homology Between the Identified RA-Peptide and Human Proteins

The 12 amino acid sequence of RA-peptide obtained by the peptide library was compared with known human proteins in a protein data bank (Swiss-Prot database) using BLASTP program *via* the NCBI BLAST network service (https://blast.ncbi.nlm.nih.gov/). In the protein selection, we considered the extent of the homology by measuring the length of the homologous stretch and the number of matched amino acids (both identities and conservative substitutions). Through this approach it is possible

to identify areas of homology between the RA-peptide and known human proteins We found that the RA-peptide shares homology with different self-antigens, such as: Protein-tyrosine kinase 2 beta (PYK2/FADK2), B cell scaffold protein with ankyrin repeats (BANK-1), Liprin-alpha 1 (LIPRIN-1), Cytotoxic T-lymphocyte protein 4 (CTLA-4) (**Figure 3A**).

## Direct or Competitive Elisa Assays

Fifteen sera from seronegative RA patients were used to obtain specific IgG antibodies directed against the RA-peptide and against an irrelevant peptide. These affinity-purified anti-RA-peptide Abs were able to bind the RA-peptide in a direct ELISA test (**Figure 3B**), while the anti-irrelevant-peptide Abs did not have this ability. Furthermore, RA-peptide was able to inhibit direct binding of purified anti-RA-peptide Abs. Such inhibition was not present using the irrelevant control peptide (**Figure 3C**).

Antibodies directed against the other peptides were obtained from the same sera of 15 seronegative RA patients (see **Supplementary Material and Methods**). We investigated the ability of these affinity-purified IgG antibodies to bind to their specific peptides (RA, PYK2/FADK2, BANK-1, LIPRIN-1 and CTLA-4 peptide) and to cross-react with the other peptides in ELISA assays, using different concentrations of anti-peptide antibodies (from 20 to 1.25 μg/ml).

All the purified antibodies bind their specific peptide with high affinity and in a dose dependent manner, whereas antibodies against the irrelevant peptide do not show similar behavior (**Figure 3D**).

Anti-CTLA-4 peptide purified antibodies displayed a lower affinity binding to PYK2/FADK2, BANK-1 and LIPRIN-1 peptides, even when we used different concentrations of antibodies. Moreover, anti-CTLA-4 and anti-RA-peptide purified antibodies showed a similar binding to CTLA-4 peptide while the other Abs displayed a lower recognition of this peptide (**Figure 3D**). We supposed that these differences in affinity binding derive from the differences in the sequence homology, as shown in **Figure 3A**.

We check whether the serum samples from the validation cohort were able to cross-react with the other four peptides (arbitrary cutoff = 0.090)

In **Figure 4A** we showed the direct reactivity of RA patients' sera to PYK2/FADK2, BANK-1, LIPRIN-1 and CTLA-4 peptides. In the cohort of seronegative RA patients, 23 out of 30 patients' sera (76.7%) had IgG antibodies directed against the peptide PYK2/FADK2, while 20 out of 30 (66.7%) against the BANK-1 peptide. Interestingly, 80% of patients positively reacted with LIPRIN-1, that is the percentage of patients' sera positive to RA-peptide. No difference between seronegative and seropositive patients (56.7%) was observed in CTLA-4 peptide recognition. In the 30 seropositive patients' sera (validation cohort), IgG antibodies directed against PYK2/FADK2, BANK-1, LIPRIN-1 peptides were present in 19 (63.3%), 17 (56.7%) and 18 (60%) patients, respectively (**Figure 4A**). None of the 25 healthy donors, used as a control, had IgG antibodies directed against all these peptides.

## Recognition of Auto-Antigens by Anti-RA-Peptide Purified Antibodies

In order to investigate whether the affinity purified antibodies against the RA-peptide can recognize autoantigens, we performed western blot and immunoprecipitation analysis. By the data obtained from the direct binding of purified CTLA-4 Abs to the different peptides (**Figure 3D**) and from the direct binding of patients' sera (**Figure 4A**), we excluded the CTLA-4 antigen.

First, we considered the protein-tyrosine kinase 2 beta (also known as PKB, PTK, PYK2, FADK2, FAK2, CAKB, CADTK, RAFTK), a non-receptor protein-tyrosine kinase implicated in many different cellular functions. Some authors reported that PYK2 exhibits the same domain organization and structural characteristics of FAK and they share a high degree of sequence similarity (48% amino acid identity and 65% similar at the protein level). However, the two proteins have differences in their C-terminal domains, which are probably implicated in their different regulation (33). For this reason, FAK and PYK2 are defined as a distinct family of non-receptor tyrosine kinases (34).

We identified two bands of approximately 125/150 kDa and 116 kDa corresponding to FAK and PYK2, respectively (**Figure 4B**), as reported also by other investigators (33, 35, 36).

We found the presence of FAK in PBMCs, but not in T or B cells or in HL60 or DAUDI cell lines. We found also a band at 125 kDa in normal human fibroblast-like synoviocytes and RA human fibroblast-like synoviocytes (HLFS and HLFS-RA, respectively), but we did not find it in normal dermal fibroblast (NHDF). These findings have been already reported by other authors (33, 37).

As regards PYK2, we did not find any band at 116 kDa in normal PBMCs, T or B cells (although some investigators reported that PYK2 band was slightly present in PBMCs from healthy donors (38)), while the expression was present in HLFS and HLFS-RA, and was absent in NHDF.

We then considered the BANK-1 protein, a cytoplasmic scaffold protein expressed in B but not in T cells or myeloid cell lines (39). The expected molecular weight of BANK-1 is around 85 kDa, but it may run at 97-105 kDa in SDS-PAGE.

We found a band approximately around 95/100 kDa only in PBMCs, B cells and DAUDI cell line, which was used as positive control. These data have been already described by other authors, who have used DAUDI as positive control (40). In our analysis, no bands were detected in the HLFS, HLFS-RA and in NHDF.

Finally, we evaluated the Liprin-alpha1, a subclass of liprins, which are cytosolic scaffold dimeric proteins, widely expressed and involved in cell motility. Liprin-alpha and beta usually homodimerize, but they can also form heterodimers or large molecular complexes interacting with other proteins, such as LAR (leukocyte common antigen-related) or integrins (41). We identified two bands of 55 kDa and 76 kDa,corresponding to homodimer and heterodimer of liprin-alpha1 protein, and a band between 110-130 kDa, corresponding to a heterodimer of liprin-alpha 1 with an unknown protein.

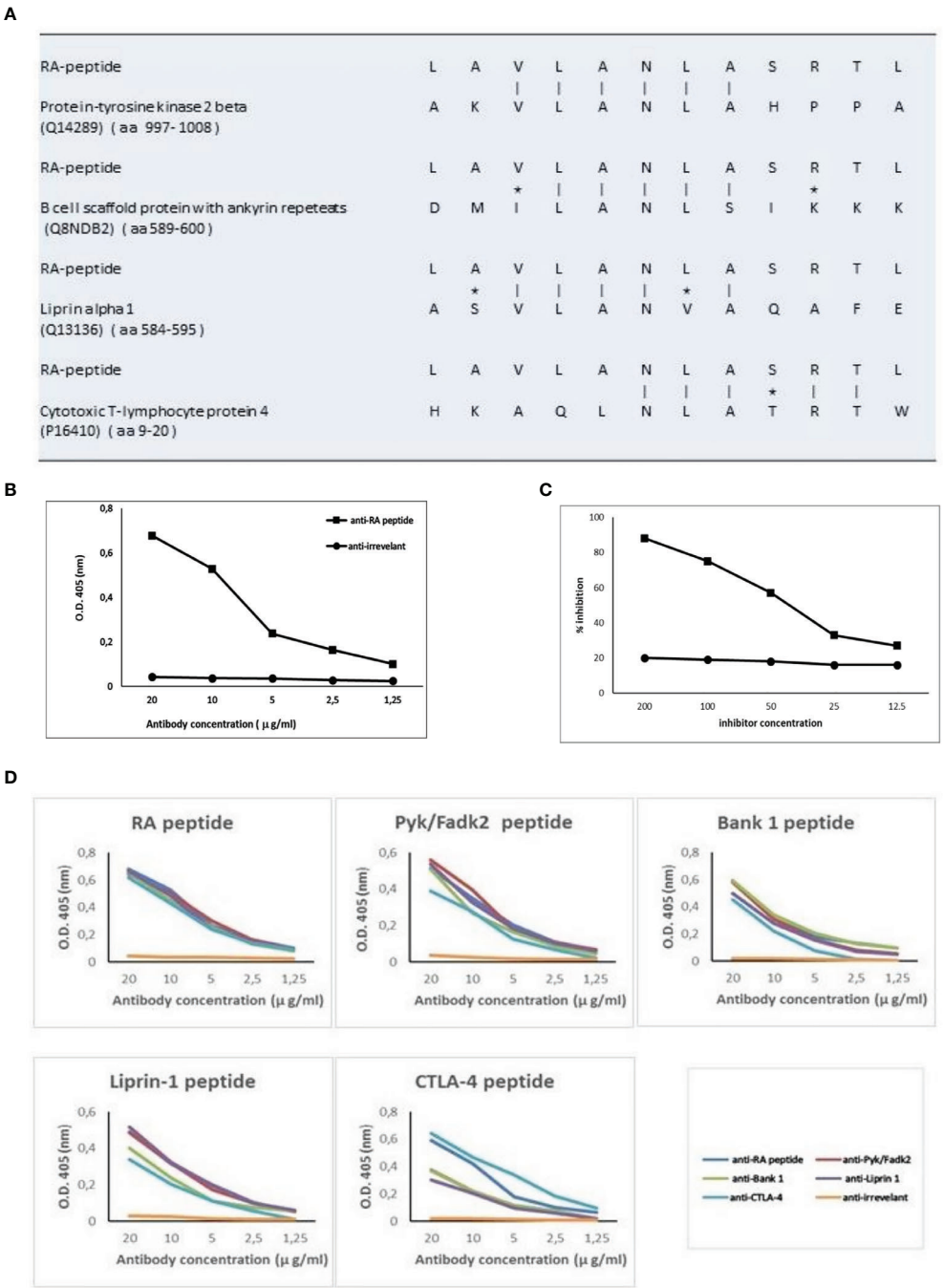


**FIGURE 3** | RA-peptide shares sequence homology with human self-proteins and anti-RA-peptide antibodies are cross-reactive. **(A)** Sequence homology between RA-peptide and autoantigens, analyzed by the basic local alignment search tool using the National Center for Biotechnology Information (NCBI) network service. Vertical line = identical amino acids; asterisk= conservative substitutions. **(B)** Direct binding of affinity purified anti-RA-peptide antibodies or anti-irrelevant peptide antibodies to RA synthetic peptide evaluated by ELISA assay. Data are plotted in a graph displaying absorbance (O.D.) at 405 nm on the vertical axis and antibody concentration expressed in µg/ml on horizontal axis. **(C)** Inhibition ELISA test: the binding of affinity-purified antibodies to RA-peptide was inhibited by RA synthetic peptide (■), but not by an irrelevant peptide (●). On vertical axis the inhibition percentage is reported, while on horizontal axis the inhibitor concentration (µg/ml) is shown. **(D)** Specific binding of cross-reactive IgG antibodies to peptides: affinity-purified antibodies against RA (blue line), Pyk/Fadk2 (red line), Bank-1 (green line), Liprin-1 (purple line), CTLA-4 (light blue line) and irrelevant (orange line) peptides were used in a direct binding assay with each one of the 5 synthetic peptides. Graph displays absorbance (O.D.) at 405 nm on vertical axis and antibody concentration µg/ml on horizontal axis.

A

| | RA-PEPTIDE | Pyk/Fadk2-PEPTIDE | BANK 1-PEPTIDE | LIPRIN 1-PEPTIDE | CTLA-4-PEPTIDE |
|---|---|---|---|---|---|
| RA - | 24/30<br>80% | 23/30<br>76.7% | 20/30<br>66.7% | 24/30<br>80% | 17/30<br>56.7% |
| RA + | 20/30<br>66.7% | 19/30<br>63.3% | 17/30<br>56.7% | 18/30<br>60% | 17/30<br>56.7% |

B

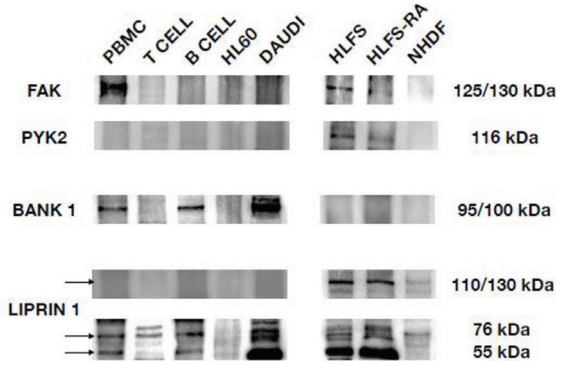


**FIGURE 4** | Peptides cross-reaction of RA patients' sera and recognition of auto-antigens by purified anti-RA-peptide Abs. **(A)** Frequency of the direct binding of seronegative and seropositive patients' sera to RA, Pyk/Fadk2, Bank-1, Liprin-1 and CTLA-4 peptides evaluated by ELISA assays. No binding was observed using serum IgG of healthy donors. The arbitrary cutoff of 0.090 was used. **(B)** Immunoblot analysis of cell lysates, after immunoprecipitation with affinity purified anti-RA-peptide antibodies detected with commercial antibodies: mouse polyclonal antibodies directed against PYK2 or rabbit polyclonal antibodies directed against BANK, or rabbit polyclonal antibodies directed against Liprin. PBMCs, T or B cells were obtained by a normal healthy donor; HL60 (Human promyelocytic leukemia) and DAUDI (Burkitt lymphoma cell) lines were used as controls; HLFS (Normal Human Fibroblast-Like Synoviocytes) and HLFS-RA (Human Fibroblast-Like Synoviocytes: Rheumatoid Arthritis) and NHDF (Normal human dermal fibroblast) were commercially obtained.

As shown in **Figure 4B** the homodimeric form was identified in PBMCs, B cells and DAUDI cell line, and it was present in synoviocytes but absent in dermal fibroblasts. The heterodimeric form was reveled in PBMCs, T and B cells, DAUDI cell line, and in HLFS, HLFS-RA and in NHDF. The higher molecular weight band was only present in HLFS, HLFS-RA and weakly detectable in NHDF. The presence of the high molecular weight band in fibroblasts has also been described by other authors (42, 43).

## DISCUSSION

Since the comprehension of pathogenesis of autoimmune diseases is crucial both for early diagnosis and treatment, the identification of novel autoantigens may play fundamental role.

The diagnosis of seropositive RA is based on the detection of serological markers such as rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPAs) (15), which may be present years before the onset of the disease (2). ACPAs can be also detected in the serum of subjects with undifferentiated arthritis, who later on will develop RA (44, 45). On the other hand, seronegative RA does not have the diagnostic help of any laboratory biomarkers so far and its definition still remains a clinical challenge.

The random peptide library is a well-known and useful molecular biology tool for the identification of possible immunodominant peptides in autoimmune disorders (26–28). The screening of peptide library with pooled immunoglobulins from patients affected by an autoimmune disease leads firstly to the identification of immunodominant peptides acting as autoantigens. Secondly, it leads to the identification of antibodies able to bind such peptides and autoantigens, in the patients' sera. Therefore, the presence of these auto-antibodies in patients' sera may be helpful in the diagnosis of the disease and, if they are functionally active, they may provide new relevant information on the pathogenesis of the studied autoimmune disease (26–28).

In the present work, by using this approach in seronegative RA patients, we could identify a peptide, named RA-peptide, which is specifically recognized by both seronegative and seropositive RA patients' sera (70.0% and 64.0%, respectively) but not by any sera from healthy donors. Such findings were confirmed in a second validation cohort of RA patients with similar results (83.3% and 66.7%, respectively).

In a second step, we verify whether the recognition of RA-peptide is truly specific for RA. To achieve this aim, we enrolled three other cohorts of patients affected by different immune-mediated diseases. We observed that, in these three groups many patients' sera show an absorbance value close to the standard cutoff (means ± 3 s.d. of the healthy control group). The ROC curve and contingency table analyses induced us to apply a second arbitrary cutoff (0.090). Using this cutoff, we observed that 73.8% and 63.3% of all seronegative or seropositive RA patients' sera, respectively, recognised the RA-peptide, while a

greater number of patients with other immune-mediated diseases resulted negative.These data indicate that this RA-peptide sequence is recognized by the sera of patients affected by RA disease at high frequency, independent of RF and ACPAs positivity, but not by the sera obtained from healthy donors or from patients with other immune-mediated diseases, thereby suggesting that antibodies directed against this epitope are highly specific for RA.

Within the group of seronegative RA patients, we found no correlation of anti-RA-peptide Abs with disease activity or inflammatory markers. This apparent discrepancy may be consistent with the findings in the different context of seropositive RA, where some authors observed that many patients treated with disease-modifying agents showed a decrease in inflammation and pain but not in ACPA levels (46).

As regards corticosteroid therapy, by analysing the quartile distribution of anti-RA-peptide antibody levels among our RA patients, we observed that patients on corticosteroid therapy were mostly in the second and/or third quartiles, while those never treated with steroid were mostly in the fourth quartile. This observation suggests that corticosteroid therapy may lead to a reduction in the levels of anti-RA-peptide Abs in RA patients' sera. Interestingly, in other autoimmune diseases we showed that corticosteroid therapy lowers the levels of Abs specifically in good responder patients (47).

However, even if corticosteroid therapy may lower Abs levels, it is worthy to note that such level remains higher than in healthy subjects, thus confirming that anti-RA-peptide Abs may be a biomarker for RA.

As above mentioned, RA-peptide shares sequence homology with proteins expressed by cells that are considered to be important in RA, such as Protein-tyrosine kinase 2 beta (PYK2/FADK2), B cell scaffold protein (BANK-1), Liprin-alpha 1 (LIPRIN-1), and Cytotoxic T-lymphocyte protein 4 (CTLA-4). For this reason, we wanted to evaluate whether affinity purified anti-RA-peptide Abs were also able to recognize these four proteins. To this aim, we selected four peptides (one of each protein) based on the sequence homology shared with RA-peptide. We then purified Abs from patients' sera directed against each one of these peptides and test their ability to cross react. We observed that these affinity-purified Abs, not only were able to recognize the peptide used for the purification, but showed a dose-dependent cross reactivity with all the other peptides. On the contrary, the Abs purified against the irrelevant peptide were not able to bind any of the five peptides, even at high concentration. We also confirm that the sera of RA patients can positively react with all the peptides.

Notably, the identified four proteins are present in cells involved in RA pathogenesis, such as B and T lymphocytes, macrophage/monocytes, fibroblast-like synoviocytes, fibroblasts, osteoclasts and endothelial cells, thereby being consistent with a potential biological role in this immune-mediated disease.

Several studies on RA have described differences in B cell subsets distribution in both peripheral blood and bone marrow before and after B cell depletion treatments (48). No differences were found at peripheral blood level between healthy donors and RA patients. Moreover, B cell subpopulations showed the same distribution among seronegative-, seropositive- and non-RA patients both in peripheral blood and synovial fluid (49). A study by Michelutti et al. showed that specific B cell subsets are present in the synovium of both seronegative and seropositive RA patients. Moreover, a recruitment of immature B cells into synovial fluid from peripheral blood was observed and, according to authors' hypothesis, a persistent inflammation may promote a B cell maturation directly in the joints (50).

Fibroblasts and related different subpopulations play a role in RA pathophysiology. Fibroblast-like synoviocytes (FLS) are aggressively proliferating in the joint synovium of RA patients and such proliferation leads to the invasion and destruction of adjacent cartilage (51, 52). Moreover, the inflammatory environment present in rheumatoid joints may trigger the proliferation of synovial fibroblasts (RA-SF) leading to an increase of the synovial pannus (53) with invasion of healthy cartilage (54, 55). We confirmed that the anti-RA-peptide Abs were able to recognize the three auto-antigens PYK2/FADK2, BANK-1 and LIPRIN-1, which are expressed in the above mentioned cell lines and may be involved in RA pathogenesis. We found that the anti-RA-peptide Abs are able to bind PYK2 and FAK proteins in PBMCs, HLFS, HLFS-RA cells (fibroblast-like synoviocytes).

PYK2 and FAK form a unique group of non-receptor tyrosine kinases. The expression of protein-tyrosine kinase 2 beta (Pyk2) is limited to epithelial cells, neurons, osteoclasts and hematopoietic cells (56, 57), while the focal adhesion kinase (FAK) is expressed in all tissues and, interestingly, in fibroblasts or synovial fibroblasts (37).

*In vitro* it has been shown that FAK plays a central role in arthritic synovial fibroblast invasion and cytokines production. In FAK-knockout mice, the FAK depletion does not reduce murine inflammatory arthritis (37). The authors suggest that FAK's homologue, Pyk2, may counterbalance the absence of FAK in this mice model. Phosphorylation of Pyk2 can be induced by TNF-α in rheumatoid synovial fibroblasts and in rheumatoid synovial tissue, a large amount of phosphorylated Pyk2 has been found. For this reason, it is likely that these two homologous proteins may equally contribute to inflammatory arthritis. It has been shown that FAK is crucial for synovial fibroblast invasion while it is not for TNF-α-driven erosive arthritis. Because of the homology with FAK and because of Pyk2 phosphorylation is induced by TNF-α, Pyk2 may play a role in erosive arthritis. Even if further studies are needed to better clarify the role of Pyk2 in inflammatory arthritis, it is likely that both proteins are involved in the pathophysiology of RA (37).

We found that anti-RA-peptide Abs can bind Bank-1 protein in PBMCs, B cells and DAUDI cell line. Bank-1 is involved in the regulation of B cell responses, such as CD40-mediated Akt activation and can prevent an excessive B cell activation (39). It is associated with increased anti-nuclear Abs levels (58) and with many different autoimmune diseases, such as RA (59). In collagen-induced arthritis in mice, a decreased expression of

Bank-1 facilitates B cell response, increasing antigen presentation and leading to the production of autoantibodies (60).

Finally, anti-RA-peptide Abs can recognize both the homodimeric and heterodimeric forms but also large molecular complexes of Liprin-alpha 1 protein in the extract of different cell types. The higher molecular weight band was identified in fibroblast-like synoviocytes (HLFS, HLFS-RA) and to a lower extent in dermal fibroblasts (NHDF), but it is not present in PBMCs, B, T, HL60 and DAUDI cells. Evident bands (homodimeric and heterodimeric forms) could be observed in DAUDI cells, a tumor B cell line, and in fibroblast-like synoviocytes. Liprin-alpha 1, as other members of the liprin family proteins, is involved in forming and maintaining the cellular cytosolic scaffold (41, 61, 62) and in the formation of adhesion foci. Notably, it is present in the cytoskeletal core of podosomes, essential elements for cell motility (63–65).

This study had some strength and limitations, which should be acknowledged. The random peptide library is a method that allows the screening of a huge number of different 12 amino acid peptides in a faster and easier way than a phage library. Those peptides are obtained by a random sequence in the flagellin of cultured E. Coli. When this method is used to search for unknown autoantibodies the criteria driving the choice of the patients' sera to be used are crucial. The use of healthy donors' sera as a prescreening step helps to eliminate from subsequent analysis those peptides that have shown some reactivity, although it may potentially lead to loss of data. Nonetheless, this can save time and decrease the number of screening steps required to finally recognize new etiopathological relevant epitopes. Our results show that RA peptide is recognized by the sera of seronegative RA patients. Speculatively, an *ad hoc* ELISA test can improve the diagnosis of this disease. However, more studies need to be done before such ELISA test could be considered as a validated tool. Larger cohorts of patients, possibly including different ethnicity, need to be investigated to check the specificity and accuracy of the test.

## CONCLUSION

This study investigates potential candidate biomarkers of seronegative RA by peptide library screening. Here we show that RA-peptide is recognized by antibodies present in the sera of RA patients, particularly in the seronegative subjects.

RA-peptide shows similarity with four epitopes contained in four different proteins which may be involved in RA pathogenesis such as PYK2/FADK2, BANK-1, LIPRIN-1, and CTLA-4.

The anti-RA-peptide Abs are able to bind both the homologous epitopes and the full proteins. Therefore, we can consider the new identified autoantibodies as a specific biomarker of seronegative RA and RA-peptide could be used as a tool for the development of diagnostic assays for seronegative RA.

## 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 study was approved by the local Ethical Committee of the Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy (protocol number: 1538, version number 3) and by Comitato Universitario di Bioetica of Perugia, Italy (identification code: 2013-012 approved 4-4-2013). All clinical investigations were conducted according to the principles expressed in the Helsinki declaration. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

CB conceived and designed the study, performed the experiments, collected, analyzed and interpreted data and wrote the manuscript. AB provided useful suggestions and wrote the manuscript. NM did the statistical analysis. BO, EB, ET, and AP recruited patients and collected the clinical and demographic data. GA, RB and GJ collected the samples and provided reagents. ET and CL provided funding acquisition. CL conceived the study, supervised the work, wrote and gave the final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.753400/full#supplementary-material

## REFERENCES

1. Gibofsky A. Overview of Epidemiology, Pathophysiology, and Diagnosis of Rheumatoid Arthritis. *Am J Manag Care* (2012) 18:S295–302.
2. van Delft MAM, Huizinga TWJ. An Overview of Autoantibodies in Rheumatoid Arthritis. *J Autoimmun* (2020) 110:102392. doi: 10.1016/j.jaut.2019.102392
3. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO3rd, et al. Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62:2569–81. doi: 10.1002/art.27584
4. Monti S, Montecucco C, Bugatti S, Caporali R. Rheumatoid Arthritis Treatment: The Earlier the Better to Prevent Joint Damage. *RMD Open* (2015) 1:e000057. doi: 10.1136/rmdopen-2015-000057

5. Somers K, Geusens P, Elewaut D, De Keyser F, Rummens JL, Coenen M, et al. Novel Autoantibody Markers for Early and Seronegative Rheumatoid Arthritis. *J Autoimmun* (2011) 36:33–46. doi: 10.1016/j.jaut.2010.10.003
6. Choi S, Lee KH. Clinical Management of Seronegative and Seropositive Rheumatoid Arthritis: A Comparative Study. *PloS One* (2018) 13:e0195550. doi: 10.1371/journal.pone.0195550
7. Vander Cruyssen B, Peene I, Cantaert T, Hoffman IE, De Rycke L, Veys EM, et al. Anti-Citrullinated Protein/Peptide Antibodies (ACPA) in Rheumatoid Arthritis: Specificity and Relation With Rheumatoid Factor. *Autoimmun Rev* (2005) 4:468–74. doi: 10.1016/j.autrev.2005.04.018
8. Avouac J, Gossec L, Dougados M. Diagnostic and Predictive Value of Anti-Cyclic Citrullinated Protein Antibodies in Rheumatoid Arthritis: A Systematic Literature Review. *Ann Rheum Dis* (2006) 65:845–51. doi: 10.1136/ard.2006.051391
9. Greiner A, Plischke H, Kellner H, Gruber R. Association of Anti-Cyclic Citrullinated Peptide Antibodies, Anti-Citrullin Antibodies, and IgM and IgA Rheumatoid Factors With Serological Parameters of Disease Activity in Rheumatoid Arthritis. *Ann NY Acad Sci* (2005) 1050:295–303. doi: 10.1196/annals.1313.031
10. Bukhari M, Thomson W, Naseem H, Bunn D, Silman A, Symmons D, et al. The Performance of Anti-Cyclic Citrullinated Peptide Antibodies in Predicting the Severity of Radiologic Damage in Inflammatory Polyarthritis: Results From the Norfolk Arthritis Register. *Arthritis Rheum* (2007) 56:2929–35. doi: 10.1002/art.22868
11. Machold KP, Stamm TA, Nell VP, Pflugbeil S, Aletaha D, Steiner G, et al. Very Recent Onset Rheumatoid Arthritis: Clinical and Serological Patient Characteristics Associated With Radiographic Progression Over the First Years of Disease. *Rheumatol (Oxford)* (2007) 46:342–9. doi: 10.1093/rheumatology/kel237
12. Sakkas LI, Bogdanos DP, Katsiari C, Platsoucas CD. Anti-Citrullinated Peptides as Autoantigens in Rheumatoid Arthritis-Relevance to Treatment. *Autoimmun Rev* (2014) 13:1114–20. doi: 10.1016/j.autrev.2014.08.012
13. Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadell G, Ronnelid J, et al. Antibodies of IgG, IgA and IgM Isotypes Against Cyclic Citrullinated Peptide Precede the Development of Rheumatoid Arthritis. *Arthritis Res Ther* (2011) 13:R13. doi: 10.1186/ar3237
14. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody Epitope Spreading in the Pre-Clinical Phase Predicts Progression to Rheumatoid Arthritis. *PloS One* (2012) 7:e35296. doi: 10.1371/journal.pone.0035296
15. Martinez-Prat L, Nissen MJ, Lamacchia C, Bentow C, Cesana L, Roux-Lombard P, et al. Comparison of Serological Biomarkers in Rheumatoid Arthritis and Their Combination to Improve Diagnostic Performance. *Front Immunol* (2018) 9:1113. doi: 10.3389/fimmu.2018.01113
16. de Brito Rocha S, Baldo DC, Andrade LEC. Clinical and Pathophysiologic Relevance of Autoantibodies in Rheumatoid Arthritis. *Adv Rheumatol* (2019) 59:2. doi: 10.1186/s42358-018-0042-8
17. Trouw LA, Rispens T, Toes REM. Beyond Citrullination: Other Post-Translational Protein Modifications in Rheumatoid Arthritis. *Nat Rev Rheumatol* (2017) 13:331–9. doi: 10.1038/nrrheum.2017.15
18. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies Recognizing Carbamylated Proteins Are Present in Sera of Patients With Rheumatoid Arthritis and Predict Joint Damage. *Proc Natl Acad Sci USA* (2011) 108:17372–7. doi: 10.1073/pnas.1114465108
19. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Toes RE, Trouw LA, et al. Anti-Carbamylated Protein Antibodies are Present in Arthralgia Patients and Predict the Development of Rheumatoid Arthritis. *Arthritis Rheum* (2013) 65:911–5. doi: 10.1002/art.37830
20. Bell DA, Elhayek S, Cairns E, Barra L. Anti-Homocitrullinated Protein Antibody Isotype Usage in Rheumatoid Arthritis and Their Unaffected First-Degree Relatives. *Clin Exp Rheumatol* (2017) 35:948–53.
21. Ajeganova S, van der Helm-van Mil A. Disease-Modifying Treatment in Rheumatoid Arthritis: Treat Early and do Not Forget to Try to Taper and Stop. *Ann Rheum Dis* (2017) 76:e17. doi: 10.1136/annrheumdis-2016-210892
22. Alessandri C, Bartosiewicz I, Pendolino M, Mancini R, Colasanti T, Pecani A, et al. Anti-Carbamylated Protein Antibodies in Unaffected First-Degree Relatives of Rheumatoid Arthritis Patients: Lack of Correlation With Anti-Cyclic Citrullinated Protein Antibodies and Rheumatoid Factor. *Clin Exp Rheumatol* (2015) 33:824–30.
23. Auger I, Charpin C, Balandraud N, Martin M, Roudier J. Autoantibodies to PAD4 and BRAF in Rheumatoid Arthritis. *Autoimmun Rev* (2012) 11:801–3. doi: 10.1016/j.autrev.2012.02.009
24. Harris ML, Darrah E, Lam GK, Bartlett SJ, Giles JT, Grant AV, et al. Association of Autoimmunity to Peptidyl Arginine Deiminase Type 4 With Genotype and Disease Severity in Rheumatoid Arthritis. *Arthritis Rheum* (2008) 58:1958–67. doi: 10.1002/art.23596
25. Colasanti T, Sabatinelli D, Mancone C, Giorgi A, Pecani A, Spinelli FR, et al. Homocysteinylated Alpha 1 Antitrypsin as an Antigenic Target of Autoantibodies in Seronegative Rheumatoid Arthritis Patients. *J Autoimmun* (2020) 113:102470. doi: 10.1016/j.jaut.2020.102470. 102470.
26. Lunardi C, Bason C, Navone R, Millo E, Damonte G, Corrocher R, et al. Systemic Sclerosis Immunoglobulin G Autoantibodies Bind the Human Cytomegalovirus Late Protein UL94 and Induce Apoptosis in Human Endothelial Cells. *Nat Med* (2000) 6:1183–6. doi: 10.1038/80533
27. Bason C, Lorini R, Lunardi C, Dolcino M, Giannattasio A, d'Annunzio G, et al. In Type 1 Diabetes a Subset of Anti-Coxsackievirus B4 Antibodies Recognize Autoantigens and Induce Apoptosis of Pancreatic Beta Cells. *PloS One* (2013) 8:e57729. doi: 10.1371/journal.pone.0057729
28. Lunardi C, Bason C, Leandri M, Navone R, Lestani M, Millo E, et al. Autoantibodies to Inner Ear and Endothelial Antigens in Cogan's Syndrome. *Lancet* (2002) 360:915–21. doi: 10.1016/S0140-6736(02)11028-2
29. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. Classification Criteria for Systemic Sclerosis: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2013) 65:2737–47. doi: 10.1002/art.38098
30. Raychaudhuri SP, Deodhar A. The Classification and Diagnostic Criteria of Ankylosing Spondylitis. *J Autoimmun* (2014) 48-49:128–33. doi: 10.1016/j.jaut.2014.01.015
31. Mease P. Psoriatic Arthritis and Spondyloarthritis Assessment and Management Update. *Curr Opin Rheumatol* (2013) 25:287–96. doi: 10.1097/BOR.0b013e32835fd8d5
32. Bason C, Pagnini I, Brucato A, Maestroni S, Puccetti A, Lunardi C, et al. Congenital Heart Block and Immune Mediated Sensorineural Hearing Loss: Possible Cross Reactivity of Immune Response. *Lupus* (2017) 26:835–40. doi: 10.1177/0961203316682099
33. Avraham H, Park SY, Schinkmann K, Avraham S. RAFTK/Pyk2-Mediated Cellular Signalling. *Cell Signal* (2000) 12:123–33. doi: 10.1016/s0898-6568(99)00076-5
34. Hall JE, Fu W, Schaller MD. Focal Adhesion Kinase: Exploring Fak Structure to Gain Insight Into Function. *Int Rev Cell Mol Biol* (2011) 288:185–225. doi: 10.1016/B978-0-12-386041-5.00005-4
35. Zhu X, Bao Y, Guo Y, Yang W. Proline-Rich Protein Tyrosine Kinase 2 in Inflammation and Cancer. *Cancers (Basel)* (2018) 10(5):139. doi: 10.3390/cancers10050139
36. Wang Q, Xie Y, Du QS, Wu XJ, Feng X, Mei L, et al. Regulation of the Formation of Osteoclastic Actin Rings by Proline-Rich Tyrosine Kinase 2 Interacting With Gelsolin. *J Cell Biol* (2003) 160:565–75. doi: 10.1083/jcb.200207036
37. Shelef MA, Bennin DA, Yasmin N, Warner TF, Ludwig T, Beggs HE, et al. Focal Adhesion Kinase Is Required for Synovial Fibroblast Invasion, But Not Murine Inflammatory Arthritis. *Arthritis Res Ther* (2014) 16:464. doi: 10.1186/s13075-014-0464-6
38. Wang M, Sun H, Zhang W, Zhang Y. Functional Activation of Proline-Rich Tyrosine Kinase2 (PYK2) in Peripheral Blood Mononuclear Cells From Patients With Systemic Lupus Erythematosus. *BMC Musculoskelet Disord* (2009) 10:141. doi: 10.1186/1471-2474-10-141
39. Aiba Y, Yamazaki T, Okada T, Gotoh K, Sanjo H, Ogata M, et al. BANK Negatively Regulates Akt Activation and Subsequent B Cell Responses. *Immunity* (2006) 24:259–68. doi: 10.1016/j.immuni.2006.01.002
40. Bernal-Quiros M, Wu YY, Alarcon-Riquelme ME, Castillejo-Lopez C. BANK1 and BLK Act Through Phospholipase C Gamma 2 in B-Cell Signaling. *PloS One* (2013) 8:e59842. doi: 10.1371/journal.pone.0059842
41. de Curtis I. Function of Liprins in Cell Motility. *Exp Cell Res* (2011) 317:1–8. doi: 10.1016/j.yexcr.2010.09.014
42. Asperti C, Astro V, Totaro A, Paris S, de Curtis I. Liprin-Alpha1 Promotes Cell Spreading on the Extracellular Matrix by Affecting the Distribution of Activated Integrins. *J Cell Sci* (2009) 122:3225–32. doi: 10.1242/jcs.054155

43. Asperti C, Pettinato E, de Curtis I. Liprin-Alpha1 Affects the Distribution of Low-Affinity Beta1 Integrins and Stabilizes Their Permanence at the Cell Surface. *Exp Cell Res* (2010) 316:915–26. doi: 10.1016/j.yexcr.2010.01.017
44. Alexiou I, Germenis A, Ziogas A, Theodoridou K, Sakkas LI. Diagnostic Value of Anti-Cyclic Citrullinated Peptide Antibodies in Greek Patients With Rheumatoid Arthritis. *BMC Musculoskelet Disord* (2007) 8:37. doi: 10.1186/1471-2474-8-37
45. Jilani AA, Mackworth-Young CG. The Role of Citrullinated Protein Antibodies in Predicting Erosive Disease in Rheumatoid Arthritis: A Systematic Literature Review and Meta-Analysis. *Int J Rheumatol* (2015) 2015:728610. doi: 10.1155/2015/728610
46. Kastbom A, Forslind K, Ernestam S, Geborek P, Karlsson JA, Petersson IF, et al. Changes in the Anticitrullinated Peptide Antibody Response in Relation to Therapeutic Outcome in Early Rheumatoid Arthritis: Results From the SWEFOT Trial. *Ann Rheum Dis* (2016) 75:356–61. doi: 10.1136/annrheumdis-2014-205698
47. Berti E, Vannucci G, Lunardi C, Bianchi B, Bason C, Puccetti A, et al. Identification of Autoantibodies Against Inner Ear Antigens in a Cohort of Children With Idiopathic Sensorineural Hearing Loss. *Autoimmunity* (2013) 46:525–30. doi: 10.3109/08916934.2013.822074
48. Palanichamy A, Roll P, Theiss R, Dorner T, Tony HP. Modulation of Molecular Imprints in the Antigen-Experienced B Cell Repertoire by Rituximab. *Arthritis Rheum* (2008) 58:3665–74. doi: 10.1002/art.24141
49. Anolik JH, Looney RJ, Lund FE, Randall TD, Sanz I. Insights Into the Heterogeneity of Human B Cells: Diverse Functions, Roles in Autoimmunity, and Use as Therapeutic Targets. *Immunol Res* (2009) 45:144–58. doi: 10.1007/s12026-009-8096-7
50. Michelutti A, Gremese E, Morassi F, Petricca L, Arena V, Tolusso B, et al. B-Cell Subsets in the Joint Compartments of Seropositive and Seronegative Rheumatoid Arthritis (RA) and No-RA Arthritides Express Memory Markers and ZAP70 and Characterize the Aggregate Pattern Irrespectively of the Autoantibody Status. *Mol Med* (2011) 17:901–9. doi: 10.2119/molmed.2011.00034
51. Vital EM, Emery P. The Development of Targeted Therapies in Rheumatoid Arthritis. *J Autoimmun* (2008) 31:219–27. doi: 10.1016/j.jaut.2008.04.006
52. Bauer S, Jendro MC, Wadle A, Kleber S, Stenner F, Dinser R, et al. Fibroblast Activation Protein Is Expressed by Rheumatoid Myofibroblast-Like Synoviocytes. *Arthritis Res Ther* (2006) 8:R171. doi: 10.1186/ar2080
53. Korb-Pap A, Bertrand J, Sherwood J, Pap T. Stable Activation of Fibroblasts in Rheumatic Arthritis-Causes and Consequences. *Rheumatol (Oxford)* (2016) 55:ii64–7. doi: 10.1093/rheumatology/kew347
54. Lefevre S, Knedla A, Tennie C, Kampmann A, Wunrau C, Dinser R, et al. Synovial Fibroblasts Spread Rheumatoid Arthritis to Unaffected Joints. *Nat Med* (2009) 15:1414–20. doi: 10.1038/nm.2050
55. Klein K, Ospelt C, Gay S. Epigenetic Contributions in the Development of Rheumatoid Arthritis. *Arthritis Res Ther* (2012) 14:227. doi: 10.1186/ar4074
56. Ostergaard HL, Lysechko TL. Focal Adhesion Kinase-Related Protein Tyrosine Kinase Pyk2 in T-Cell Activation and Function. *Immunol Res* (2005) 31:267–82. doi: 10.1385/IR:31:3:267
57. Pfaff M, Jurdic P. Podosomes in Osteoclast-Like Cells: Structural Analysis and Cooperative Roles of Paxillin, Proline-Rich Tyrosine Kinase 2 (Pyk2) and Integrin Alphavbeta3. *J Cell Sci* (2001) 114:2775–86. doi: 10.1242/jcs.114.15.2775
58. Alkaissi H, Havarinasab S, Nielsen JB, Soderkvist P, Hultman P. Bank1 and NF-KappaB as Key Regulators in Anti-Nucleolar Antibody Development. *PloS One* (2018) 13:e0199979. doi: 10.1371/journal.pone.0199979
59. Cantaert T, Yeremenko NG, Teitsma CA, van Duivenvoorde LM, Paramarta JE, Tak PP, et al. Altered BANK1 Expression Is Not Associated With Humoral Autoimmunity in Chronic Joint Inflammation. *Rheumatol (Oxford)* (2013) 52:252–60. doi: 10.1093/rheumatology/kes247
60. Yang J, Ren J, Yang Y, Sun J, Zhou X, Zheng S, et al. BANK1 Alters B Cell Responses and Influences the Interactions Between B Cells and Induced T Regulatory Cells in Mice With Collagen-Induced Arthritis. *Arthritis Res Ther* (2018) 20:9. doi: 10.1186/s13075-017-1503-x
61. Nachat R, Cipolat S, Sevilla LM, Chhatriwala M, Groot KR, Watt FM. KazrinE Is a Desmosome-Associated Liprin That Colocalises With Acetylated Microtubules. *J Cell Sci* (2009) 122:4035–41. doi: 10.1242/jcs.047266
62. Wei Z, Zheng S, Spangler SA, Yu C, Hoogenraad CC, Zhang M. Liprin-Mediated Large Signaling Complex Organization Revealed by the Liprin-Alpha/CASK and Liprin-Alpha/Liprin-Beta Complex Structures. *Mol Cell* (2011) 43:586–98. doi: 10.1016/j.molcel.2011.07.021
63. Spinardi L, Marchisio PC. Podosomes as Smart Regulators of Cellular Adhesion. *Eur J Cell Biol* (2006) 85:191–4. doi: 10.1016/j.ejcb.2005.08.005
64. Seano G, Chiaverina G, Gagliardi PA, di Blasio L, Puliafito A, Bouvard C, et al. Endothelial Podosome Rosettes Regulate Vascular Branching in Tumour Angiogenesis. *Nat Cell Biol* (2014) 16:931–41, 1-8. doi: 10.1038/ncb3036
65. Pehkonen H, von Nandelstadh P, Karhemo PR, Lepikhova T, Grenman R, Lehti K, et al. Liprin-Alpha1 Is a Regulator of Vimentin Intermediate Filament Network in the Cancer Cell Adhesion Machinery. *Sci Rep* (2016) 6:24486. doi: 10.1038/srep24486


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# Identification of Diagnostic Signatures and Immune Cell Infiltration Characteristics in Rheumatoid Arthritis by Integrating Bioinformatic Analysis and Machine-Learning Strategies

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**Background:** Rheumatoid arthritis (RA) refers to an autoimmune rheumatic disease that imposes a huge burden on patients and society. Early RA diagnosis is critical to preventing disease progression and selecting optimal therapeutic strategies more effectively. In the present study, the aim was at examining RA's diagnostic signatures and the effect of immune cell infiltration in this pathology.

**Methods:** Gene Expression Omnibus (GEO) database provided three datasets of gene expressions. Firstly, this study adopted R software for identifying differentially expressed genes (DEGs) and conducting functional correlation analyses. Subsequently, we integrated bioinformatic analysis and machine-learning strategies for screening and determining RA's diagnostic signatures and further verify by qRT-PCR. The diagnostic values were assessed through receiver operating characteristic (ROC) curves. Moreover, this study employed cell-type identification by estimating relative subsets of RNA transcript (CIBERSORT) website for assessing the inflammatory state of RA, and an investigation was conducted on the relationship of diagnostic signatures and infiltrating immune cells.

**Results:** On the whole, 54 robust DEGs received the recognition. Lymphocyte-specific protein 1 (LSP1), Granulysin (GNLY), and Mesenchymal homobox 2 (MEOX2) (AUC = 0.955) were regarded as RA's diagnostic markers and showed their statistically significant difference by qRT-PCR. As indicated from the immune cell infiltration analysis, resting NK cells, neutrophils, activated NK cells, T cells CD8, memory B cells, and M0 macrophages may be involved in the development of RA. Additionally, all diagnostic signatures might be different degrees of correlation with immune cells.

**Conclusions:** In conclusion, LSP1, GNLY, and MEOX2 are likely to be available in terms of diagnosing and treating RA, and the infiltration of immune cells mentioned above may critically impact RA development and occurrence.

**Keywords: rheumatoid arthritis, immune cells, diagnostic marker, bioinformatic analysis, machine-learning strategies**

## INTRODUCTION

Rheumatoid arthritis (RA) is recognized as a general chronic autoimmune connective tissue disease, which primarily covers the joints and eventually leads to chronic disability, joint destruction, and shortened life span (1–3). Rheumatoid arthritis affects 5 to 10 per 1,000 people (3). Ultimately, RA irreversibly damages joints, imposing a great adverse effect onto individual and society. Nevertheless, detection of RA at an early stage offers the opportunity for an effective treatment response, and this preclinical stage may be as short as a few months (4). At present, diagnosed rheumatoid factor and anticyclic citrullinated peptide antibody are serum biomarkers for the diagnosis of rheumatoid (5, 6). Yet early RA, particularly negative serum rheumatoid factor and anticyclic citrullinated antibodies, cannot easily be diagnosed for insufficient feasible biomarker. Therefore, finding novel and feasible biomarker is very important to early diagnose and treat RA.

Recently, increasing articles revealed that infiltration of immune cells critically impacts RA occurrence and progresses. For instance, RA exhibits a unique pattern of macrophage infiltration. The degree of macrophage infiltration in joint tissues and the level of monocyte-derived cytokines in serum show positive correlations to disease severity (7). CD8+ T cell exhibits anti-inflammation characteristic and is likely to contribute to the reduction of persistent autoimmune responses in rheumatoid joints (8, 9). B cells impact bone remodeling in RA (10). Nevertheless, the molecular system allowing different immune cells to impact RA occurrence and progresses should be clarified (11). For the mentioned reason, according to the aspect of immune systems, evaluating immune cells' infiltration and ascertaining the distinctions within the infiltrating immune cells' components are critical in elucidating RA molecular system and finding novel immunotherapeutic target. Cell-type identification by estimating relative subsets of RNA transcript (CIBERSORT) is a computational method for quantifying cell composition from tissue gene expression profiles obtained by RNA sequencing (12). Thus far, no studies have used CIBERSORT to analyze immune cell infiltration in whole blood of rheumatoid arthritis.

This study obtained RA microarray datasets in the GEO database to conduct investigations for differential expression gene. Besides, to screen and identify diagnostic markers of RA in depth, bioinformatics analysis, and machine-learning strategies were combined. Next, CIBERSORT was adopted for investigating the differences in whole-blood immune infiltrates in 22 immune cell subsets between RA and normal samples. Furthermore, the associations of diagnostic markers and infiltrating immune cells were investigated for gaining more insights into the molecular immune mechanisms involved in RA development.

**Abbreviations:** RA, rheumatoid arthritis; ROC, receiver operating characteristic; CIBERSORT, cell-type identification by estimating relative subsets of RNA transcript; GEO, Gene Expression Omnibus; RRA, Robust Rank Aggregation; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; RF, fandom forests; LASSO, least absolute shrinkage and selection operator; SVM-RFE, vector machine-recursive feature elimination; WGCNA, weighted gene co-expression network analysis; AUC, area under the curve; LSP1, lymphocyte-specific protein 1; GNLY, granulysin; MEOX2, mesenchymal homobox 2.

## MATERIALS AND METHODS

### Data Collection and Data Processing

Here, datasets received the search from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) with the keywords: "Arthritis, Rheumatoid" [MeSH Terms] OR "Arthritis, Rheumatoid" [All Fields] AND "Homo sapiens" [porgn: txid9606] AND "Expression profiling by array" [All Fields]. The screening standards included the following: the microarray datasets referred to profiles of gene expressions with genome-wide of whole blood; the microarray datasets contained samples from RA and samples from healthy state; all included samples were not treated with drugs. Eventually, three datasets received the screening to achieve the in-depth investigation: GSE100191 (13), GSE17755 (14), and GSE93272 (15). The table of the clinical information for the samples of RA patients and healthy subjects is provided in **Supplementary Table 1**. According to the inclusion criteria, only RA and healthy samples were selected for further analysis, including 50 normal controls and 119 RA patients. Next, the present study conducted the data preprocessing based on RMA (16) (e.g., expression calculation, normalization, and background correction).

### Differential Expression Analysis

The present study adopted LIMMA (http://www.bioconductor.org/packages/release/bioc/html/limma.html) package for identifying DEGs through the comparison of the expression datasets of GSE100191 and GSE17755, and the volcano plot was drawn to present the differential expression of DEGs. DEGs with $P < 0.05$ and $|\log_2 FC| > 1$ were considered statistically significant. Next, the DEGs were further identified based on the "RobustRankAggreg" package in R to obtain robust DEGs. This method of Robust Rank Aggregation (RRA) can minimize the deviation and error between multiple datasets (17).

### Functional Correlation Analysis

For the exploration of the function and pathway of the identified feature gene, this study conducted the gene ontology (GO) and

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment investigations with the use of the "clusterProfiler" package (18). $P$ <0.05 was considered to show the statistical significance. In order to more intuitively clarify the gene expression level of significantly enriched functional pathways, gene set enrichment analysis (GSEA) was performed in R software (19).

## Screening and Verification of Diagnostic Markers

The four algorithms were adopted for screening of novel and key biomarkers for RA, including random forests (RF) (20, 21), least absolute shrinkage and selection operator (LASSO) logistic regression (22), support vector machine-recursive feature elimination (SVM-RFE) (23), and weighted gene co-expression network analysis (WGCNA) (24). This study adopted the random forest algorithm with R package "randomForest." This study carried out LASSO logistic regression investigation with R package "glmnet," and minimal lambda was considered optimal. This study conducted the featured gene selection with the RFE function within the caret package based on five-fold cross-validation. The SVM classifier was constructed using R package e1071 with five-fold cross-validation. WGCNA was performed by R package "WGCNA" (25). Then, this study selected the overlapping genes from the mentioned four classification models for further analysis. For the in-depth test of the efficacy of key biomarkers, the dataset of GSE93272 was combined with GSE100191 and GSE17755 as the validation set. It was assessed based on the investigation of receiver operating characteristic (ROC) curves (MedCalc software), and the area under the curve (AUC) was calculated for evaluating the predictive effect achieved by the algorithms. A two-sided $P$ < 0.05 showed statistical significance.

## Quantitative PCR Analysis

A total of 34 whole blood samples (including 16 RA without drug treatments and 18 control samples with healthy state) were collected from Fuzhou Second Hospital affiliated to Xiamen University. The Ethical Committee of Fuzhou Second Hospital affiliated to Xiamen University approved this study, and the respective patient provided informed consent in a written form. All whole blood samples were immediately frozen in liquid nitrogen after the collecting process and stored at −80°C. The extraction of total RNA was performed with the use of Trizol reagent (TAKARA, Dalian, China). With a miRNA First Strand cDNA Synthesis Kit (Sangon, China), the reverse transcription of total RNA and miRNAs was performed. Besides, this study adopted the MicroRNAs qPCR Kit (Sangon, China) for examining miRNA and mRNA expressions, with the following primers: GAPDH (forward: 5′-GACAGTCAGCCGCATCTTCT-3′, reverse: 5′-ACCAAATCCGTTGACTCCGA-3′), LSP1 (forward: 5′-CTGTTAGCTTGGGAAGAGG-3′, reverse: 5′-ATAGCCCCTCTCAGATAGTC-3′), MEOX2 (forward: 5′-ATACTAGGGGAGATTCTCGC-3′, reverse: 5′-TAGGACTTTGGAGGGCTTAG-3′), and GNLY (forward: 5′-TCTGGTCCTAACTCTACTGG-3′, reverse: 5′-CAATCCTAGACAGTGTAGGC-3′) synthesized by Sangon Biotech. GAPDH was then handled as an internal reference. The relative expression was calculated using the 2−ΔΔCT method. $P$ values < 0.05 showed statistical significance.

## Evaluation and Correlation Analysis of Infiltration-Related Immune Cells

The CIBERSORT website was used to filter 22 kinds of the immune cell matrix. According to $P$ < 0.05, the immune cell infiltration matrix was obtained. The "ggplot2" package was used for PCA cluster investigation of the immune cell infiltration matrix. The present study adopted "corrplot" package for drawing the correlation heatmap for visualizing the correlation of 22 kinds of infiltrating immune cells. The "ggstatsplot" and "ggplot2" packages were adopted for analyzing the Spearman relationship between characteristic diagnostic markers and immune infiltrating cells and visualizing the result.

# RESULTS

## Screening of DEGs in Different Datasets

**Figure 1** illustrates a workflow of this study. There were 1,226 DEGs in GSE100191, including 207 upregulated and 1,019 downregulated genes (**Supplementary Table 2** and **Figure 2A**). Meanwhile, 58 DEGs were screened from the GSE17755 datasets, including 33 upregulated and 25 downregulated genes (**Supplementary Table 3** and **Figure 2B**). Next, 54 robust DEGs were screened in total with the RRA method (including 29 upregulated and 25 downregulated genes) (**Supplementary Table 4**).

## Functional Enrichment Analyses

Based on the results of the present study, the significantly enriched biological processes included immune response, regulation of natural killer cell–mediated immunity, regulation of chronic inflammatory response, adaptive immune response, innate immune response, etc. (**Figure 3A**). Moreover, antigen processing and presentation, endocytosis, natural killer cell–mediated cytotoxicity, primary immunodeficiency, and oxidative phosphorylation were considered to be the most remarkably enriched pathways (**Figure 3B**), and GSEA results presented the enriched mainly pathways (**Figure 3C**). The above results suggest that the immune system is critical to RA.

## Screening and Verification of Diagnostic Markers

The present study adopted LASSO logistic regression algorithm to identify 16 key biomarkers from DEGs (**Figures 4A, B**). Sixteen genes were identified as key biomarkers from DEGs by the SVM-RFE algorithm (**Figure 4C**). Moreover, 15 genes were identified as vital biomarkers with RF algorithm (**Figure 4D**). When 0.92 acted as the correlation coefficient threshold, the soft-thresholding power was selected as 20 (**Figures 5A, B**). In accordance with WGCNA analysis, six remarkable co-expression modules were built. As indicated from the

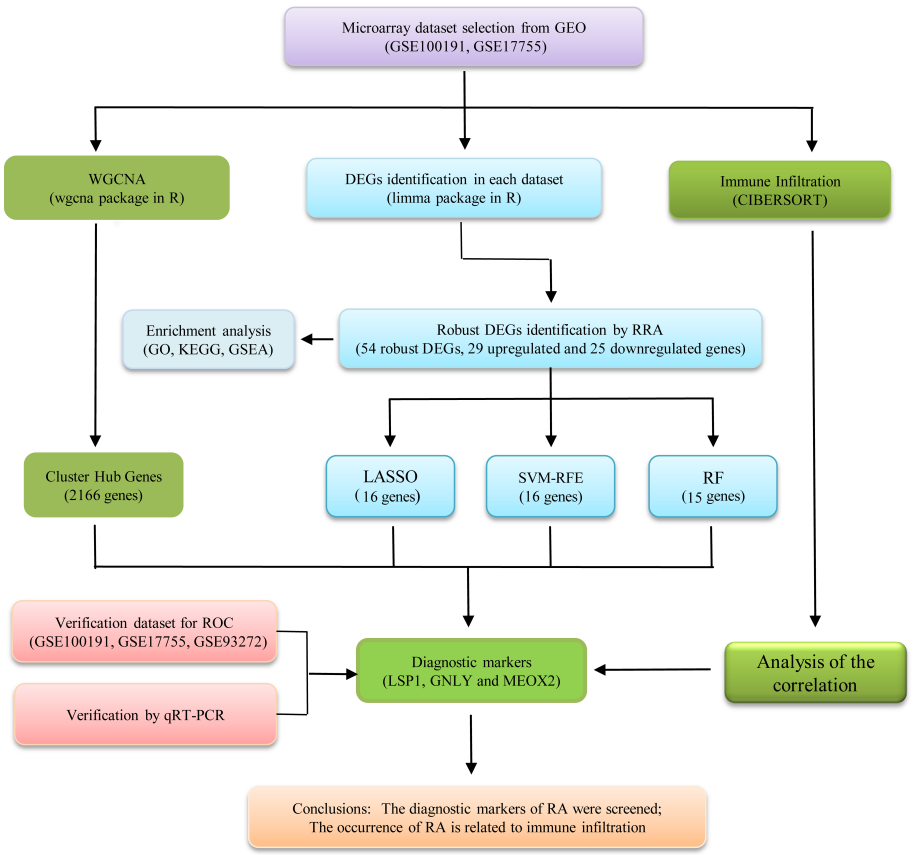


**FIGURE 1** | The flowchart of the analysis process.

investigations of module-trait correlations, multiple modules were related to RA (**Figure 5C**), and the turquoise module was the most significant one, with 2,166 genes included in total (**Figures 5D, E**). LSP1, GNLY, and MEOX2 were overlapping genes by the four algorithms including one upregulated (MEOX2) and two downregulated (LSP1 and GNLY) genes (**Figure 6A**). The ROC curves of LSP1, GNLY, and MEOX2 revealed their probability as valuable biomarkers with AUCs of 0.967, 0.854, and 0. 923, respectively (**Figure 6B**), indicating that the three biological markers had a high accuracy of predictive value.

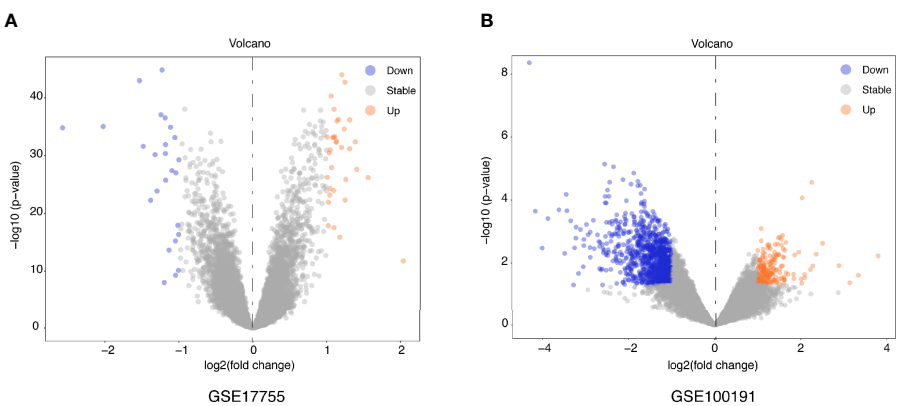


**FIGURE 2** | Volcano plots of DEGs distribution in GSE17755 **(A)** and GSE100191 **(B)**. Orange represented a high expression of robust DEG, while blue represented a low expression of robust DEG.

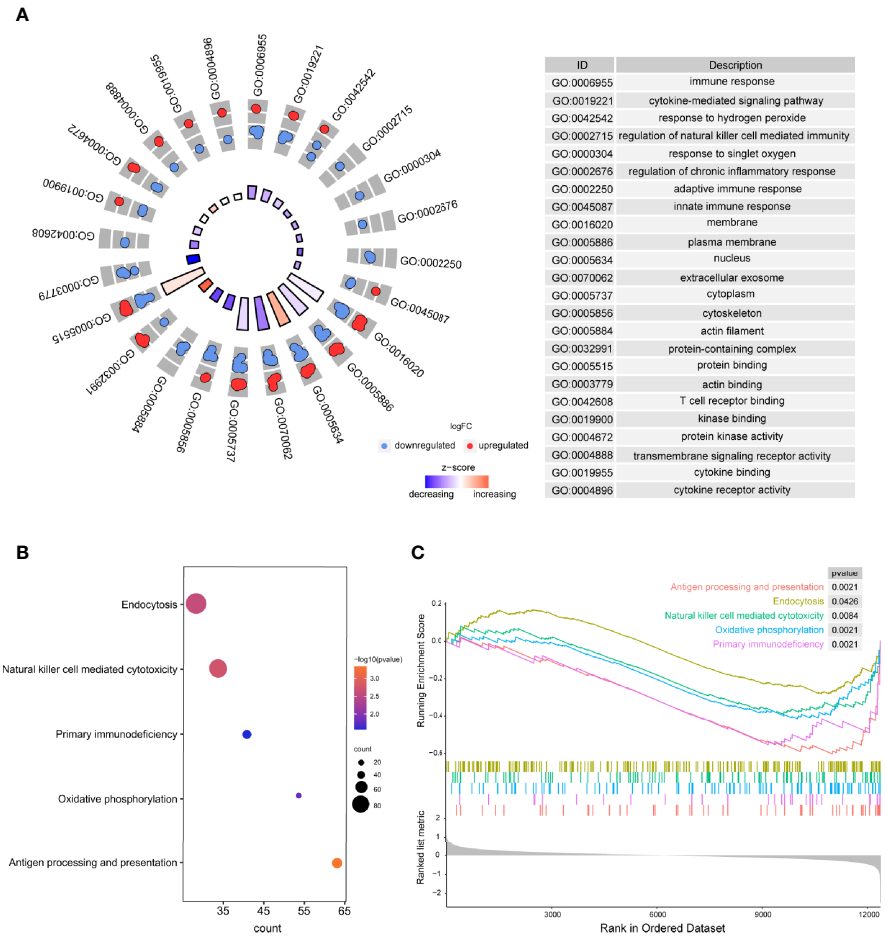


**FIGURE 3** | The results of functional enrichment analyses. **(A)** GO analyses results of DEGs; **(B)** Pathway analysis results of DEGs; **(C)** GSEA profiles depicting the five significant GSEA sets.

The expression levels of the three biomarkers were examined by qRT-PCR in 34 whole blood samples. Three biomarkers (LSP1, GNLY, and MEOX2) were reported to be significantly dysregulated in RA compared with healthy samples. LSP1 and GNLY showed the significant downregulation, while MEOX2 showed a significant upregulation in RA ($P$<0.005) (**Figures 6C–E**), indicating that the results were reproducible and reliable.

## Infiltration of Immune Cells Results

With the CIBERSORT algorithm, we first summarized the results obtained from 50 normal controls and 119 RA patients. By PCA, the proportions of immune cells from the whole blood of RA patients and normal controls displayed distinct group-bias clustering and individual differences (**Figure 7A**). As indicated from the correlation heatmap of the 22 types of immune cells, eosinophils and M0 macrophages, resting T cells CD4 memory and naive T cells CD4, T cells follicular helper and naive T cells CD4, and activated NK cells and resting NK cells displayed a significant negative correlation, respectively. M1 macrophages and monocytes, M1 macrophages and T cells CD8, resting mast cells and naive B cells, eosinophils and resting dendritic cells displayed significant positive correlations, respectively (**Figure 7B**). In comparison with normal samples, RA samples generally contained a higher proportion of resting NK cells, neutrophils, whereas the proportions of B cells memory, T cells CD8, activated NK cells, and M0 macrophages were relatively lower ($P$ < 0.05) (**Figure 7C**).

## Correlation Analysis Between Key Biomarkers and Infiltration-Related Immune Cells

Based on the results of correlation analysis, LSP1 displayed a positive correlation with memory B cells (r = 0.512, p = 0.011) and activated mast cells (r = 0.423, p = 0.024) and showed a negative correlation with activated dendritic cells (r = –0.382, p = 0.026) and activated T cells CD4 memory (r = –0.341, p = 0.037) (**Figure 8A**). GNLY showed a positive correlation with neutrophils (r = 0.321, p = 0.025) and showed a negative correlation with resting mast cells (r = –0.292, p = 0.012) and resting NK cells (r = 0.242, p = 0.026) (**Figure 8B**). MEOX2

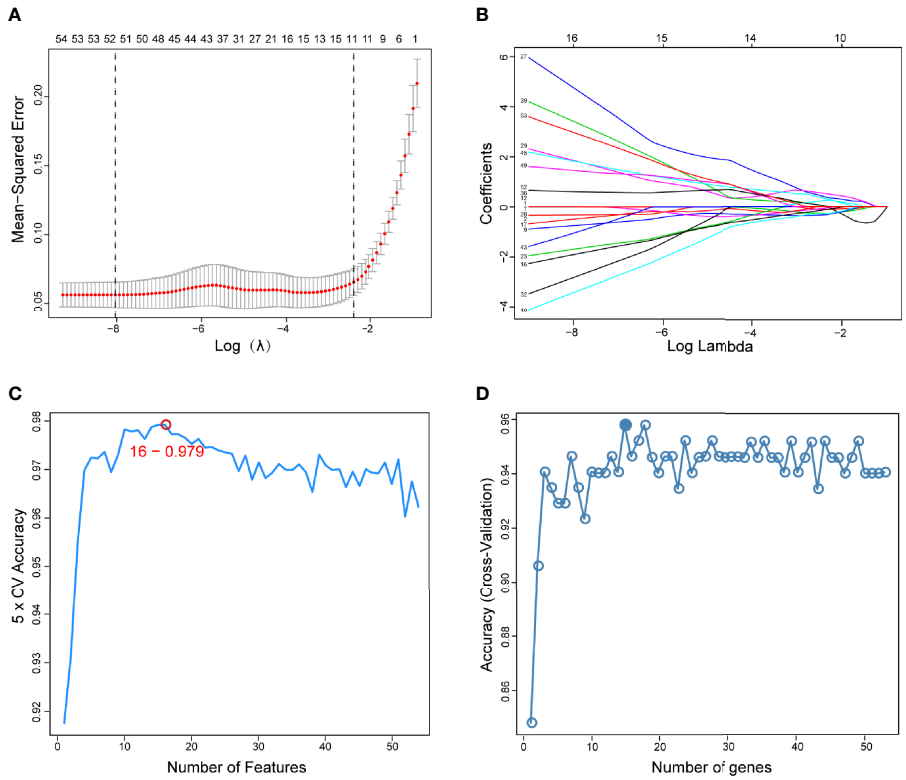


**FIGURE 4** | Screening of diagnostic markers *via* the comprehensive strategy. **(A)** Least absolute shrinkage and selection operator (LASSO) logistic regression algorithm to screen diagnostic markers; **(B)** Different colors represent different genes; **(C, D)** Based on support vector machine-recursive feature elimination (SVM-RFE) and random forest (RF) algorithm to screen biomarkers.

showed a positive correlation with M2 macrophages (r = 0.382, p = 0.033) and activated T cells CD4 memory (r = 0.282, p = 0.045) and showed a negative correlation with monocytes (r = 0.202, p = 0.039) (**Figure 8C**).

## DISCUSSION

RA primarily features chronic synovitis, systemic inflaming process, as well as the arrival of autoantibodies, leading to chronic inflammation, joint damage, and dysfunction of other vital organs. Moreover, according to existing articles, infiltration of immune cells noticeably impacts on RA's occurrence and progresses (8, 26). Therefore, it is of profound significance to search for particular diagnosis marker and analyze the infiltration patterns pertaining to RA immune cells in terms of facilitating RA cases' prognosis. Here, an attempt was made for finding diagnosis marker pertaining to RA and delving into the effect exerted by infiltration of immune cells within RA.

In our study, we identified 54 robust DEGs, covering 29 risen and 25 declined DEGs, by comparing genes expressed in RA and normal samples. Afterwards, the DEGs underwent the annotation based on function-related enrichment study. The mentioned genes displayed tight associations to immune responses and inflaming signals (e.g., immune responses, regulation of natural killer cell–mediated immunity, responses to singlet oxygen, regulation of chronic inflammatory response and adaptive immune response). KEGG channels undergoing the enriching process covered endocytosis, cytotoxicity under the mediation of natural killer cell, antigen-presenting process and processing, primary immunodeficiency, and oxidative phosphorylation. Based on GO and KEGG enrichment study, RA achieved robust immune activating process and immune cell involvements, largely causing RA synovial inflaming process, thereby inducing arthralgia and arthritis. Generally, arthralgia and arthritis refer to the major RA clinically related reflections (3).

The model of random forest (RF) refers to a non-parametric approach to achieve the classifying process under the supervision (21). RF covers decision tree respectively originating from data subdivided set. The present work conducted the training and analysis for one RF classifying model for identifying descriptors that could discriminate RA from general sample. LASSO logistic regression, one machine-learning algorithm, determined variables by searching for λ under the smallest probability of classification error (22). SVM Recursive Feature Elimination (SVM-RFE) refers to an approach for machine learning and achieves extensive applications to rank features and to select the significant ones for classification (23). WGCNA refers to an

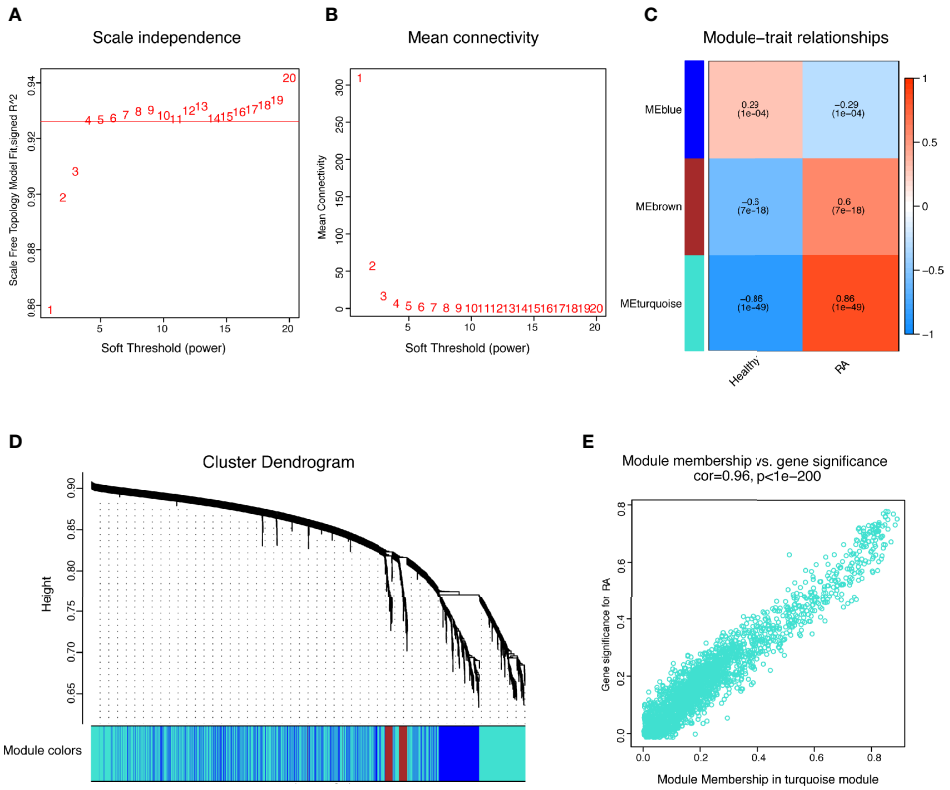


**FIGURE 5** | WGCNA revealed gene co-expression networks in the whole peripheral blood of 169 RA patients. **(A, B)** Analysis of the scale-free fit index and the mean connectivity for various soft-thresholding powers; **(C)** Relationships of consensus modules with samples. It contains a set of highly linked genes. Each specified color represents a specific gene module; **(D)** Clustering dendrogram of differentially expressed genes related to RA; **(E)** The gene significance for RA in the turquoise module (one dot represents one gene in the turquoise module).

approach for investigating gene expressing modes within sample. Gene exhibiting consistent expressing modes underwent the clustering process, and the relations of the module with particular trait or phenotype can be determined (24). We integrated the four different algorithms, each of which had its own inherent characteristics. Finally, LSP1, GNLY, and MEOX2 were selected and were accurate for in-depth verifications here, which suggested that our prediction exhibited the feasibility by the integration strategy.

Lymphocyte-specific protein 1 (LSP1) is capable of encoding intracellular F-actin-binding protein (27), achieving the expressions within endothelial cell, macrophage, neutrophil, and lymphocyte and regulating neutrophils' movement, fibrinogen matrix protein adhesion, and transendothelial migration (28). The F-actin-bound cytoskeleton protein LSP1 has been identified as a regulator of neutrophil chemotaxis during inflammation (29, 30). Hwang et al. reported that cases with RA had reduced LSP1 expression in peripheral blood T cell, but improved migration ability, indicating that defects in the LSP1 signaling pathway lowered T-cell activation threshold (cell migrating process) in RA cases (27). LSP1 regulates a variety of biological processes in immune cells. However, immune cell, largely comprising macrophage, T cell, and B cell, to be autoimmune disease, critically impacts the pathogenesis of RA. Granulysin (GNLY), a member of the saponin family, has a location within the cytotoxic granules pertaining to T cells and is released in response to antigen stimulation. GNLY is a cytotoxic granuloprotein secreted by cytotoxic T lymphocytes and natural killer cells (31, 32). Although many studies have evaluated serum GNLY as a biomarker in cases with solid or hematological malignancies (33, 34), few studies have reported serum GNLY concentrations in RA cases (35). Mesenchymal homobox 2 (MEOX2) encodes a member of a non-aggregated, divergent, tentacle-like homobox gene subfamily. The MEOX family includes two homologous domain proteins, MEOX1 and MEOX2, which have 95% sequence homology in the homologous domain and are required for the normal development of bone and muscle in mouse embryos (36). MEOX2 expression is inhibited by zinc finger binding protein (37), and the abnormal expression of zinc finger protein displays a tight relation with RA occurrence and progresses (38, 39). Accordingly, this work infers that MEOX2 is likely to critically impact RA progresses. Considering the above findings, LSP1, GNLY, and MEOX2 are likely to impact RA progresses and act as diagnosis markers, whereas a large number of clinically related articles are further required for the verification of the diagnosis significance for LSP1, GNLY, as well as MEOX2.

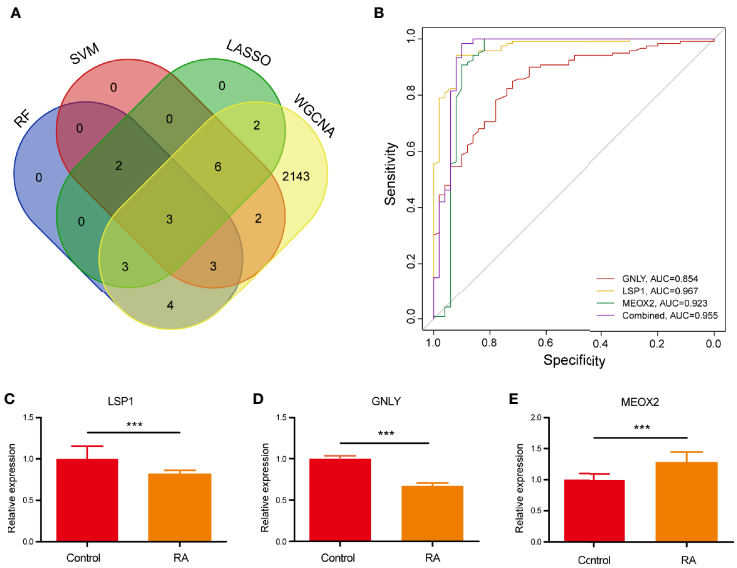


**FIGURE 6** | **(A)** Venn diagram showed the intersection of diagnostic markers obtained by the four algorithms. **(B)** The ROC curve of the diagnostic efficacy verification after fitting three diagnostic markers to one variable. **(C–E)** The miRNA expressions of potential diagnostic markers were validated by qRT-PCR. ***P < 0.001.

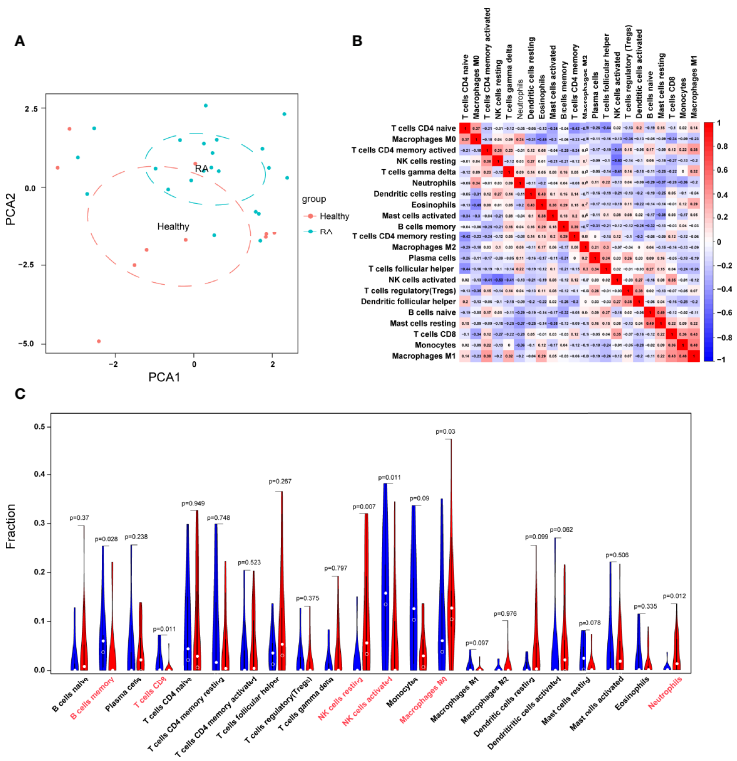


**FIGURE 7** | Evaluation and visualization of immune cell infiltration. **(A)** PCA cluster plot of immune cell infiltration between RA samples and control samples. **(B)** Heatmap of correlation in 22 types of immune cells. The size of the colored squares represents the strength of the correlation; red represents a positive correlation, and blue represents a negative correlation. Darker color implies stronger association. **(C)** Violin diagram of the proportion of 22 types of immune cells. The red marks represent the difference in infiltration between the two groups of samples.

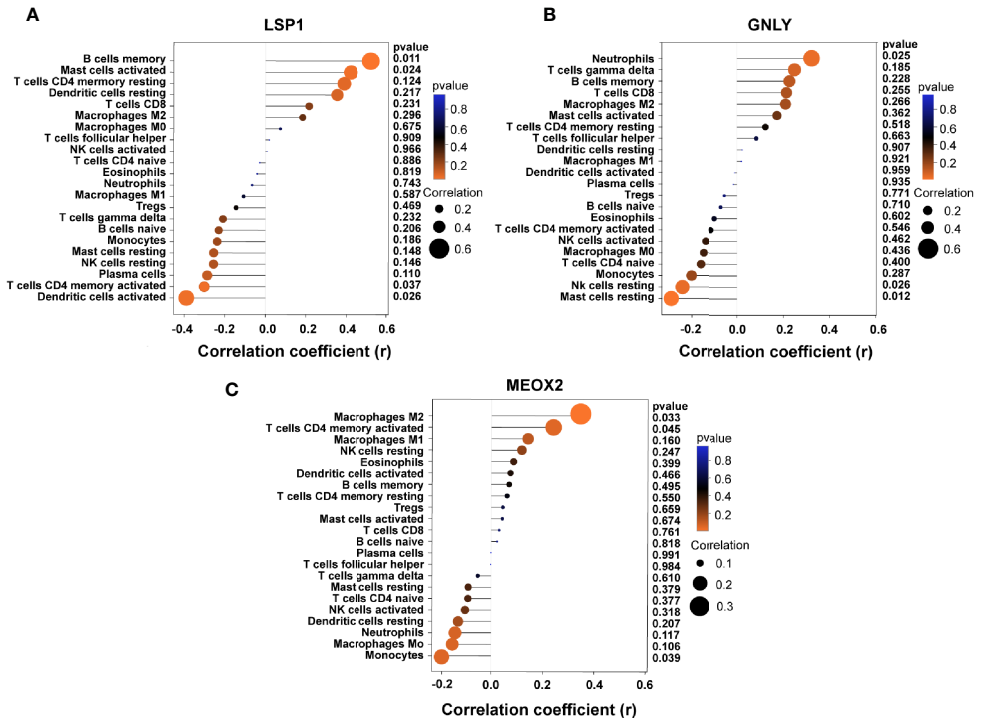


**FIGURE 8** | Correlation between diagnostic markers and infiltrating immune cells. **(A)** Correlation between LSP1 and infiltrating immune cells. **(B)** Correlation between GNLY and infiltrating immune cells. **(C)** Correlation between MEOX2 and infiltrating immune cells. The size of the dots represents the strength of the correlation between genes and immune cells; the larger the dots, the stronger the correlation, and the smaller the dots, the weaker the correlation. The color of the dots represents the *p*-value; the greener the color, the lower the *p*-value; and the red the color, the larger the *p*-value. $P < 0.05$ was considered statistically significant.

To more specifically examine effects exerted by infiltration of immune cell in RA, the present study applied CIBERSORT for assessing the immune infiltrating process within RA. The infiltration of resting NK cells and neutrophils increased, while the infiltration of B cell memory, T cell CD8, activated NK cells, and M0 macrophages decreased, probably showing associations with RA occurrence and progresses. It is well known that B cells are a vital part pertaining to human adaptive immunity, whereas these cells under RA become a possible factor in RA pathogenesis (40). Local synthesizing process for cytokine (e.g., IL-1α, IL-23, IL-12, IL-6, and TNF-α) under the induction from local autoreactive B cell was suggested to impact pathology-associated RA cells, triggering bone injury, inflammation, and immune disorder (41, 42). It is currently evidenced that CD4+ T helper cells impact the pathogenesis of RA largely *via* the secreting process for cytokine and chemokine. Type 1 T-helper cells achieve the significant activation within RA and secrete pro-inflammation cytokines (e.g., IFN-γ, IL-2, and TNF-α) (43). CD8+ T cells exhibit anti-inflammation characteristic and are likely to contribute to the reduction of ongoing autoimmune responses in rheumatoid joints (8). CD56+ NK cells were overexpressed and produced higher levels of IFN-γ in inflammatory joints compared with NK cell of peripheral blood (44). Nevertheless, NK cell's exact mechanism continues to be unclear. Under normal conditions (45), most macrophages exist in the tissue in a resting state. However, in inflammatory joints, they conduct the regulation of the secreting process for pro-inflammatory cytokine and injury-associated enzyme under the relation to the inflaming response and afterwards trigger joint destructing process (45). Although this has been mentioned many times, further research into the molecular mechanisms and functions of immune cell infiltration in rheumatoid arthritis is urgently needed.

Based on the investigation of the correlations of immune cell and diagnostic signatures, LSP1 suggested a positive correlation with memory B cell and mast cell under the activation and negative correlations to activated dendritic cell and activated T cell CD4 memory. GNLY showed positive correlations to neutrophils and showed negative correlations to resting mast cell and resting NK cell. MEOX2 showed positive correlations to M2 macrophage and activated T cell CD4 memory and a negative correlation with monocytes. Interestingly, a study reported found that cases with RA achieved declining LSP1 expressing state within peripheral T cell with improved migratory ability, thereby demonstrating that defects within LSP1 signaling lead to the decline of T-cell activation threshold (46). Kulkarni et al. reported that LSP1 underwent the interacting process with the interferon-inducible protein inside dendritic cell for facilitating surface-bound HIV-1 endocytosis and early endosome forming processes (47). Granulysin refers to

one protein in the granules of natural killer cell and human cytotoxic T lymphocyte, exhibiting cytolysis activity against tumor and microbe (32), whereas there is no information concerning the mechanisms involved in RA. Due to a relatively small amount of research, the sophisticated interacting processes of gene and immune cell should be investigated in depth based on the mentioned assumption.

New science approaches (e.g., RF, LASSO logistic regression, WGCNA, and SVM-RFE algorithm) were used for identifying RA diagnosis-related markers. Besides, CIBERSORT was used for investigating infiltration of immune cells. Nevertheless, this study is subject to some limits. The CIBERSORT investigation complies with confined genetic information probably deviating from cellular heterogeneity interacting process, disease-induced diseases, or phenotypic plastic property. Moreover, this study indicates a 2$^{nd}$ mining and investigation for existing datasets. Though the results of several existing studies show no consistency with the result of this analysis, whether the results here are reliable should receive in-depth verification by experiments with large samples.

## CONCLUSIONS

In brief, this study reported that LSP1, GNLY, and MEOX2 refer to diagnostic markers of RA. This study also reported that resting NK cells, neutrophils, memory B cells, T cells CD8, activated NK cells, and M0 macrophages are likely to participate in the occurrence and progress of RA. In addition, LSP1 was significantly associated with memory B cells, activated mast cells, activated dendritic cells, activated T cells CD4 memory; GNLY was significantly associated with neutrophils, resting mast cells, resting NK cells; MEOX2 was significantly associated with M2 macrophages, activated T cells CD4 memory, monocytes. The mentioned immune cells are likely to critically impact RA development, and the in-depth exploration of the immune cells is likely to ascertain the targets in immunotherapy and help optimize immunomodulatory therapy for RA patient.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Committee of Fuzhou Second Hospital affiliated to Xiamen University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

YYZ and RY: conception and design of the study and funding acquisition. RY, JZ, and YGZ: data acquisition, bioinformatics analysis, and drafting and critical revision of the manuscript. RY, XH, ST, and JY: visualization and validation. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724934/full#supplementary-material

**Supplementary Table 1 |** The characteristic of three datasets.

**Supplementary Table 2 |** The list of differentially expressed genes of GSE100191.

**Supplementary Table 3 |** The list of differentially expressed genes of GSE17755.

**Supplementary Table 4 |** Fifty-four robust DEGs were screened with the RRA method from the two lists of differentially expressed genes.

## REFERENCES

1. Müller-Ladner U, Pap T, Gay RE, Neidhart M, Gay S. Mechanisms of Disease: The Molecular and Cellular Basis of Joint Destruction in Rheumatoid Arthritis. *Nat Clin Pract Rheumatol* (2005) 1:102–10. doi: 10.1038/ncprheum0047
2. van der Woude D, van der Helm-van Mil AHM. Update on the Epidemiology, Risk Factors, and Disease Outcomes of Rheumatoid Arthritis. *Best Pract Res Clin Rheumatol* (2018) 32:174–87. doi: 10.1016/j.berh.2018.10.005
3. Smolen JS, Aletaha D, McInnes IB. Rheumatoid Arthritis. *Lancet (London England)* (2016) 388:2023–38. doi: 10.1016/s0140-6736(16)30173-8
4. Aletaha D, Smolen JS. Diagnosis and Management of Rheumatoid Arthritis: A Review. *Jama* (2018) 320:1360–72. doi: 10.1001/jama.2018.13103
5. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Ann Rheum Dis* (2010) 69:1580–8. doi: 10.1136/ard.2010.138461
6. Won P, Kim Y, Jung H, Rim YA, Sohn DH, Robinson WH, et al. Pathogenic Role of Circulating Citrullinated Antigens and Anti-Cyclic Monoclonal Citrullinated Peptide Antibodies in Rheumatoid Arthritis. *Front Immunol* (2021) 12:692242. doi: 10.3389/fimmu.2021.692242

7. Kinne RW, Stuhlmüller B, Burmester GR. Cells of the Synovium in Rheumatoid Arthritis. Macrophages. *Arthritis Res Ther* (2007) 9:224. doi: 10.1186/ar2333
8. Carvalheiro H, da Silva JA, Souto-Carneiro MM. Potential Roles for CD8(+) T Cells in Rheumatoid Arthritis. *Autoimmun Rev* (2013) 12:401–9. doi: 10.1016/j.autrev.2012.07.011
9. Higashioka K, Yoshimura M, Sakuragi T, Ayano M, Kimoto Y, Mitoma H, et al. Human PD-1(Hi)CD8(+) T Cells Are a Cellular Source of IL-21 in Rheumatoid Arthritis. *Front Immunol* (2021) 12:654623. doi: 10.3389/fimmu.2021.654623
10. Bugatti S, Bogliolo L, Montecucco C, Manzo A. B Cell Autoimmunity and Bone Damage in Rheumatoid Arthritis. *Reumatismo* (2016) 68:117–25. doi: 10.4081/reumatismo.2016.914
11. Huang J, Fu X, Chen X, Li Z, Huang Y, Liang C. Promising Therapeutic Targets for Treatment of Rheumatoid Arthritis. *Front Immunol* (2021) 12:686155. doi: 10.3389/fimmu.2021.686155
12. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust Enumeration of Cell Subsets From Tissue Expression Profiles. *Nat Methods* (2015) 12:453–7. doi: 10.1038/nmeth.3337
13. Macías-Segura N, Castañeda-Delgado JE, Bastian Y, Santiago-Algarra D, Castillo-Ortiz JD, Alemán-Navarro AL, et al. Transcriptional Signature Associated With Early Rheumatoid Arthritis and Healthy Individuals at High Risk to Develop the Disease. *PloS One* (2018) 13:e0194205. doi: 10.1371/journal.pone.0194205
14. Lee HM, Sugino H, Aoki C, Nishimoto N. Underexpression of Mitochondrial-DNA Encoded ATP Synthesis-Related Genes and DNA Repair Genes in Systemic Lupus Erythematosus. *Arthritis Res Ther* (2011) 13:R63. doi: 10.1186/ar3317
15. Tasaki S, Suzuki K, Kassai Y, Takeshita M, Murota A, Kondo Y, et al. Multi-Omics Monitoring of Drug Response in Rheumatoid Arthritis in Pursuit of Molecular Remission. *Nat Commun* (2018) 9:2755. doi: 10.1038/s41467-018-05044-4
16. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, Normalization, and Summaries of High Density Oligonucleotide Array Probe Level Data. *Biostatistics (Oxford England)* (2003) 4:249–64. doi: 10.1093/biostatistics/4.2.249
17. Naorem LD, Muthaiyan M, Venkatesan A. Identification of Dysregulated miRNAs in Triple Negative Breast Cancer: A Meta-Analysis Approach. *J Cell Physiol* (2019) 234:11768–79. doi: 10.1002/jcp.27839
18. Yu G, Wang LG, Han Y, He QY. Clusterprofiler: An R Package for Comparing Biological Themes Among Gene Clusters. *Omics* (2012) 16:284–7. doi: 10.1089/omi.2011.0118
19. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles. *Proc Natl Acad Sci U S A* (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
20. Strobl C, Boulesteix AL, Zeileis A, Hothorn T. Bias in Random Forest Variable Importance Measures: Illustrations, Sources and a Solution. *BMC Bioinformatics* (2007) 8:25. doi: 10.1186/1471-2105-8-25
21. Wang H, Yang F, Luo Z. An Experimental Study of the Intrinsic Stability of Random Forest Variable Importance Measures. *BMC Bioinformatics* (2016) 17:60. doi: 10.1186/s12859-016-0900-5
22. Tibshirani R. Regression Shrinkage and Selection via the Lasso. *J R Stat Soc Series B (Methodol)* (1996) 58:267–88. doi: 10.1111/j.2517-6161.1996.tb02080.x
23. Suykens JAK, Vandewalle J. Least Squares Support Vector Machine Classifiers. *Neural Process Lett* (1999) 9:293–300. doi: 10.1023/A:1018628609742
24. Zhang B, Horvath S. A General Framework for Weighted Gene Co-Expression Network Analysis. *Stat Appl Genet Mol Biol* (2005) 4:Article17. doi: 10.2202/1544-6115.1128
25. Langfelder P, Horvath S. WGCNA: An R Package for Weighted Correlation Network Analysis. *BMC Bioinformatics* (2008) 9:559. doi: 10.1186/1471-2105-9-559
26. Ge Y, Zhou L, Chen Z, Mao Y, Li T, Tong P, et al. Identification of Differentially Expressed Genes, Signaling Pathways and Immune Infiltration in Rheumatoid Arthritis by Integrated Bioinformatics Analysis. *Hereditas* (2021) 158:5. doi: 10.1186/s41065-020-00169-3
27. Jongstra-Bilen J, Jongstra J. Leukocyte-Specific Protein 1 (LSP1): A Regulator of Leukocyte Emigration in Inflammation. *Immunol Res* (2006) 35:65–74. doi: 10.1385/ir:35:1:65
28. Wu Y, Zhan L, Ai Y, Hannigan M, Gaestel M, Huang CK, et al. MAPKAPK2-Mediated LSP1 Phosphorylation and FMLP-Induced Neutrophil Polarization. *Biochem Biophys Res Commun* (2007) 358:170–5. doi: 10.1016/j.bbrc.2007.04.104
29. Jongstra-Bilen J, Misener VL, Wang C, Ginzberg H, Auerbach A, Joyner AL, et al. LSP1 Modulates Leukocyte Populations in Resting and Inflamed Peritoneum. *Blood* (2000) 96:1827–35. doi: 10.1182/blood.V96.5.1827
30. Wang C, Hayashi H, Harrison R, Chiu B, Chan JR, Ostergaard HL, et al. Modulation of Mac-1 (CD11b/CD18)-Mediated Adhesion by the Leukocyte-Specific Protein 1 Is Key to Its Role in Neutrophil Polarization and Chemotaxis. *J Immunol (Baltimore Md 1950)* (2002) 169:415–23. doi: 10.4049/jimmunol.169.1.415
31. Krensky AM, Clayberger C. Biology and Clinical Relevance of Granulysin. *Tissue Antigens* (2009) 73:193–8. doi: 10.1111/j.1399-0039.2008.01218.x
32. Clayberger C, Krensky AM. Granulysin. *Curr Opin Immunol* (2003) 15:560–5. doi: 10.1016/s0952-7915(03)00097-9
33. Nagasawa M, Ogawa K, Nagata K, Shimizu N. Serum Granulysin as a Possible Biomarker of Natural Killer Cell Neoplasms. *Br J Haematol* (2010) 148:812–4. doi: 10.1111/j.1365-2141.2009.07999.x
34. Sekiguchi N, Asano N, Ito T, Momose K, Momose M, Ishida F. Elevated Serum Granulysin and Its Clinical Relevance in Mature NK-Cell Neoplasms. *Int J Hematol* (2012) 96:461–8. doi: 10.1007/s12185-012-1159-x
35. Zhang F, Wei K, Slowikowski K, Fonseka CY, Rao DA, Kelly S, et al. Defining Inflammatory Cell States in Rheumatoid Arthritis Joint Synovial Tissues by Integrating Single-Cell Transcriptomics and Mass Cytometry. *Nat Immunol* (2019) 20:928–42. doi: 10.1038/s41590-019-0378-1
36. Mankoo BS, Skuntz S, Harrigan I, Grigorieva E, Candia A, Wright CV, et al. The Concerted Action of Meox Homeobox Genes Is Required Upstream of Genetic Pathways Essential for the Formation, Patterning and Differentiation of Somites. *Development (Cambridge England)* (2003) 130:4655–64. doi: 10.1242/dev.00687
37. Chen Y, Banda M, Speyer CL, Smith JS, Rabson AB, Gorski DH. Regulation of the Expression and Activity of the Antiangiogenic Homeobox Gene GAX/MEOX2 by ZEB2 and microRNA-221. *Mol Cell Biol* (2010) 30:3902–13. doi: 10.1128/mcb.01237-09
38. Liu Q, Niu N, Wada Y, Liu J. The Role of Cdkn1A-Interacting Zinc Finger Protein 1 (CIZ1) in DNA Replication and Pathophysiology. *Int J Mol Sci* (2016) 17:212. doi: 10.3390/ijms17020212
39. Fattah SA, Abdel Fattah MA, Mesbah NM, Saleh SM, Abo-Elmatty DM, Mehanna ET. The Expression of Zinc Finger 804a (ZNF804a) and Cyclin-Dependent Kinase 1 (CDK1) Genes Is Related to the Pathogenesis of Rheumatoid Arthritis. *Arch Physiol Biochem* (2020) 16:1–6. doi: 10.1080/13813455.2020.1716810
40. Bugatti S, Vitolo B, Caporali R, Montecucco C, Manzo A. B Cells in Rheumatoid Arthritis: From Pathogenic Players to Disease Biomarkers. *BioMed Res Int* (2014) 2014:681678. doi: 10.1155/2014/681678
41. Schlegel PM, Steiert I, Kötter I, Müller CA. B Cells Contribute to Heterogeneity of IL-17 Producing Cells in Rheumatoid Arthritis and Healthy Controls. *PloS One* (2013) 8:e82580. doi: 10.1371/journal.pone.0082580
42. Yan L, Liang M, Yang T, Ji J, Jose Kumar GSSreena, Hou X, et al. The Immunoregulatory Role of Myeloid-Derived Suppressor Cells in the Pathogenesis of Rheumatoid Arthritis. *Front Immunol* (2020) 11:568362. doi: 10.3389/fimmu.2020.568362
43. Meednu N, Zhang H, Owen T, Sun W, Wang V, Cistrone C, et al. Production of RANKL by Memory B Cells: A Link Between B Cells and Bone Erosion in Rheumatoid Arthritis. *Arthritis Rheumatol (Hoboken NJ)* (2016) 68:805–16. doi: 10.1002/art.39489
44. Shegarfi H, Naddafi F, Mirshafiey A. Natural Killer Cells and Their Role in Rheumatoid Arthritis: Friend or Foe? *ScientificWorldJournal* (2012) 2012:491974. doi: 10.1100/2012/491974
45. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester GR. Macrophages in Rheumatoid Arthritis. *Arthritis Res* (2000) 2:189–202. doi: 10.1186/ar86
46. Hwang SH, Jung SH, Lee S, Choi S, Yoo SA, Park JH, et al. Leukocyte-Specific Protein 1 Regulates T-Cell Migration in Rheumatoid Arthritis. *Proc Natl Acad Sci U S A* (2015) 112:E6535–6543. doi: 10.1073/pnas.1514152112
47. Kulkarni R, Jiang S, Birrane G, Prasad A. Lymphocyte-Specific Protein 1 (LSP1) Regulates Bone Marrow Stromal Cell Antigen 2 (BST-2)-Mediated

Intracellular Trafficking of HIV-1 in Dendritic Cells. *FEBS Lett* (2020) 594:1947–59. doi: 10.1002/1873-3468.13788

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# Anti-Carbamylated Fibrinogen Antibodies Might Be Associated With a Specific Rheumatoid Phenotype and Include a Subset Recognizing *In Vivo* Epitopes of Its γ Chain One of Which Is Not Cross Reactive With Anti-Citrullinated Protein Antibodies

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To identify the targets recognized by anti-carbamylated protein antibodies (anti-CarP) in patients with early Rheumatoid Arthritis (RA), to study the cross-reactivity between anti-CarP and anti-citrullinated protein antibodies (ACPA) and to evaluate their prognostic value. 331 patients (184 RA and 147 other rheumatisms) from the Very Early Arthritis (VErA) French cohort were analyzed. We performed mass spectrometry analysis of RA sera displaying anti-CarP activity and epitope mapping of the carbamylated fibrinogen γ chain to identify immunodominant peptides. The specificity of these targets was studied using competition assays with the major antigens recognized by ACPA. The prognostic value of anti-carbamylated fibrinogen IgG antibodies (ACa-Fib IgG) was compared to that of anti-cyclic citrullinated peptide antibodies (anti-CCP) and anti-CarP using an in-house ELISA. Besides the α chain, the γ chain of fibrinogen, particularly one immunodominant epitope that has a specific reactivity, was identified as a circulating carbamylated target in sera. The prevalence of ACa-Fib was 37% at baseline and 10.9% for anti-CCP-negative RA. In anti-CCP-negative patients, ACa-Fib positivity was associated with a more inflammatory and erosive disease at baseline but not with rapid radiological progression, which remains strongly related to anti-CCP antibodies. Fibrinogen seems to be one of the antigens recognized *in vivo* by the anti-CarP response, particularly 2 epitopes of the γ chain, one of which is not cross reactive with ACPA. This specificity might be associated with a distinct clinical phenotype since ACa-Fib IgG were shown to be linked to systemic inflammation in very early RA but not to rapid radiological progression.

**Keywords: very early rheumatoid arthritis, anti-carbamylated protein antibodies (anti-CarP), fibrinogen, ACPA, prognosis**

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## INTRODUCTION

Anti-citrullinated protein antibodies (ACPA), the hallmark of rheumatoid arthritis (RA), are of both diagnostic and prognostic interest (1, 2). Besides citrulline, other post-translational modifications (PTMs) are currently being studied, including carbamylation. In 2011, Shi et al. detected anti-carbamylated protein antibodies (anti-CarP) in the serum of RA patients, 45% of IgG isotype and 43% of IgA isotype. These antibodies might display diagnostic value since, among ACPA-negative patients, 16% and 30% had anti-CarP antibodies of IgG and IgA isotype respectively (3). They also have a prognostic value since their presence signals a form of RA with more severe radiological damage (4). Carbamylated Fetal Calf Serum (FCS) is the substrate currently used for enzyme-linked immunosorbent assay (ELISA) tests, which represents the gold standard. However, FCS contains many antigenic targets that remain undetermined. To better understand the clinical interest of anti-CarP, it seems relevant to identify proteins that are spontaneously carbamylated *in vivo*. Among the antigens tested (vimentin, GRP78, albumin, etc.) (5–7), carbamylated fibrinogen was the most recognized by these antibodies (8, 9). Moreover, Jones et al. showed that the anti-carbamylated fibrinogen response primarily targeted the fibrinogen β-chain (10). Finally, some studies suggest that there is cross-reactivity between ACPA and anti-CarP (11, 12).

The primary objective of this study was to identify the targets recognized by anti-CarP in the in the serum of RA patients. Secondary objectives were to determine whether the anti-carbamylated fibrinogen (ACa-Fib) antibody response was specific and different to anti-cyclic citrullinated peptide (anti-CCP) antibody response, in other words, to study the cross-reactivity between ACa-Fib and anti-citrullinated fibrinogen antibodies, and to evaluate their prognostic value in the Very Early Arthritis (VErA) cohort.

## PATIENTS AND METHODS

### Patients and Serum Samples

VErA is a multicenter cohort that prospectively included patients with early inflammatory rheumatism. The inclusion criteria were that participants were at least 18 years old and had at least 2 swollen joints for at least 4 weeks, which had been evolving for less than 6 months (median symptom duration of 4 months). Patients had to be corticosteroid and disease-modifying anti-rheumatic drug (DMARD) naïve. Patients with inflammatory back pain were excluded. A biological assessment and X-rays of the wrists, hands, and forefeet (and all other painful joints) were performed at inclusion and regular intervals. Patients were followed up every 6 months for 10 years. A total of 331 patients were included from 1998 to 2002, 184 patients with RA and 147 patients classified as having "other rheumatic diseases" (systemic lupus erythematosus, ANCA associated vasculitis, Gougerot Sjögren's syndrome, systemic sclerosis, psoriatic arthritis, mixed connective tissue diseases, crystal-induced arthritis) or "undifferentiated rheumatic disease". Sera used as controls (n=100) were obtained from a serum bank of healthy individuals at the Établissement Français du Sang (EFS). For the clinical part of the present study, only sera from the 184 RA patients meeting the ACR 2010 criteria (which were applied when available) were used (13). This study was approved by the Upper Normandy Ethics Committee (file: 95/138/HP).

### Proteomic Analysis: Enzymatic Digestion of Proteins and Nano LC-MS/MS Analysis

To perform proteomic analysis from VErA sera samples known to be ACa-FCS-positive, protein concentration was determined using the standard Bradford method (Biorad). Enzymatic digestion of proteins in sera samples has been described previously (14). Identification of carbamylated peptides was performed with a classic setup of mass spectrometry analysis using a Q-exactive Plus (ThermoScientific) equipped with a nanoESI source, as described previously (15). All spectra obtained were exported in "raw" format to identify peptides and proteins with Proteome Discoverer 1.4 software (Thermo Scientific). Peak lists were searched using MASCOT search software (Matrix Science) against the *human* Swissprot database with the following parameters: one miss cleavage site allowed, carbamidomethylation on cysteine, oxidation on methionine, and carbamylation on lysine as variable modifications. The parent-ion and daughter-ion tolerances were 5ppm and 0.02Da respectively.

Proteomic data deposit on ProteomeXchange *via* the PRIDE database (Project accession: PXD028121).

### Antigens and Carbamylation

Fetal calf serum (Sigma-Aldrich) and peptides of the γ chain of fibrinogen were incubated with potassium cyanate 1 M (Sigma-Aldrich) at 4 mg/mL and 2 to 4 mg/mL respectively during 12 hours at 37°C and dialyzed against water. Human fibrinogen, purified from human plasma (F3879, Sigma-Aldrich), was incubated with potassium cyanate 0.5 M for 3 days and dialyzed against water. Carbamylation of protein/peptide was determined by ELISA (OxiSelec Protein Carbamylation Sandwich ELISA Kit, Cell Biolabs).

### Detection of Anti-CarP Antibodies of IgG Isotype

Detection of IgG autoantibodies recognizing carbamylated FCS (ACa-FCS), carbamylated fibrinogen (ACa-Fib), and carbamylated peptides of the γ chain of fibrinogen (ACa-Fib γ chain) was performed using home-made ELISA. Carbamylated and native proteins/peptides were coated at 10 µg/mL in carbonate-bicarbonate buffer on Nunc Maxisorp plates (Thermo Scientific) and incubated overnight at 4°C. For blocking, plates were incubated with PBS BSA 1% for 5 hours at 4°C. Sera from healthy controls (from Etablissement Français du Sang, EFS) or patients were diluted (1/50) in PBS BSA 1% Tween 0.05% and incubated overnight. Plates were incubated with biotinylated anti-human IgG (SouthernBiotech) diluted (1/3000) in PBS BSA 1% Tween 0.05% for 1h15 and HRP-conjugated streptavidin (1/25000) (Thermo Scientific) for 30 minutes at 4°C before the addition of

tetramethylbenzidine substrate solution and STOP buffer (Sigma-Aldrich). Washing Washing with PBS Tween 0.05% was carried out between steps. The reactivity to native proteins/peptides was subtracted from that of the corresponding carbamylated protein/peptide. The absorbance was measured at 415 nm and converted to arbitrary units (AU): we performed a range with an anti-carbamylation (homocitrulline) monoclonal antibody (Cayman Chemical) (serial dilution at ½ with a first range point dilution at 1/2000) with the same protocol as previously described. The threshold of positivity was determined at 38.12 AU for ACa-FCS IgG (healthy controls (n=100) mean + 3 standard deviations) and 19.47 AU for ACa-Fib IgG (after performing a ROC curve using data obtained from healthy controls (n=100) and RA patients (n=184) of the VErA cohort).

## Detection of Anti-CCP Antibodies

Detection of anti-CCP antibodies was performed using second generation commercially available kits (EuroImmun). Anti-CCP antibodies levels ≥ 10 arbitrary units (AU) were considered as positive.

## Epitope Mapping of the γ Chain of Carbamylated Fibrinogen

Fifteen peptides of the γ chain of fibrinogen were synthesized by Eurogentec (27 amino acids overlapping on 3 amino acids, containing at least one lysine residue) and reconstituted according to the protocol described above (**Table 1**). ACa-Fib γ chain peptides were detected according to the previously described protocol. Sixteen positive sera of RA patients with ACa-Fib and 30 sera of healthy controls (EFS) were tested. The threshold of positivity was determined at 0.25 OD (Optical Density), which was the cut off used in other studies (3).

## Cross-Reactivity Assay With Immunodominant Peptides of α and β Chains of Citrullinated Fibrinogen

Patient sera positive for ACa-Fib γ chain peptides 5 (n=4) and 13 (n=3) were selected for the competition experiments. Carbamylated and native peptides 5 or 13 were coated as described above. Sera were diluted (1/50) in PBS BSA 1% Tween 0.05% and incubated for 2 hours with increasing concentrations (0, 0.1, 0.5, 1, 5 and 10 µg) of peptide 5, peptide 13, immunodominant peptide of citrullinated fibrinogen α chain (GV(cit)GP(cit)VVE(cit)HQSACKDSDWP) or β chain (PSL(cit)PAPPPISGGGY(cit)A(cit)PAK). These two peptides were synthesized by Eurogentec. Arginine residues were replaced by citrulline residues during the synthesis. The protocol for antibody detection is the same as described above.

## Statistics

Prism7 (GraphPad) was used for statistical analysis. The statistical differences in antibody levels between healthy controls and patients were determined by one-way ANOVA and Tukey post-test.

# RESULTS

## Titer, Prevalence, and Distribution of ACa-FCS and Anti-CCP Antibodies in Healthy Donors and the VErA Cohort

In the healthy population (n=100), the prevalence of ACa-FCS IgG antibodies was estimated at 1% while in RA and other rheumatism (OR) patients of the VErA cohort, it was calculated at 23.9% and 12.2% respectively. The distribution of ACa-FCS positivity according to anti-CCP status is summarized in **Figures 1A, B**.

## One of the Antigens Targeted *In Vivo* by the Anti-CarP Response in RA Is Located in the γ Chain of Fibrinogen

To attempt to identify the specific targets *in vivo* of the anti-CarP response, 15 sera of RA patients were selected according to the presence of ACa-FCS antibodies and the detection of carbamylated proteins in the sera. Then, they were analyzed using mass spectrometry. According to this approach, 2 chains of fibrinogen were identified, more precisely the α chain and the γ chain in 13 and 2 sera respectively of patients with very early RA (**Figure 2A**). For these 2 fibrinogen chains, the same peptide

**TABLE 1** | Native or carbamylated peptides of fibrinogen γ chain (27 mer-straddling 3 amino acids) containing at least one lysine.

| Peptide | Sequence | Concentration native form (mg/mL) | Concentration carbamylated form (mg/mL) |
|---|---|---|---|
| 1 | CGIADFLSTYQTKVDKDLQSLEDILHQ | 4 | 4 |
| 2 | LHQVENKTSEVKQLIKAIQLTYNPDES | 4 | 4 |
| 3 | DESSKPNMIDAATLKSRKMLEEIMKYE | 4 | 2 |
| 4 | KYEASILTHDSSIRYLQEIYNSNNQKI | 4 | 4 |
| 5 | QKIVNLKEKVAQLEAQCQEPCKDTVQI | 4 | 4 |
| 6 | VQIHDITGKDCQDIANKGAKQSGLYFI | 4 | 4 |
| 7 | YFIKPLKANQQFLVYCEIDGSGNGWTV | 4 | 2 |
| 8 | WTVFQKRLDGSVDFKKNWIQYKEGFGH | 4 | 4 |
| 9 | FGHLSPTGTTEFWLGNEKIHLISTQSA | 2 | 2 |
| 10 | AMFKVGPEADKYRLTYAYFAGGDAGDA | 4 | 4 |
| 11 | GDAFDGFDFGDDPSDKFFTSHNGMQFS | 4 | 2 |
| 12 | QFSTWDNDNDKFEGNCAEQDGSGWWMN | 4 | 4 |
| 13 | WMNKCHAGHLNGVYYQGGTYSKASTPN | 4 | 4 |
| 14 | TPNGYDNGIIWATWKTRWYSMKKTTMK | 4 | 4 |
| 15 | TMKIIPFNRLTIGEGQQHHLGGAKQVR | 4 | 4 |

*Name, sequences and concentrations.*

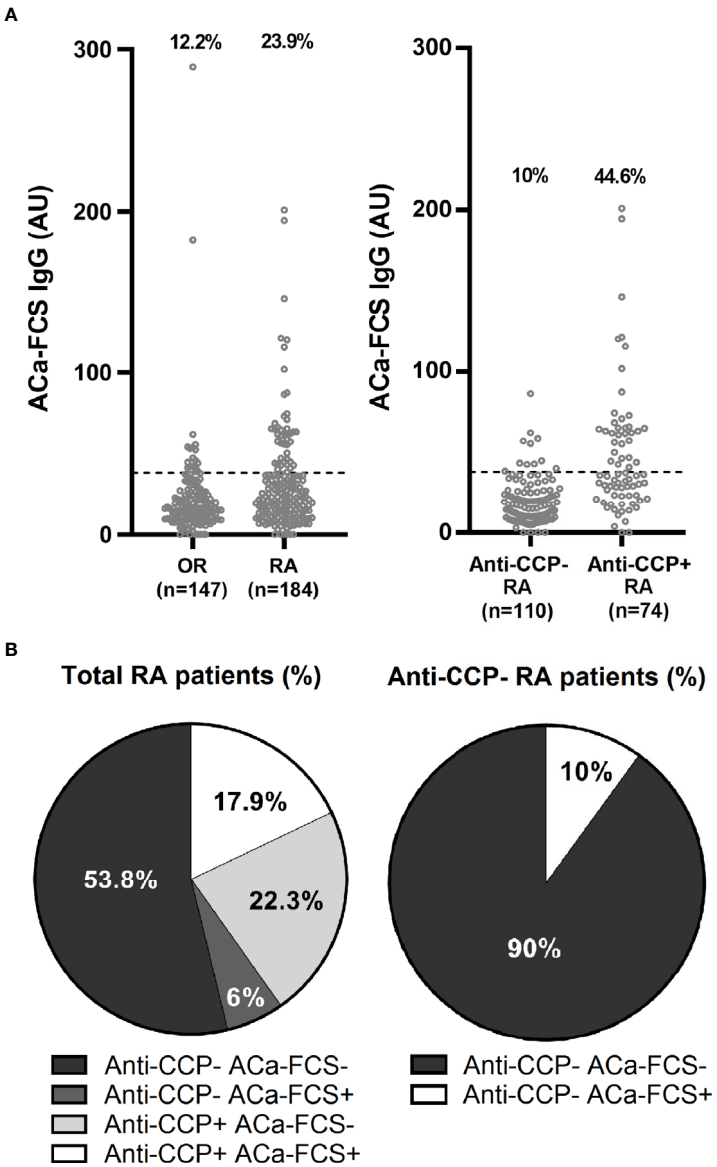


**FIGURE 1** | Titer, prevalence, and distribution of ACa-FCS and anti-CCP antibodies in the VErA cohort at baseline. **(A)** ELISA test for detection of ACa-Fib IgG based on RA status (n=184) and other rheumatism (OR) (n=147) and anti-CCP antibodies status in RA patients (n=184). **(B)** Distribution of anti-CCP antibodies and ACa-FCS in RA patients from the VErA cohort. Anti-CCP, anti-cyclic citrullinated peptide antibodies; ACa-FCS, anti-carbamylated fetal calf serum antibodies.

sequences, containing a carbamylated lysine residue, were found in these patients.

## Epitope Mapping of Antibody Response Directed Against the γ Chain of Carbamylated Fibrinogen

We performed epitope mapping of the γ chain of carbamylated fibrinogen. We selected RA sera that were immunopositive for ACa-Fib IgG (n=54). Sera were tested by ELISA for reactivity towards the 15 linear peptides of the γ chain of fibrinogen, that were carbamylated *in vitro*. Among these 54 sera, 16 recognized at least one of the 15 peptides of the γ chain of carbamylated fibrinogen (data not shown). These 16 sera reacted with a restricted set of epitopes (**Figure 2B**). Interestingly, it seems that ACa-Fib IgG recognized mostly 2 peptides (peptides 5 and 13). Of the 16 RA patients, 7 and 8 specifically recognized peptides 5 and 13 respectively; 2 sera bound to the 2 peptides while 13 were directed against at least one of the two.

## At Least a Part of the ACa-Fib response Does Not Overlap With the Anti-Citrullinated Fibrinogen Response

ACa-Fib response, in part, targeted peptides 5 and 13 of the γ chain of carbamylated fibrinogen. Based on inhibition tests with

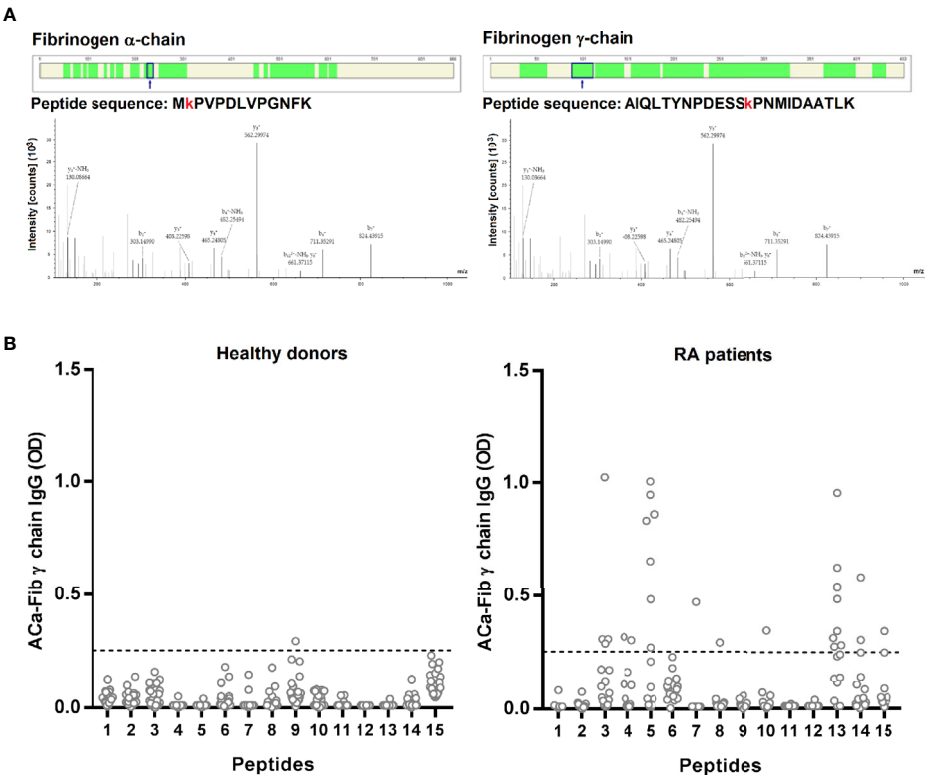


**FIGURE 2** | Identification by MS/MS of carbamylated target of anti-CarP *in situ* and epitope mapping of the antibody response directed against the γ chain of carbamylated fibrinogen. **(A)** Identified peptides on the α chain (40% of coverage rate) and γ chain (65% of coverage rate) have a carbamyl residue on lysine. Filters used for identification are as follows: high peptide confidence; peptide rank 1 and a peptide score > 20. **(B)** ELISA test for detection of ACa-Fib γ chain IgG from RA patients with Aca-Fib IgG (n=16) and healthy donors (n=30), expressed as OD (Optical Density) at 450 nm. ACa-Fib γ chain, anti-carbamylated fibrinogen γ chain antibodies. The horizontal line corresponds to the threshold of positivity (OD ≥ 0.25) and each point represents a patient.

their corresponding peptides, we investigated whether there was cross-reactivity with the most representative immunodominant peptides of the anti-citrullinated response, as suggested in some studies (11). We analyzed the ability of peptides 5 and 13 of the γ chain of carbamylated fibrinogen to compete at increasing concentrations up to saturating concentrations of 10 µg/ml binding to themselves (positive control) using the sera of RA patients (n=4 and n=3 for peptide 5 and 13 respectively) who had a strong reactivity towards either of these 2 peptides (**Figure 3A**). Each peptide completely inhibited its binding to RA sera from a saturating concentration of 5µg/ml. The same competition experiment was performed with the immunodominant peptide of the citrullinated fibrinogen α chain (cit-Fib α chain peptide) (**Figure 3B**) and the citrullinated fibrinogen β chain (cit-Fib β chain peptide) (**Figure 3C**). There was cross-reactivity between peptide 5 of the γ chain of carbamylated fibrinogen and an α chain peptide of citrullinated fibrinogen. A saturating dose of 10 µg/ml of cit-Fib α chain peptide resulted in a strong inhibition of >30% of the 4 sera tested (**Figure 3D**). In contrast, a weak inhibition was observed for the cit-Fib α chain peptide on peptide 13 (**Figure 3B**). This weak inhibition represents a percentage of <25% for one of the 3 sera tested and <15% for the two other sera (**Figure 3D**). There was also cross-reactivity between γ chain peptide 5 of carbamylated fibrinogen and cit-Fib β chain peptide (**Figure 3C**), the binding of three out of four sera was inhibited at the saturating dose of 10 µg/ml. This degree of competition is important because all sera had at least a percentage inhibition of > 50% (**Figure 3D**). In contrast, concerning peptide 13, inhibition by the cit-Fib β chain peptide seems less important than for peptide 5 because the binding of the 3 sera tested was not completely inhibited at a concentration of 10 µg/ml (**Figure 3C**). Furthermore, for the serum of two patients, this inhibition was <50% and the other serum was not inhibited at all by the cit-Fib β chain peptide (**Figure 3D**). These data suggest that peptide 13 (which we will name Ca-Fibγ361-387) contains a specific epitope of the anti-carbamylated fibrinogen response that is able to lead to an immunization distinct from citrullination.

## Titer, Prevalence, and Distribution of ACa-Fib Antibodies in Healthy Donors and the VErA Cohort According to Anti-CCP and/or ACa-FCS Status

The α and γ chains of carbamylated fibrinogen might be two targets of the anti-CarP response in RA. Jones et al. showed that this response was also directed against the fibrinogen β chain.

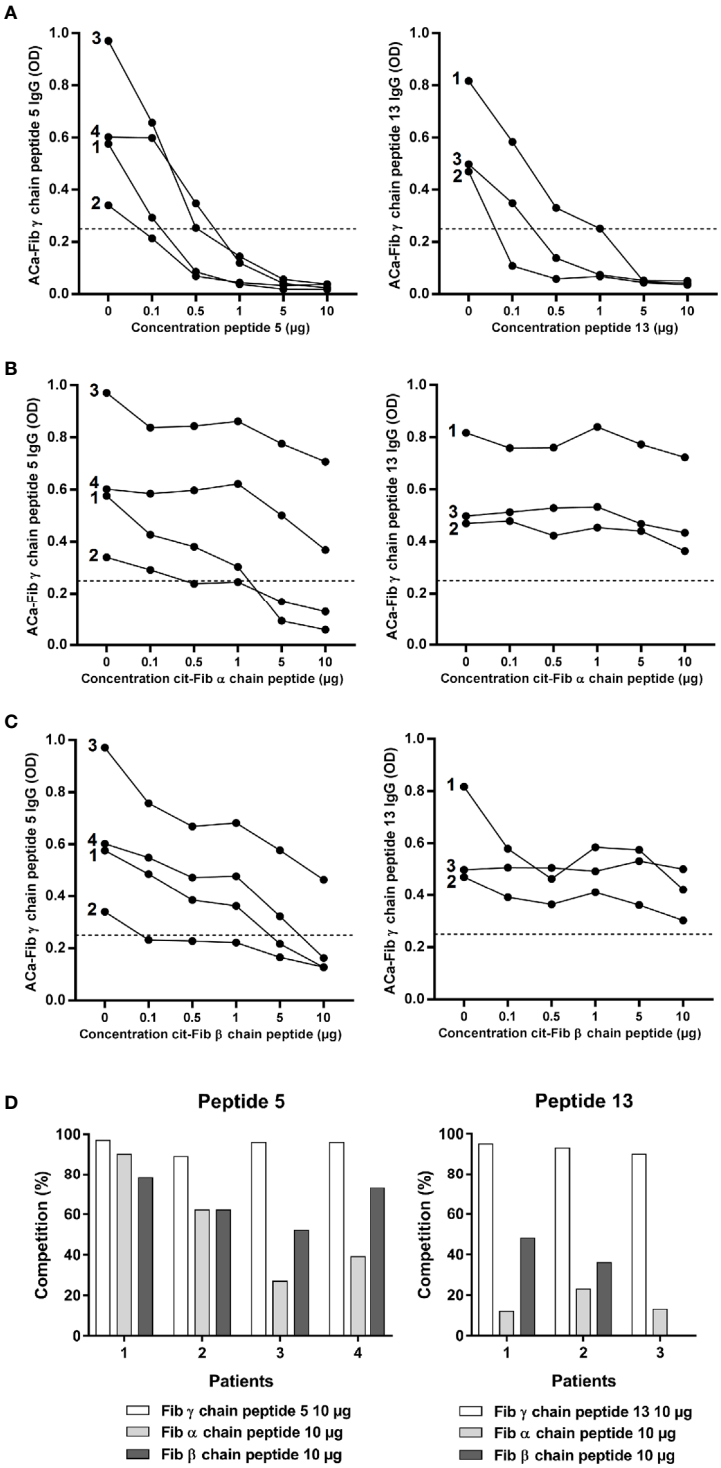


**FIGURE 3** | Cross reactivity assay of carbamylated γ chain of fibrinogen peptides 5 and 13 with major epitopes of the anti-citrullinated fibrinogen response. **(A)** ELISA inhibition test of carbamylated γ chain of fibrinogen peptides 5 or 13 with their corresponding peptides at increasing concentrations (0, 0.1, 0.5, 1, 5 or 10 µg/mL). **(B)** ELISA inhibition test of carbamylated γ chain of fibrinogen peptides 5 or 13 with an immunodominant epitope of citrullinated fibrinogen α chain at increasing concentrations (0, 0.1, 0.5, 1, 5 or 10 µg/mL). **(C)** ELISA inhibition test of carbamylated γ chain of fibrinogen peptides 5 or 13 with an immunodominant epitope of citrullinated fibrinogen β chain at increasing concentrations (0, 0.1, 0.5, 1, 5 or 10 µg/mL). **(D)** Percentages of inhibition for each peptide. ELISA tests expressed as OD (Optical Density) at 450 nm. Sera highly positive for carbamylated γ chain of fibrinogen peptide 5: n=4; sera highly positive for carbamylated γ chain of fibrinogen peptide 13: n=3. ACa-Fib γ chain, anti-carbamylated fibrinogen γ chain antibodies.

Thus, the 3 chains making up fibrinogen appear to comprise immunodominant epitopes of the anti-CarP response in RA. Thus, we developed an ELISA using the whole fibrinogen protein as a substrate to detect ACa-Fib IgG. While no ACa-Fib IgG antibodies were measured in the healthy population, our ELISA test revealed that patients classified as having RA and OR in the VErA cohort were ACa-Fib IgG-positive in a proportion of 29.3% and 8.2% respectively. Considering all RA in the cohort, 56.8% and 10.9% of patients were immunopositive for ACa-Fib IgG in anti-CCP-positive and anti-CCP-negative patients respectively (**Figure 4A**). The serological profiles for both populations of autoantibodies in the VErA cohort are illustrated in **Figure 4B**. Of all patients with very early RA, 53.3% were immunonegative for both anti-CCP antibodies and ACa-Fib, 6.5% were immunopositive for ACa-Fib, 17.4% were immunopositive for anti-CCP antibodies, and 22.8% were positive for both anti-CCP antibodies and ACa-Fib. Among anti-CCP negative patients, 10.9% of sera were ACa-Fib immunopositive. Although these profiles were close to those obtained with the ELISA test using carbamylated FCS as a substrate (**Figure 1B**), these findings were not strictly similar. The autoantibody profiles with ACa-FCS, ACa-Fib, and anti-CCP status in RA patients from the VErA cohort are shown in **Figure 4C**. Values are expressed in absolute numbers and percentages and represent the positivity of each autoantibody. Of the RA patients who were seronegative for anti-CCP antibodies at baseline (n=115), 11 and 12 patients had ACa-FCS and ACa-Fib IgG respectively. When carbamylated FCS was used as a substrate for the ELISA test, some epitopes of carbamylated fibrinogen were not recognized (n=8 patients in the cohort). Conversely, when carbamylated fibrinogen was used as a substrate for the ELISA test, other carbamylated targets within the FCS were not detected in 7 patients of the cohort.

## Clinical Interest of ACa-Fib Autoantibodies and Their Role in Prognostic Strategies in Relation to Anti-CCP Antibodies and ACa-FCS

To evaluate potential clinical interest in ACa-Fib IgG from 184 RA patients of the VErA cohort who met the 2010 ACR/EULAR criteria, three follow-up time-points were considered, i.e., baseline (M0), 6 months (M6), and 2 years (M24). The DAS (Disease Activity Score) 44 (index based on 44 joints and including levels of ESR) was calculated and its mean values are shown in **Figure 5**. In the early stages of RA, a small subgroup of patients with only ACa-Fib (n=12) was characterized by a significantly higher disease activity than that observed with

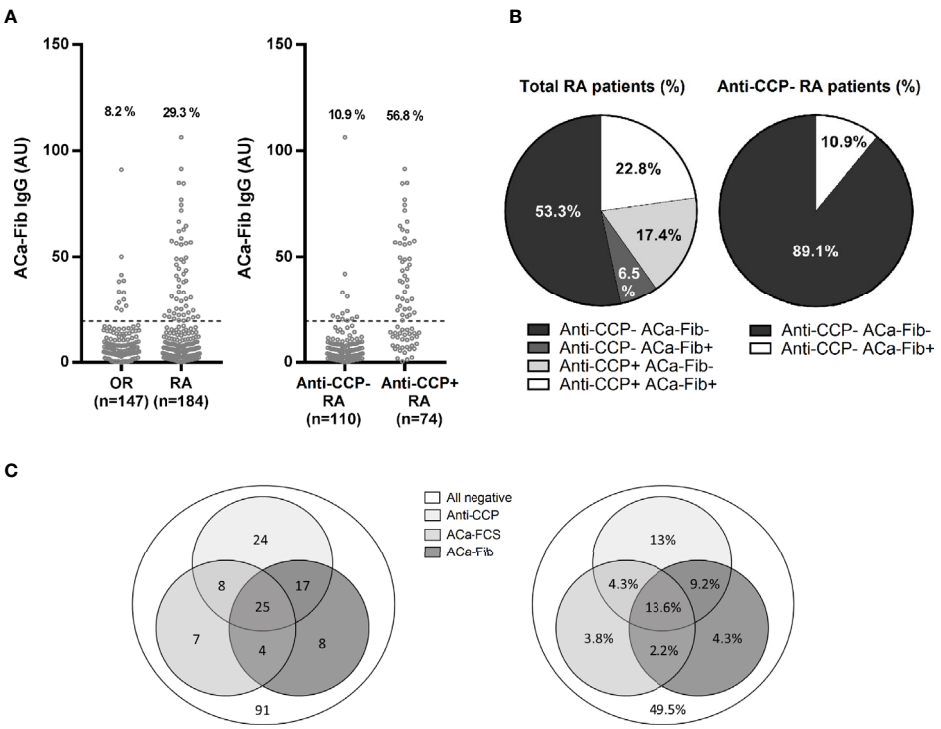


**FIGURE 4** | Titer, prevalence and distribution of ACa-Fib and anti-CCP antibodies in the VErA cohort at baseline and distribution according to the triple status ACa-Fib, ACa-FCS, and anti-CCP antibodies. **(A)** ELISA test for detection of ACa-Fib IgG based on RA status (n=184) and other rheumatism (n=147) and anti-CCP antibodies status in RA patients (n=184). **(B)** Distribution of anti-CCP antibodies and ACa-FCS in RA patients from the VErA cohort. **(C)** Distribution of immunological profile in RA patients considering triple status anti-CCP antibodies, ACa-Fib, ACa-FCS. Values are expressed in absolute numbers and percentages and represent the positivity of each auto-antibody. Anti-CCP, anti-cyclic citrullinated peptide antibodies; ACa-Fib, anti-carbamylated fibrinogen antibodies; ACa-FCS, anti-carbamylated fetal calf serum antibodies.

other autoimmune profiles (**Figure 5A**). However, this link between ACa-Fib and DAS 44 was only observed prior to DMARD initiation. The DAS 44 is a composite index reflecting both joint activity and systemic inflammation. In this respect, we wondered whether the relationship between disease activity and ACa-Fib IgG positivity at baseline was due to systemic inflammation. This latter was determined by C reactive protein (CRP) levels (**Figure 6**). Once again, compared to ACa-FCS status, CRP values at the onset of RA were higher in the ACa-Fib-positive subgroup than those measured in the other subsets, notably characterized by single positivity of anti-CCP antibodies ($p<0.001$) (**Figure 6A**). Finally, no link between ACa-FCS and DAS44 was found at any time points (**Figure 5B**) and this also applied to CRP levels (**Figure 6B**). Compared to double negative patients, those who were immunopositive for anti-CCP antibodies and/or ACa-FCS antibodies had a significantly higher DAS 44 (**Figure 5B**). However, such a relationship was not found with CRP levels, which means that seropositivity of anti-CCP antibodies and/or ACa- FCS autoantibodies are related to disease articular activity rather than systemic inflammation. These data are consistent with those of Truchetet et al. (4).

Structural impairment of RA patients was assessed according to data on *Van der Heijde* modified Sharp scores that were collected at M0, M6, and M24, the erosive status at M0 and M24 and the degree of structural progression and more precisely rapid radiological progression defined by a 5-point change in the total Sharp score over the first year of evolution. As shown in **Figure 7**, all ACPA-positive subgroups, regardless of ACa-FCS and/or ACa-Fib status, had a higher proportion of erosive RA at M24 and were associated with rapid radiological progression. The small ACa-Fib-positive subgroup which had more inflammatory disease at RA onset showed a tendency to be not associated with rapid radiological progression.

## DISCUSSION

The characteristics of the ACa-FCS profile obtained in our regional VErA cohort are comparable to those found in the French national cohort ESPOIR (*Etude et Suivi des POlyarthrites Indifférenciées Récentes*), which comprised 720 patients with the same inclusion criteria (4). Indeed, 36% and 32% of patients in these 2 cohorts, regardless of their RA or OR diagnosis, were immunopositive for ACa-FCS respectively, supporting the external validity of our results (**Figure 1**). When we consider ACa-Fib, their prevalence in VErA is 37%, whatever the patient's status (RA or not), which is similar to ACa-FCS (targeting multiple, not clearly identified, antigens). This has reinforced the idea that fibrinogen is probably one of the main targets of the anti-CarP response in RA. When anti-CarP fine specificity is studied, Brink et al. have shown there was a clear increase of antibodies against fibrinogen (Fibß36-52) during the early stage of the disease compared to other PTMs (9).

We were able to directly demonstrate with mass spectrometry the presence of an *in vivo* carbamylated target by analysis of RA sera, known to be ACa-FCS-positive and to contain carbamylated proteins (**Figure 2A**). In addition to the α and β chains of fibrinogen that are already known as targets of citrullination and carbamylation in RA, we have identified a new target, the γ chain of fibrinogen, that contains 2 immunodominant peptides, peptide 5 and peptide 13 (Ca-Fibγ361-387) (**Figure 2B**). However, the fact that fibrinogen was detected in a carbamylated format in the circulation does not mean that the protein is complexed to autoantibodies since the anti-CarP response was shown to be of overall low avidity as compared to that of ACPA-IgG (16). Furthermore, our findings suggest that the pool of carbamylated antigens present in FCS does not include all epitopes of carbamylated fibrinogen and that other carbamylated proteins are targeted in this biological fluid. In this regard, another team has identified, using immunochemistry or mass spectrometry analysis, the presence of a large number of carbamylated proteins in synovial tissue and synovial fluid, that could be recognized by anti-CarP antibodies present in the sera of RA patients (17, 18).

The fact that the response to the γ chain is restricted to two main epitopes suggests that the amino acids surrounding homocitrulline play a key role in this recognition, as shown for ACPA that targets citrulline residues located in a particular amino acid environment (19). Nevertheless, among patients with ACa-Fib, a high proportion (38/54) did not recognize any of these peptides, suggesting that some epitopes may be conformational and/or that the other targets are located on the α and β chains of fibrinogen, as suggested previously (10).

We focused our study on the γ chain of fibrinogen which is a different target of citrullination (20). There are many data suggesting cross-reactivity between ACPAs and anti-CarP (21). In this regard, our competition experiments performed with the immunodominant peptides of the anti-citrullinated fibrinogen response have shown that only ACa-Fib that recognize peptide 13 of the γ chain (Ca-Fibγ361-387) has a specific reactivity distinct from anti-citrullinated fibrinogen (**Figure 3**). This finding could be explained by the fact that its sequence does not contain any arginine residue that could be the site of citrullination. This point is of particular relevance to better understand the profile of RA serum autoantibodies against the citrullinome and the homocitrullinome. In this regard, despite a lower reactivity of RA serum samples against homocitrullinated peptides using a high density peptide array to screen the entire proteome, Lo et al. showed that extensive anti-CarP antibody reactivity was seen in a restricted set (4/18) of RA samples (22). Although these RA sera also showed ACPA reactivity and did not constitute an anti-CarP-positive and ACPA-negative subgroup, it is important to remember that this study defined ACPA as a family of autoantibodies likely to recognize a larger panel of citrullinated peptides in which some of them are not targeted by anti-CCP antibodies, whose main antigen is citrullinated fibrinogen, including a large number of proteins that are not known to be associated with RA.

Even if we have discovered a new target, distinct from ACPA response, it is important to recall that multiple antigenic targets are recognized by ACPA, anti-CarP, and more recently by anti-acetylated protein antibodies with broad reactivity to various antigens (21, 23). Indeed, several investigations suggest that monoclonal ACPA antibodies can exhibit multireactivity that extends to other PTMs (carbamylation, acetylation) with a

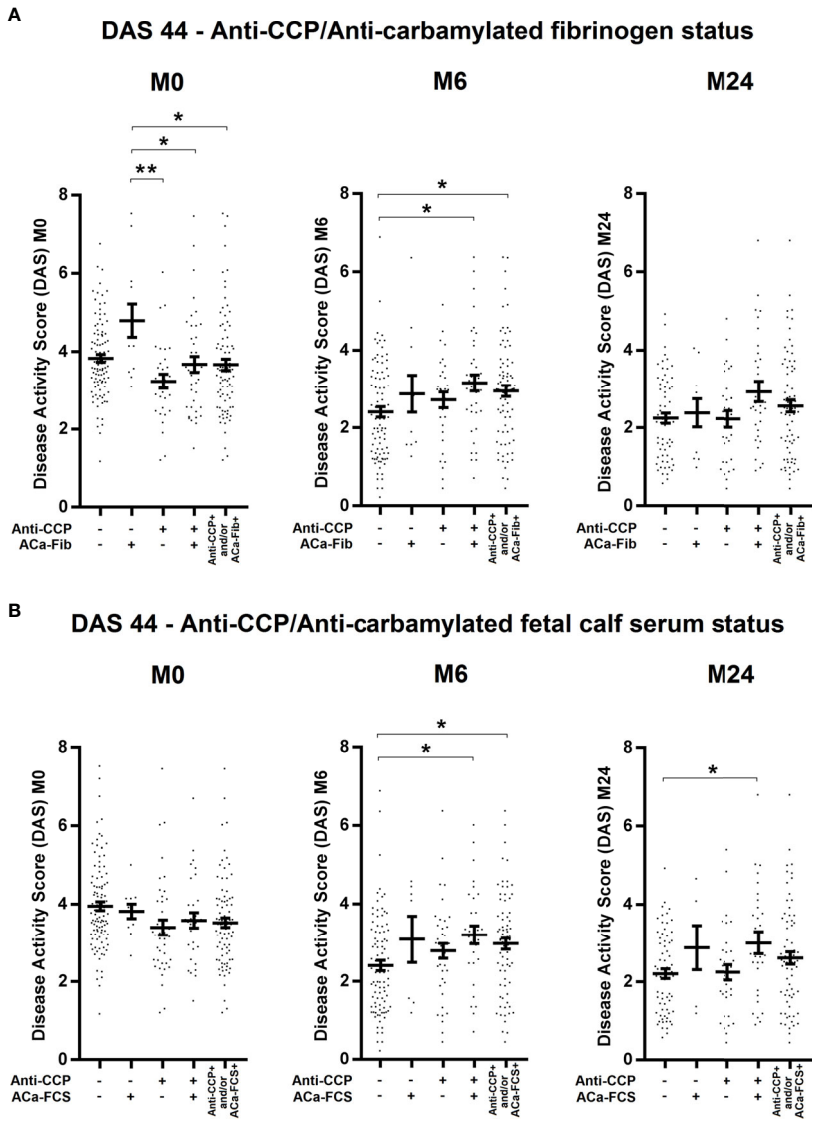


**FIGURE 5** | Evolution of disease activity of RA patients at baseline, 6 months, and 2 years according to anti-CCP antibodies and ACa-Fib status or ACa-FCS status. Disease Activity Score (DAS) 44 of RA patients according to **(A)** Anti-CCP/ACa-Fib status or **(B)** Anti-CCP/ACa-FCS status at baseline (M0), 6 months (M6), and 2 years (M24). Averages with SEM are shown. Groups compared using one-way ANOVA and Tukey post-test, *p-value<0.05, **p-value<0.01. Anti-CCP, anti-cyclic citrullinated peptide antibodies; ACa-Fib, anti-carbamylated fibrinogen antibodies; ACa-FCS, anti-carbamylated fetal calf serum antibodies.

hierarchy of these reactivities comprising multireactivity restricted to citrulline, multireactivity to citrullinated and carbamylated antigens, and multireactivity that covers all 3 PTMs. For some of these clones, it has been demonstrated that the carbamylated protein or acetylation protein binding is of higher apparent affinity than citrullinated protein binding. Taken together, all these findings suggest that anti-CarP should be considered as a different entity to ACPA/anti-modified proteins antibodies profiles (24).

In the VErA cohort, we have identified a small subgroup of RA patients, who are anti-CCP-negative/ACa-Fib-positive and who have a higher disease activity at disease onset than patients who are immunopositive for anti-CCP antibodies (**Figure 5A**). Our data suggest that this relationship is rather due to systemic inflammation than to joint activity. Thus, ACa-Fib antibodies are possibly associated with initial systemic inflammation (**Figure 6A**). This link between ACa-Fib and CRP levels prior to DMARD initiation raises the question of a potential relationship with structural damage since systemic inflammation is often associated with a higher risk of radiological progression (25). Previous studies showed that the presence of ACa-FCS was associated with more severe radiological damage in RA, regardless of anti-CCP status but also in the subgroup of anti-CCP-negative/ACa-FCS-positive patients who displayed a significantly higher *van der Heijde*

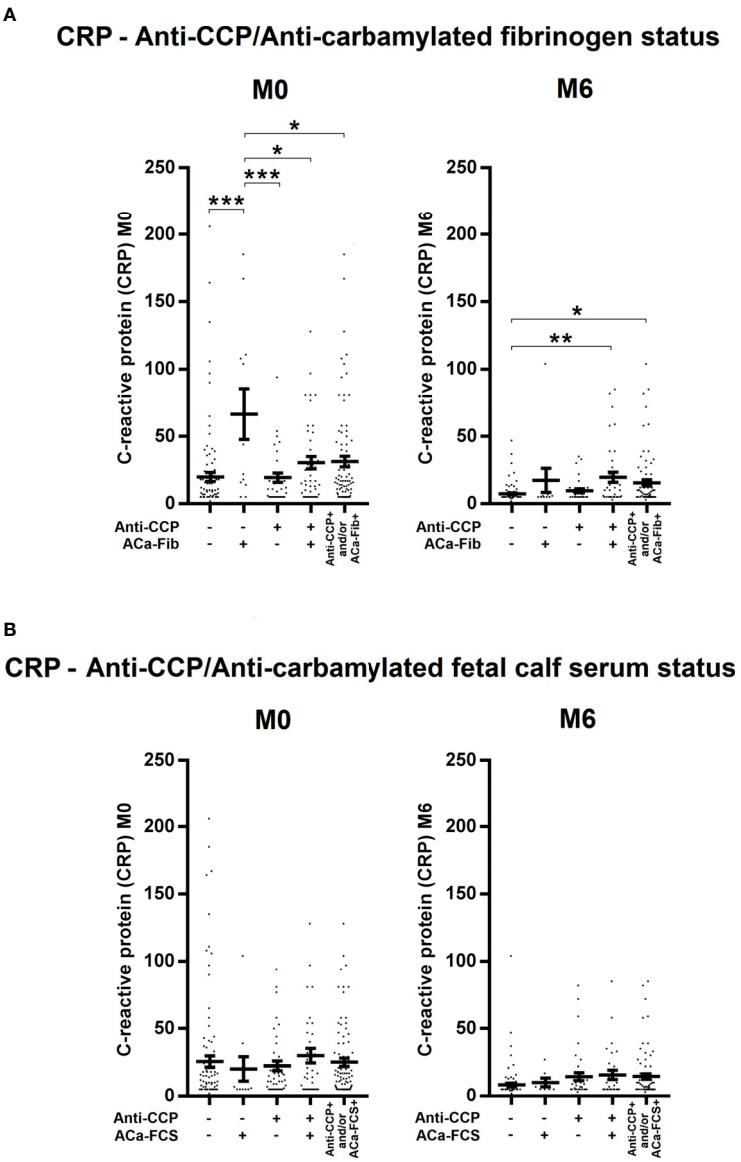


**FIGURE 6** | Evolution of systemic inflammation of RA patients at baseline and 6 months according to anti-CCP antibodies and ACa-Fib or ACa-FCS status. C reactive protein (CRP) levels of RA patients according to **(A)** Anti-CCP/ACa-Fib status or **(B)** Anti-CCP/ACa-FCS status at baseline (M0), 6 months (M6), and 2 years (M24). Averages with SEM are shown. Groups were compared using one-way ANOVA and Tukey post-test, *p-value<0.05, **p-value<0.01, ***p-value<0.001. Anti-CCP, anti-cyclic citrullinated peptide antibodies; ACa-Fib, anti-carbamylated fibrinogen antibodies; ACa-FCS, anti-carbamylated fetal calf serum antibodies.

modified total Sharp score compared to anti-CCP-negative and ACa-FCS-negative patients (**Figure 7**). In addition, Ajeganova et al. tested the added value of ACa-FCS to ACPA and RF serological status in matrices predicting joint destruction. This did not make it possible to better rank the severity of radiological damage at 5 years compared to the information provided by ACPA alone (26). In our study, when patients were stratified according to autoantibody profile in relation to anti-CCP antibodies, ACa-FCS and ACa-Fib, rapid radiological progression was associated with the positivity of anti-CCP antibodies and not with the presence of anti-CarP (**Figure 7**).

To conclusion, in the specific subgroup of patients negative for ACPA for whom we lack biomarkers to predict the structural outcome, the presence of anti-CarP, more specifically in ACa-Fib-positive patients, might be related to a more benign form since it is not related to rapid radiological progression but is characterized by a more active disease at presentation. Due to the limited size of the different subgroups that were defined according to anti-CCP/anti-CarP profiles, this potential relationship between anti-ACa-Fib antibodies and initial systemic inflammation needs to be replicated in a larger cohort of very early arthritis prior to drawing robust conclusions.

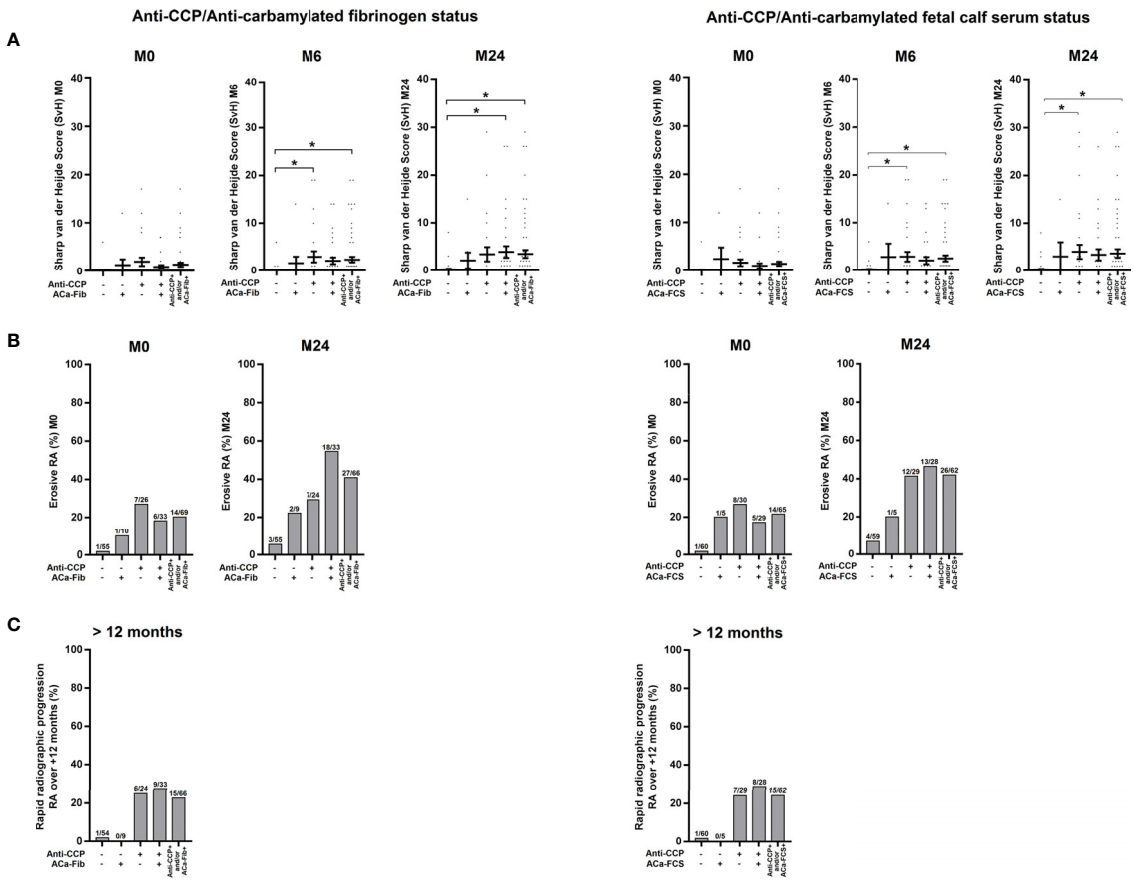


**FIGURE 7** | Evolution of structural parameters of RA patients according to anti-CCP antibodies and ACa-Fib or ACa-FCS status. **(A)** van der Heijde modified Sharp score at baseline (M0), 6 months (M6), and 2 years (M24) according to Anti-CCP/ACa-Fib status or Anti-CCP/ACa-FCS status. Averages with SEM are shown. Groups were compared using one-way ANOVA and Tukey post-test, *p-value<0.05. **(B)** Percentage of erosive RA at baseline (M0) and 2 years (M24) according to Anti-CCP/ACa-Fib status or Anti-CCP/ACa-FCS status. **(C)** Percentage of patients with rapid radiographic progression over a period of 12 months starting from M0 according to Anti-CCP/ACa-Fib status or Anti-CCP/ACa-FCS status. Anti-CCP, anti-cyclic citrullinated peptide antibodies; ACa-Fib, anti-carbamylated fibrinogen antibodies; ACa-FCS, anti-carbamylated fetal calf serum antibodies.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRIDE, PXD028121.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Upper Normandy Ethics Committee (file: 95/138/HP). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

OV and MF designed and supervised the project. XL-L, PF, and OV supervised the VErA cohort. PB, CL, CG, PR, and MF performed the experiments. PB, CL, CG, PR, TL, OB, MF, PC, and OV analyzed the data. PB, MF, and OV wrote the manuscript with the help of all authors. All authors contributed to the article and approved the submitted version.

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## REFERENCES


1. van Nies JA, Tsonaka R, Gaujoux-Viala C, Fautrel B, van der Helm-van Mil AH. Evaluating Relationships Between Symptom Duration and Persistence of Rheumatoid Arthritis: Does a Window of Opportunity Exist? Results on the Leiden Early Arthritis Clinic and ESPOIR Cohorts. *Ann Rheum Dis* (2015) 74:806–12. doi: 10.1136/annrheumdis-2014-206047
2. van Nies JA, Krabben A, Schoones JW, Huizinga TW, Kloppenburg M, van der Helm-van Mil AH. What is the Evidence for the Presence of a Therapeutic Window of Opportunity in Rheumatoid Arthritis? A Systematic Literature Review. *Ann Rheum Dis* (2014) 73:861–70. doi: 10.1136/annrheumdis-2012-203130
3. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies Recognizing Carbamylated Proteins are Present in Sera of Patients With Rheumatoid Arthritis and Predict Joint Damage. *Proc Natl Acad Sci U S A* (2011) 108:17372–7. doi: 10.1073/pnas.1114465108
4. Truchetet ME, Dublanc S, Barnetche T, Vittecoq O, Mariette X, Richez C, et al. Association of the Presence of Anti-Carbamylated Protein Antibodies in Early Arthritis With a Poorer Clinical and Radiologic Outcome: Data From the French ESPOIR Cohort. *Arthritis Rheumatol* (2017) 69:2292–302. doi: 10.1002/art.40237
5. Martinez G, Gomez JA, Bang H, Martinez-Gamboa L, Roggenbuck D, Burmester GR, et al. Carbamylated Vimentin Represents a Relevant Autoantigen in Latin American (Cuban) Rheumatoid Arthritis Patients. *Rheumatol Int* (2016) 36:781–91. doi: 10.1007/s00296-016-3472-9
6. Yu HC, Lai PH, Lai NS, Huang HB, Koo M, Lu MC. Increased Serum Levels of Anti-Carbamylated 78-kDa Glucose-Regulated Protein Antibody in Patients With Rheumatoid Arthritis. *Int J Mol Sci* (2016) 17(9):1510. doi: 10.3390/ijms17091510
7. Nakabo S, Hashimoto M, Ito S, Furu M, Ito H, Fujii T, et al. Carbamylated Albumin is One of the Target Antigens of Anti-Carbamylated Protein Antibodies. *Rheumatol (Oxford)* (2017) 56:1217–26. doi: 10.1093/rheumatology/kex088
8. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated Fibrinogen Antibodies are Specific to Rheumatoid Arthritis and Frequently Bind Citrullinated Proteins/Peptides. *J Rheumatol* (2014) 41:270–9. doi: 10.3899/jrheum.130742
9. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-Carbamylated Protein Antibodies in the Pre-Symptomatic Phase of Rheumatoid Arthritis, Their Relationship With Multiple Anti-Citrulline Peptide Antibodies and Association With Radiological Damage. *Arthritis Res Ther* (2015) 17:25. doi: 10.1186/s13075-015-0536-2
10. Jones JD, Hamilton BJ, Rigby WFC. Brief Report: Anti-Carbamylated Protein Antibodies in Rheumatoid Arthritis Patients Are Reactive With Specific Epitopes of the Human Fibrinogen Beta-Chain. *Arthritis Rheumatol* (2017) 69:1381–6. doi: 10.1002/art.40098
11. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, et al. Antibodies to Carbamylated Alpha-Enolase Epitopes in Rheumatoid Arthritis Also Bind Citrullinated Epitopes and are Largely Indistinct From Anti-Citrullinated Protein Antibodies. *Arthritis Res Ther* (2016) 18:96. doi: 10.1186/s13075-016-1001-6
12. Lac P, Racape M, Barra L, Bell DA, Cairns E. Relatedness of Antibodies to Peptides Containing Homocitrulline or Citrulline in Patients With Rheumatoid Arthritis. *J Rheumatol* (2018) 45:302–9. doi: 10.3899/jrheum.170446
13. Le Loet X, Nicolau J, Boumier P, Daragon A, Mejjad O, Pouplin S, et al. Validation of the 2010-ACR/EULAR -Classification Criteria Using Newly EULAR-Defined Erosion for Rheumatoid Arthritis on the Very Early Arthritis Community-Based (VErA) Cohort. *Joint Bone Spine* (2015) 82:38–41. doi: 10.1016/j.jbspin.2014.03.008
14. Obry A, Hardouin J, Lequerre T, Jarnier F, Boyer O, Fardellone P, et al. Identification of 7 Proteins in Sera of RA Patients With Potential to Predict ETA/MTX Treatment Response. *Theranostics* (2015) 5:1214–24. doi: 10.7150/thno.12403
15. Gaviard C, Cosette P, Jouenne T, Hardouin J. LasB and CbpD Virulence Factors of Pseudomonas Aeruginosa Carry Multiple Post-Translational Modifications on Their Lysine Residues. *J Proteome Res* (2019) 18:923–33. doi: 10.1021/acs.jproteome.8b00556
16. van Delft MAM, Verheul MK, Burgers LE, Rantapaa-Dahlqvist S, van der Helm-van Mil AHM, Huizinga TWJ, et al. The Anti-Carbamylated Protein Antibody Response is of Overall Low Avidity Despite Extensive Isotype Switching. *Rheumatol (Oxford)* (2018) 57:1583–91. doi: 10.1093/rheumatology/key135
17. Verheul MK, Janssen GMC, de Ru A, Stoeken-Rijsbergen G, Levarht EWN, Kwekkeboom JC, et al. Mass-Spectrometric Identification of Carbamylated Proteins Present in the Joints of Rheumatoid Arthritis Patients and Controls. *Clin Exp Rheumatol* (2020) 39(3):570–7.
18. Verheul MK, Yee A, Seaman A, Janssen GM, van Veelen PA, Drijfhout JW, et al. Identification of Carbamylated Alpha 1 Anti-Trypsin (A1AT) as an Antigenic Target of Anti-CarP Antibodies in Patients With Rheumatoid Arthritis. *J Autoimmun* (2017) 80:77–84. doi: 10.1016/j.jaut.2017.02.008
19. Mahler M, Bluthner M, Pollard KM. Advances in B-Cell Epitope Analysis of Autoantigens in Connective Tissue Diseases. *Clin Immunol* (2003) 107:65–79. doi: 10.1016/S1521-6616(03)00037-8
20. Cornillet M, Sebbag M, Verrouil E, Magyar A, Babos F, Ruyssen-Witrand A, et al. The Fibrin-Derived Citrullinated Peptide Beta60-74Cit(6)(0),(7)(2),(7)(4) Bears the Major ACPA Epitope Recognised by the Rheumatoid Arthritis-Specific Anticitrullinated Fibrinogen Autoantibodies and Anti-CCP2 Antibodies. *Ann Rheum Dis* (2014) 73:1246–52. doi: 10.1136/annrheumdis-2012-202868
21. Kissel T, Reijm S, Slot LM, Cavallari M, Wortel CM, Vergroesen RD, et al. Antibodies and B Cells Recognising Citrullinated Proteins Display a Broad Cross-Reactivity Towards Other Post-Translational Modifications. *Ann Rheum Dis* (2020) 79:472–80. doi: 10.1136/annrheumdis-2019-216499
22. Lo KC, Sullivan E, Bannen RM, Jin H, Rowe M, Li H, et al. Comprehensive Profiling of the Rheumatoid Arthritis Antibody Repertoire. *Arthritis Rheumatol* (2020) 72:242–50. doi: 10.1002/art.41089
23. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of Novel Antiacetylated Vimentin Antibodies in Patients With Early Inflammatory Arthritis. *Ann Rheum Dis* (2016) 75:1099–107. doi: 10.1136/annrheumdis-2014-206785
24. Sahlstrom P, Hansson M, Steen J, Amara K, Titcombe PJ, Forsstrom B, et al. Different Hierarchies of Anti-Modified Protein Autoantibody Reactivities in Rheumatoid Arthritis. *Arthritis Rheumatol* (2020) 72:1643–57. doi: 10.1002/art.41385
25. Fautrel B, Granger B, Combe B, Saraux A, Guillemin F, Le Loet X. Matrix to Predict Rapid Radiographic Progression of Early Rheumatoid Arthritis Patients From the Community Treated With Methotrexate or Leflunomide: Results From the ESPOIR Cohort. *Arthritis Res Ther* (2012) 14:R249. doi: 10.1186/ar4092
26. Ajeganova S, van Steenbergen HW, Verheul MK, Forslind K, Hafstrom I, Toes RE, et al. The Association Between Anti-Carbamylated Protein (Anti-CarP) Antibodies and Radiographic Progression in Early Rheumatoid Arthritis: A Study Exploring Replication and the Added Value to ACPA and Rheumatoid Factor. *Ann Rheum Dis* (2017) 76:112–8. doi: 10.1136/annrheumdis-2015-208870


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# Derlin-1, as a Potential Early Predictive Biomarker for Nonresponse to Infliximab Treatment in Rheumatoid Arthritis, Is Related to Autophagy

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**Background:** The goal of this study was to identify potential predictive biomarkers for the therapeutic effect of infliximab (IFX) in Rheumatoid arthritis (RA) and explore the potential molecular mechanism of nonresponse to IFX treatment to achieve individualized treatment of RA.

**Methods:** Differential gene expression between IFX responders and nonresponders in the GSE58795 and GSE78068 datasets was identified. Coexpression analysis was used to identify the modules associated with nonresponse to IFX therapy for RA, and enrichment analysis was conducted on module genes. Least absolute shrink and selection operator (LASSO) regression was used to develop a gene signature for predicting the therapeutic effect of IFX in RA, and the area under the receiver operating characteristic curve (AUC) was used to evaluate the predictive value of the signature. Correlation analysis and single-sample gene set enrichment analysis (ssGSEA) were used to explore the potential role of the hub genes. Experimental validation was conducted in synovial tissue and RA fibroblast-like synoviocytes (RA-FLSs).

**Results:** A total of 46 common genes were obtained among the two datasets. The yellow-green module was identified as the key module associated with nonresponse to IFX therapy for RA. We identified a 25-gene signature in GSE78068, and the AUC for the signature was 0.831 in the internal validation set and 0.924 in the GSE58795 dataset (external validation set). *Derlin-1 (DERL1)* was identified as the hub gene and demonstrated to be involved in the immune response and autophagy regulation.

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DERL1 expression was increased in RA synovial tissue compared with OA synovial tissue, and DERL1-siRNA partially inhibited autophagosome formation in RA-FLSs.

**Conclusion:** The 25-gene signature may have potential predictive value for the therapeutic effect of IFX in RA at the beginning of IFX treatment, and autophagy may be involved in nonresponse to IFX treatment. In particular, DERL1 may be associated with the regulation of autophagy.

**Keywords: rheumatoid arthritis, derlin-1, infliximab, predictive biomarker, autophagy**

## INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune systemic inflammatory disease characterized by chronic inflammatory hyperplasia of the synovium (1). Persistent synovitis could lead to the destruction of joint structure, joint deformity, and loss of joint function (2). RA usually occurs in women around middle age or at the time of menopause, and it can also occur at any age (1). The ratio of males to females is 1:2 to 1:3 and the global average incidence of RA is approximately 0.3% ~ 1% (3). The pathological mechanism of RA is still unclear, and there is still no definitive cure for RA (1, 3).

Tumor necrosis factor-α (TNFα) is an important pathological inflammatory factor in RA that is closely related to synovial inflammation and joint destruction in RA. Clinical studies have shown that anti-TNFα biologic agents can significantly attenuate RA and delay the progression of RA (4). Currently, five anti- TNFα biologic agents are widely used for RA treatment: infliximab (IFX), etanercept (ETN), adalimumab (ADA), certolizumab pegol, and golimumab (5–8).IFX is a chimeric human/mouse monoclonal antibody, in which the Fc domain is of human origin and the Fab' domain is of mouse origin (4). IFX can specifically bind to biologically active TNFα, inhibit its downstream signal transduction, and plays an anti-inflammatory and immune regulatory role (9). IFX is currently mainly applied to RA, psoriasis, ankylosing spondylitis, ulcerative colitis, Crohn's disease, and other immune-inflammatory diseases (10). Studies have found that, in the early stage of RA, the combined use of IFX and methotrexate (MTX) can effectively inhibit synovial inflammation and reduce joint destruction, and its efficacy is greater than MTX therapy alone (11). In addition, IFX is also used in patients who do not respond to other anti-TNFα biologic agents, such as ADA or ETN (12). Therefore, IFX is particularly important for the control of RA, especially in the early stage. The introduction of IFX has been proven to be advantageous for many RA patients, but not all patients benefit from IFX treatment. Clinical application shows that there are still some patients who do not respond to IFX (13), and the patients have to increase the IFX dose, which leads to higher side effects and a larger economic burden. At present, the molecular mechanism of nonresponse to IFX treatment for RA is not fully understood. It has been found that the formation of anti-IFX antibodies is one of the causes of nonresponse to IFX treatment in patients with Crohn's disease and RA (14–17). It is not clear who would benefit from IFX treatment before the treatment begins. Therefore, accurate prediction of IFX efficacy by biomarkers before treatment is helpful to achieve individualized treatment of RA.

Autophagy is a physiological process that is essential for cell growth, differentiation, development, survival and homeostasis (18). It is an adaptive cell response that allows the cell to survive bioenergetic stress such as nutrient deprivation or other stresses (19). It has been found that anti- TNFα biologic agents can induce apoptosis of peripheral blood lymphocytes, and the sensitivity to apoptosis induced by the agents varies widely among RA patients (20). Further research found that the difference in sensitivity to anti- TNFα agents may be related to autophagy state of peripheral blood mononuclear cells (PBMCs). Vomero et al. found that TNFα was able to induce autophagy in RA PBMCs and RA-FLSs in a dose-dependent manner, and PBMCs from patients with RA responsive to anti- TNFα biologic agents showed a reduced autophagy and an increased apoptotic activation (21). These studies suggest that autophagy may be a potential molecular mechanism of nonresponse to IFX therapy in RA.

To understand the early molecular changes and potential molecular mechanisms of nonresponse to IFX therapy in RA, we analyzed the expression profiles of RA patients treated with IFX from the Gene Expression Omnibus (GEO) database. We used common genes in different datasets to establish a least absolute shrink and selection operator (LASSO) model in the training set to develop a gene signature for predicting the therapeutic effect of IFX in RA treatment, and used receiver operating characteristic curve (ROC) to evaluate the predictive value of the model in both internal validation set and external validation set. Weighted gene coexpression network analysis (WGCNA) was used to identify modules associated with nonresponse to IFX therapy in RA, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of module genes were performed. The same genes in the LASSO model and the WGCNA modules were the hub genes of nonresponse to IFX therapy of RA. The bioinformatic analysis was followed by clinical validation. The goal of the present study was to identify predictive biomarkers of the therapeutic effect of IFX in RA treatment and explore the potential molecular mechanism, which was conducive to the individualized treatment of RA.

## MATERIALS AND METHODS

### Human Tissue Collection and Cell Culture

A total of 9 OA and 9 RA patients' synovial tissue samples were obtained, and all the patients fulfilled the American College of

Rheumatology (ACR) criteria for OA or RA and provided informed consent. This study was approved by the human research ethics committee of Xi'an Hong Hui Hospital (Xi'an Hong Hui Hospital, Xi'an, China). RA fibroblast-like synoviocytes (RA-FLSs) were obtained according to the previous method with some modifications (22). Briefly, RA synovial tissue without adipose tissue and cartilage was washed in sterilized saline and diced thoroughly. Next, the tissue was incubated in trypsin (2.5 mg/mL) (Sigma Co, St. Louis, Missouri, USA) at 37°C for 30 minutes and then removed the trypsin solution. After that, the synovial tissue was then transferred into Type I collagenase solution (2 mg/mL) at 37°C for another 3 hours. The isolated cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies, USA), 100 μg/mL streptomycin, and 100 units/mL penicillin at 37°C under 5% $CO_2$. Following the above procedure, 3rd- to 8th-passage RA-FLSs were used for further experiments. The patients' information is shown in **Table 1**.

## Data Sources

We collected data related to IFX treatment for RA from the GEO database. Two datasets were obtained, including the GSE58795 dataset and the GSE78068 dataset, and the gene expression data in both datasets were obtained from baseline whole blood. The GSE58795 dataset included 23 nonresponders and 36 responders, and the GSE78068 dataset included 98 nonresponders and 42 responders. Three datasets GSE77298, GSE55457, and GSE89408, which gene expression data were obtained from synovium, were selected as the validation sets to verify the expression of the hub genes. The GSE77298 dataset included 7 healthy control synovium and 16 RA synovium, the GSE55457 dataset included 10 healthy synovium and 13 RA synovium, and the GSE89408 dataset included 28 healthy synovium and 152 RA synovium. The information for selected datasets is presented in **Table 2**.

**TABLE 1** | Patient characteristics.

| | OA | RA |
|---|---|---|
| Patients | 9 | 9 |
| Sex, n, female/male | 2/7 | 6/3 |
| Age[a], yr | 65 (51-75) | 59 (55-73) |
| RF[b], IU/ml | 10.7 ± 4.3 | 107.5 ± 67.2 |
| Anti-CCP–positive, n | 0/9 | 7/9 |
| ESR[b], mm/h | 15 ± 7.1 | 43 ± 12.9 |
| CRP[b], mg/L | 3.7 ± 3.5 | 19.8 ± 18.3 |
| Medications, number of patients | | |
| NSAIDs | 3 | 0 |
| NSAIDs +Steroids+ Chinese herbal medicine | 2 | 2 |
| NSAIDs +Biological agents | 0 | 1 |
| NSAIDs +Steroids+ Chinese herbal medicine +MTX+ Folic acid | 0 | 3 |
| NSAIDs + Chinese herbal medicine +MTX+ Folic acid | 0 | 3 |
| NSAIDs + Chinese herbal medicine | 4 | 0 |

*RF, rheumatoid factor; CCP, cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; NSAIDs, nonsteroidal anti-inflammatory drugs; MTX, methotrexate.*
*[a]Median (range), [b]Mean ± standard deviation.*

## Identification of Differential Gene Expression

Differentially expressed genes (DEGs) between responders and nonresponders in the GSE58795 and GSE78068 datasets were identified using the 'Limma' R package (23), and the genes whose expression differences with a *P* value less than 0.05 were defined as DEGs. All the DEGs were visualized in volcano plots using the 'ggplot2' R package, and the common DEGs were visualized in two Venn diagrams using the 'VennDiagram' R package.

## Gene Set Variation Analysis

Gene set variation analysis (GSVA) was carried out to identify the differences in the signaling pathways and biological processes between responders and nonresponders in the GSE78068 dataset using the 'GSVA' and the 'Limma' R packages (24).

## Weighted Gene Coexpression Network Analysis

The top 10000 genes with the lowest *P* values in the GSE78068 dataset were used to identify the coexpression network using the 'WGCNA' R package (25). Briefly, after extracting the 10000 genes, the scale-free distribution topology matrix was calculated. The "pickSoflThreshold" function was used to select the optimal soft threshold power (the soft threshold power was set as 9 when the scale-free $R^2$ threshold was 0.9 in the present study), and the Pearson correlation coefficient of each gene was calculated. The adjacency matrix was constructed using the weighted Pearson correlation coefficient, and then the adjacency matrix was transformed into a topological overlap matrix (TOM) to construct the hierarchical clustering tree. Different gene modules were identified based on the hierarchical clustering tree. The modules with a gene number greater than 30 were retained, and those with a similarity greater than 0.5 were merged. The modules that were highly correlated with nonresponse to IFX therapy in RA were identified as the key modules.

## Function Enrichment Analysis

GO and KEGG analyses in the key module and the GSE78068 dataset were conducted using the 'ClusterProfiler' R package (26). The top three terms of biological processes (BP), components (CC), and molecular functions (MF) with adjusted $p < 0.05$ were visualized using the 'Goplot' R package (27), and all KEGG terms with adjusted $p < 0.05$ were visualized using the "ClueGO" plugin in Cytoscape software (28). Gene set enrichment analysis (GSEA) in the GSE78068 dataset was also conducted to explore the KEGG pathways associated with the hub genes using the 'ClusterProfiler' R package, and the results with adjusted $p < 0.05$ were considered statistically significant.

## LASSO Regression

Those common DEGs in the same direction in the GSE58795 and GSE78068 datasets were used to construct a LASSO model using the 'glmnet' R package. In the GSE78068 dataset, 70% of samples were randomly selected as the training set, and the other 30% of samples were selected as the test set (internal validation set). The samples in the GSE58795 were selected as the

**TABLE 2** | Information for selected datasets.

| GEO accession | Samples | | Samples | | Source tissue | Attribute |
|---|---|---|---|---|---|---|
| | Nonresponders | Responders | Healthy | RA | | |
| GSE78068 | 98 | 42 | – | – | Whole blood | Training set and internal validation set |
| GSE58795 | 23 | 36 | – | – | Whole blood | External validation set |
| GSE77298 | – | – | 7 | 16 | Synovium | Validation set |
| GSE55457 | – | – | 10 | 13 | Synovium | Validation set |
| GSE89408 | – | – | 28 | 152 | Synovium | Validation set |

validation set (external validation set). ROC curves were constructed to evaluate the predictive value of the key genes using the 'pROC' R package (29). The same genes in the LASSO model and the key modules identified by WGCNA were defined as hub genes.

## Single-Sample Gene Set Enrichment Analysis (ssGSEA)

Autophagy-related genes were integrated from the GO_AUTOPHAGY gene set on the GSEA website (http://software.broadinstitute.org/gsea/index.jsp) and in the Human Autophagy Database (HADb, http://www.autophagy.lu/index.html) and used as marker genes of autophagy (30). The enrichment score of autophagy in the GSE78068 dataset was calculated using the ssGSEA function of the 'GSVA' R package. The differences of the autophagy score between responders and nonresponders in the GSE78068 dataset were analyzed using Student's t-test, and the correlations between the autophagy score and the hub genes in nonresponders were also detected using Pearson's correlation.

## Oligonucleotides and Cell Transfection

DERL1-siRNA targeting human DERL1 and negative control siRNA were chemically synthesized by GenePharma (GenePharma, Shanghai, China). RA-FLSs were plated in 6-well plates at a density of $1.5\times 10^5$/well. Twenty-four hours after plating, siRNA (80 nM) or negative control siRNA (80 nM) was transfected into RA-FLSs using Lipofectamine 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. The cells were cultured for another 48 hours (for protein) or 24 hours (for mRNA) and then used in the experiments. The siRNA sequence information is given in **Supplementary Table 1**.

## RNA Extraction and Real-Time PCR

Total RNA was isolated from RA and OA synovial tissue and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Two micrograms of total RNA was used to synthesize cDNA using a Transcriptor cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), followed by real-time PCR to detect mRNA expression using a SYBR Green System (Roche Diagnostics, Mannheim, Germany). β-actin expression was used as an endogenous control to normalize the target gene mRNA expression. The $2^{-\Delta\Delta CT}$ method was used to quantify the relative expression. The primer sequence information is listed in **Supplementary Table 1**.

## Western Blotting

Twenty micrograms of protein sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking in 10% milk in Tris-buffered saline/Tween-20 (TBST) for 2 h at room temperature, the nitrocellulose membranes were incubated with the following primary antibodies overnight at 4°C: anti-LC3 monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA, 1: 1000), anti-P62 monoclonal antibody (Abcam, Cambridge, UK, 1:10000), anti-β-actin monoclonal antibody (Bioss, Beijing, China, 1:1000), and anti-DERL1 polyclonal antibody (Abcam, Cambridge, UK, 1:2000). The membranes were then incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (BOSTER, Wuhan, China, 1:10000) for 2 h at room temperature, followed by expression detection with an electrochemiluminescence (ECL) system (Gene Gnome 5, Synoptics Ltd., UK).

## Immunohistochemistry

After the RA and OA synovial tissue histologic slices were dewaxed, 3% $H_2O_2$ was used to inhibit endogenous peroxide, and microwave heating was used to retrieve antigen. The slices were blocked with 5% goat serum (Beyotime Institute of Biotechnology, Shanghai, China) for 30 minutes, and then incubated in DERL1 antibodies (1:200) overnight at 4°C, and 5% bovine serum albumin (BSA) was used as the negative control. The histologic slices were then incubated with a horseradish peroxidase-conjugated secondary antibody (BOSTER, Wuhan, China) for 30 minutes, followed by staining with 3,3'- diaminobenzidine (Beyotime Institute of Biotechnology, Shanghai, China) and mounting. Then, the DERL1-positive cells (brown cells) was examined using a microscope (Olympus, Tokyo, Japan).

## Autophagic Flux Detection

RA-FLSs were plated in 6-well plates at a density of $1.5\times 10^5$/well. Twelve hours after plating, the RA-FLSs were transfected with DERL1-siRNA (80 nM) or negative control siRNA (80 nM) using Lipofectamine 2000 according to the manufacturer's recommendations. Cells were then cultured for another 48 hours, and 0.1 μM of bafilomycin A1 (Baf-A1) (Sigma-Aldrich, St. Louis, MO, USA) or matched amounts of dimethyl sulfoxide (DMSO) were added to cell cultures for the last 4 hours, and then detected the expression of LC3B, P62 and DERL1 with western blotting.

## mCherry-GFP-LC3B Adenovirus Transfection

RA-FLSs were plated in 12-well plates at a density of 5× $10^4$/well. Twelve hours after plating, the mCherry-GFP-LC3B adenovirus (Beyotime Institute of Biotechnology, Shanghai, China) at 20 multiplicity of infection (MOI) was added to the cells according to the manufacturer's protocol. Twenty-four hours after infection, the RA-FLSs were transfected with DERL1-siRNA (80 nM) or negative control siRNA (80 nM) using Lipofectamine 2000 according to the manufacturer's recommendations. Cells were then cultured for another 48 hours, and 0.1 μM of bafilomycin A1 (Sigma-Aldrich, St. Louis, MO, USA) or matched amounts of dimethyl sulfoxide (DMSO) were added to cell cultures for the last 4 hours, and then photographed under a fluorescence microscope (Leica, Germany).

## Statistical Analysis

The data of the experimental validation part are presented as the mean ± standard deviation (SD) or mean with 95% confidence interval (CI). If the data were normally distributed, Student's T-test was used to analyze the differences between two groups, whereas the Mann-Whitney U test was used. GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was used for data analysis, and $P$ values less than 0.05 were considered statistically significant.

# RESULTS

## Identification of Differentially Expressed Genes

The flowchart is shown in **Figure 1**. To identify the DEGs between responders and nonresponders treated with IFX in RA, we downloaded datasets related to IFX treatment for RA from the GEO database. A total of two datasets were obtained. In the GSE78068 dataset, there were 98 nonresponders and 42 responders, and a total of 1452 DEGs ($p < 0.05$) were identified (**Figure 2A**). In the GSE58795 dataset, there were 23 nonresponders and 36 responders, and a total of 3664 DEGs ($p < 0.05$) were identified (**Figure 2B**). Among these DEGs, 46 genes were common DEGs, including 28 concordant downregulated genes and 18 concordant upregulated genes (**Figures 2C, D**). The 46 common genes were chosen for subsequent analysis.

We also used GSVA to explore the different BP terms and KEGG pathways between responders and nonresponders in the

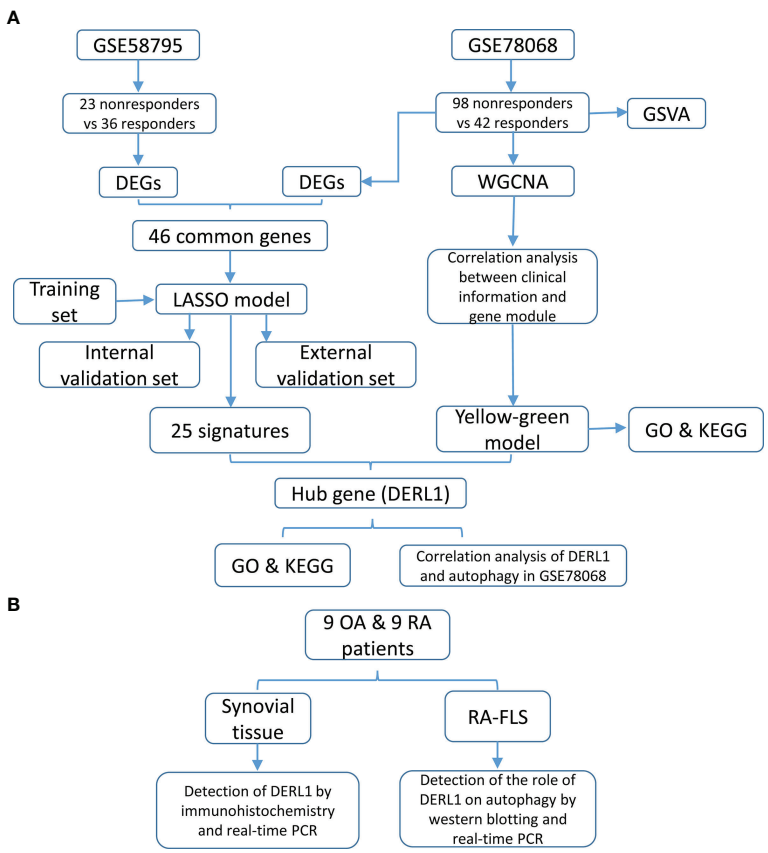



**FIGURE 1** | Study design and flowchart. **(A)** The flowchart of construction of predictive model and identification of the hub genes. **(B)** The flowchart of experimental validation of the hub genes.

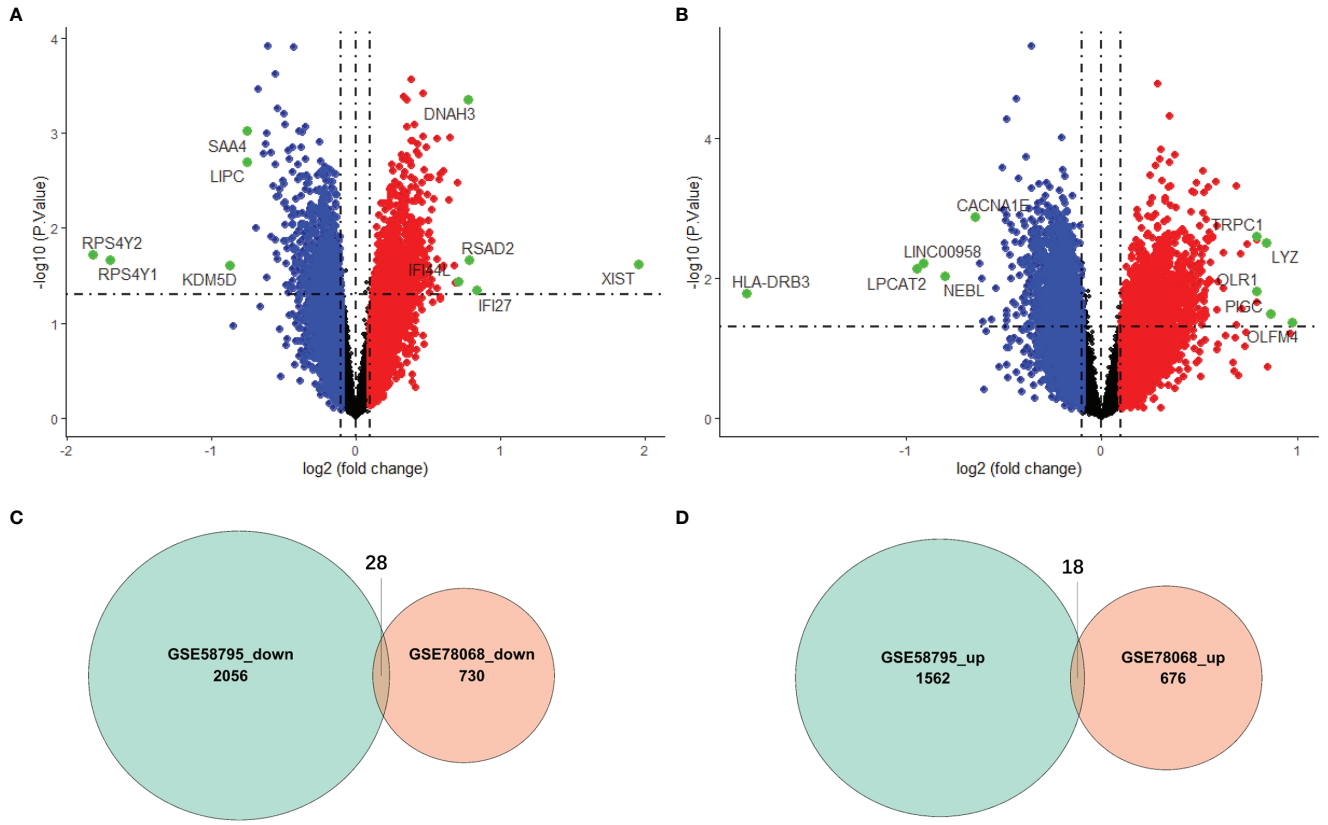



**FIGURE 2** | Identification of differentially expressed genes (DEGs). **(A)** Volcano plot of DEGs between responders and nonresponders in IFX treatment for RA in the GSE78068 dataset. **(B)** Volcano plot of DEGs between responders and nonresponders in IFX treatment for RA in the GSE58795 dataset. **(C)** The Venn diagram of the concordant downregulated genes in the two datases. **(D)** The Venn diagram of the concordant upregulated genes in the two datases.

GSE78068 dataset. Several different BP terms ($P$<0.05) relevant to immune and inflammatory responses and intracellular substance metabolism and transport were mainly enriched in nonresponders (**Figure 3A**, **Supplementary Table 2**). For the KEGG pathway, nonresponders were positively correlated with sulfur metabolism, complement and coagulation cascades, cytosolic and sensing pathways, adipocytokine signaling pathways, Toll-like receptor signaling pathways, receptor tyrosine kinase (ERBB) signaling pathways and so on (**Figure 3B**, **Supplementary Table 3**), which might be related to nonresponse to IFX therapy for RA.

## Identification of the Key Modules Associated With Nonresponse to IFX Therapy for RA

To identify the key modules associated with nonresponse to IFX therapy for RA in the GSE78068 dataset, we performed WGCNA. By setting the optimal soft power threshold for WGCNA as 9 and the cut height as 0.5, scale-free topology is preserved (**Figure 4A**). Twelve modules were identified (**Figures 4B, C**). The heat map of module–trait relationships showed that the yellow-green module was most associated with nonresponse to IFX therapy for RA (correlation coefficient = 0.21, $p$ = 0.04), and there were 674 genes in the yellow-green module. The correlation between module membership (MM) in the yellow-green module and gene significance (GS) for nonresponders was also performed, and the results showed that the yellow-green module was highly correlated with nonresponders (correlation coefficient = 0.24, p = 2.8e–10, **Figure 4D**). We also performed GO function and KEGG pathway enrichment analysis with the genes in yellow-green module. The results showed that 147 BP, 47 CC, and eight MF GO terms were enriched, and the genes were mainly enriched in processes such as antigen processing and presentation of peptide antigen *via* MHC class I in BP, integral component of endoplasmic reticulum membrane in CC, and carbohydrate derivative transmembrane transporter activity in MF (**Figure 4F**). For KEGG pathway analysis, six pathways were enriched, and the genes of the six pathways and their connectivity are shown in **Figure 4E**.

## Construction of the Prediction Model and Identification of the Hub Genes Associated With Nonresponse to IFX Therapy for RA

We constructed a LASSO model to identify the key genes associated with nonresponse to IFX therapy for RA.

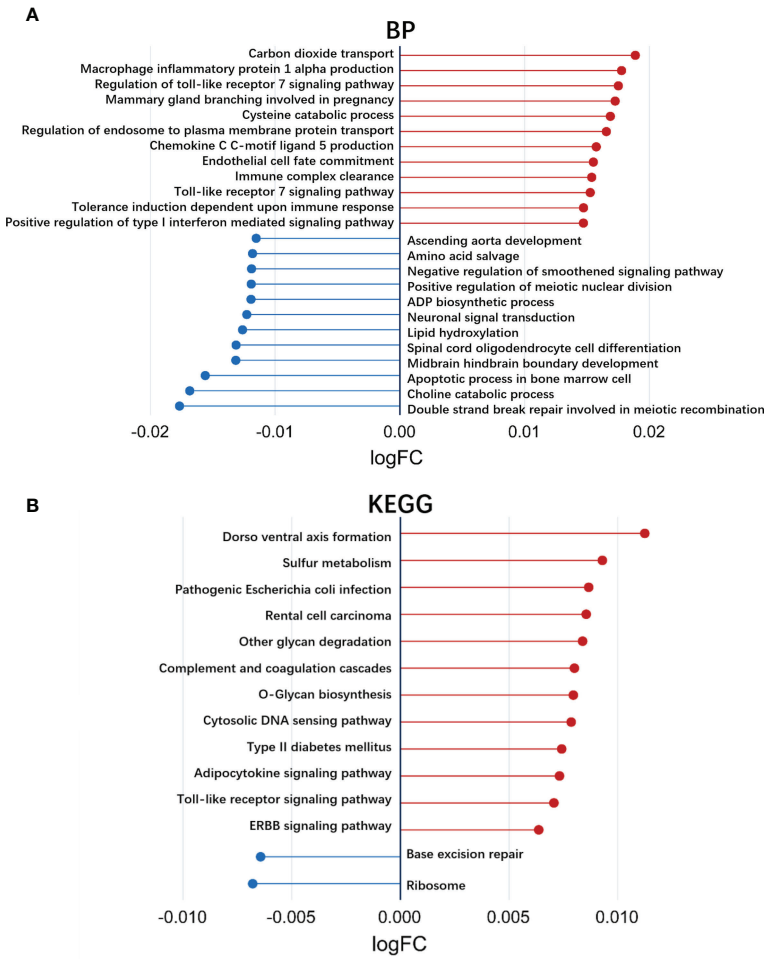


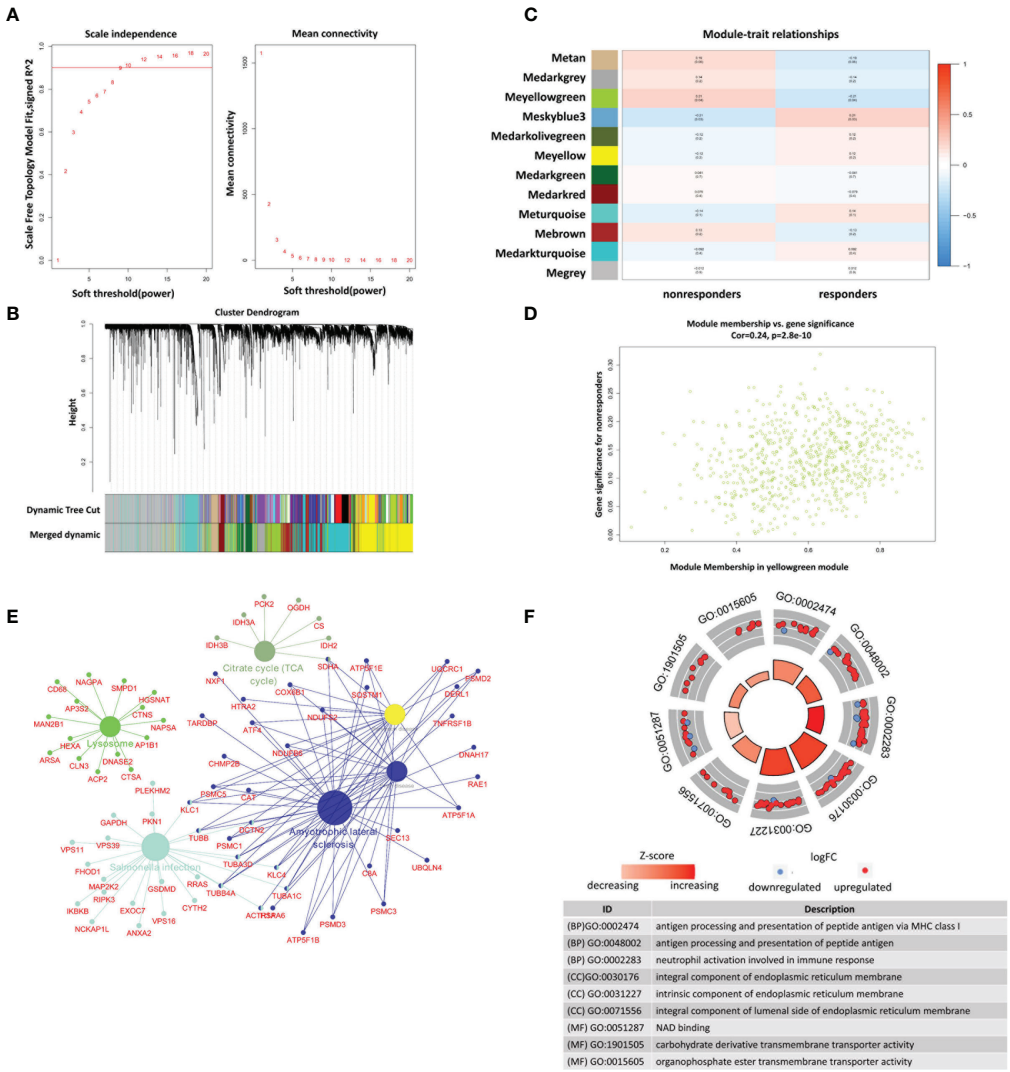

**FIGURE 3** | Exploration of the different biological processes terms and KEGG pathways using gene set variation analysis (GSVA). **(A)** Up- or down-regulated biological processes between responders and non-responders in IFX treatment for RA in the GSE78068 dataset quantified by GSVA. **(B)** Up- or down-regulated KEGG pathways between responders and non-responders in IFX treatment for RA in the GSE78068 dataset quantified by GSVA.

The samples in the GSE78068 dataset were randomly split into a training set (70%) and an internal validation set (30%). The 46 common genes identified above were used to construct a LASSO model in the training set. The LASSO results showed that when the lambda value was selected as lambda.min (0.0207), a total of 25 genes with nonzero coefficients were screened out (**Figures 5A, B**, **Supplementary Table 4**). The AUCs for the 25-gene signature were 0.967 and 0.831 in the training set and the internal validation set, respectively (**Figures 5C, D**). We also validated the signature using the external validation set and GSE58795 dataset, and the AUC was 0.924 (**Figure 5E**). The results showed that the 25-gene signature had good diagnostic ability.

The common genes in the LASSO model and the yellow-green module identified by WGCNA were further screened. Interestingly, only one gene, *DERL1*, was screened out, so *DERL1* was recognized as a hub gene for subsequent validation and analysis. The expression of DERL1 was increased in nonresponders in both the GSE78068 and GSE58795 datasets (**Figures 5F, G**).

## Potential Functions of DERL1 in Nonresponse to IFX Therapy for RA

To gain further insight into the potential role of *DRL1* in nonresponse to IFX therapy for RA, we first conducted a batch correlation analysis between *DRL1* and other genes in nonresponders of the GSE78068 dataset. A total of 8366 genes ($p < 0.05$) were screened for subsequent gene enrichment analysis. GO and KEGG analyses were performed by the clusterProfiler package. BP was mainly enriched in neutrophil immune responses, such as neutrophil-mediated immunity, neutrophil activation involved in the immune response, and neutrophil activation. The cellular component (CC) results revealed that these genes were mainly enriched in the mitochondrial matrix, cell substrate adherens junctions, focal adhesion and so on. For the MF results, the genes were mainly




| ID | Description |
|---|---|
| (BP)GO:0002474 | antigen processing and presentation of peptide antigen via MHC class I |
| (BP) GO:0048002 | antigen processing and presentation of peptide antigen |
| (BP) GO:0002283 | neutrophil activation involved in immune response |
| (CC)GO:0030176 | integral component of endoplasmic reticulum membrane |
| (CC) GO:0031227 | intrinsic component of endoplasmic reticulum membrane |
| (CC) GO:0071556 | integral component of lumenal side of endoplasmic reticulum membrane |
| (MF) GO:0051287 | NAD binding |
| (MF) GO:1901505 | carbohydrate derivative transmembrane transporter activity |
| (MF) GO:0015605 | organophosphate ester transmembrane transporter activity |

**FIGURE 4** | Identification of modules associated with nonresponse to IFX treatment in RA in GSE78068 dataset through weighted gene coexpression network analysis (WGCNA). **(A)** Correlation between scale free topology model fit and soft threshold power. **(B)** The cluster dendrogram showing the change of modules before and after merging, and different colors represent different modules. **(C)** Module-trait relationships. Each row represents a module and each column represents a clinical trait (responders and nonresponders). **(D)** Scatter plot of genes in the yellow-green module. **(E)** The network of KEGG pathway in the yellow-green module. **(F)** The GO analysis of the genes in yellow-green module.

involved in protein serine/threonine kinase activity, ubiquitin-like protein ligase binding, and transcription coactivator activity (**Figure 6A**). The KEGG analysis revealed that these genes were enriched in pathways mainly related to autophagy, lysosome, and B cell receptor signaling pathways (**Figure 6B**). Interestingly, both the BP and KEGG analysis results showed that these genes were associated with autophagy (**Supplementary Table 5, Table 6**), which indicated that there was a relationship between DERL1 and autophagy.

## Autophagy Might Play a Role in Nonresponse to IFX Therapy for RA

From the study above, *DERL1* was identified as the key gene associated with nonresponse to IFX therapy for RA, and we also found that there was a potential role of DERL1 in autophagy. Based on these results, we speculated that autophagy might also play a role in nonresponse to IFX therapy for RA. To identify the difference of the autophagy-related BP between responders and nonresponders in the GSE78068 dataset, we performed GSEA. Autophagy-related genes were integrated from the GO_AUTOPHAGY gene set in the GSEA website and the Human Autophagy Database (HADb), and the GSEA results showed that autophagy-related genes were mainly enriched in nonresponders (**Figure 6C**). Moreover, we used the above autophagy-related genes to analyze the autophagy score of the GSE78068 dataset using the ssGSEA method, and the autophagy score was significantly increased in the nonresponders (**Figure 6D**). We also performed a correlation analysis between

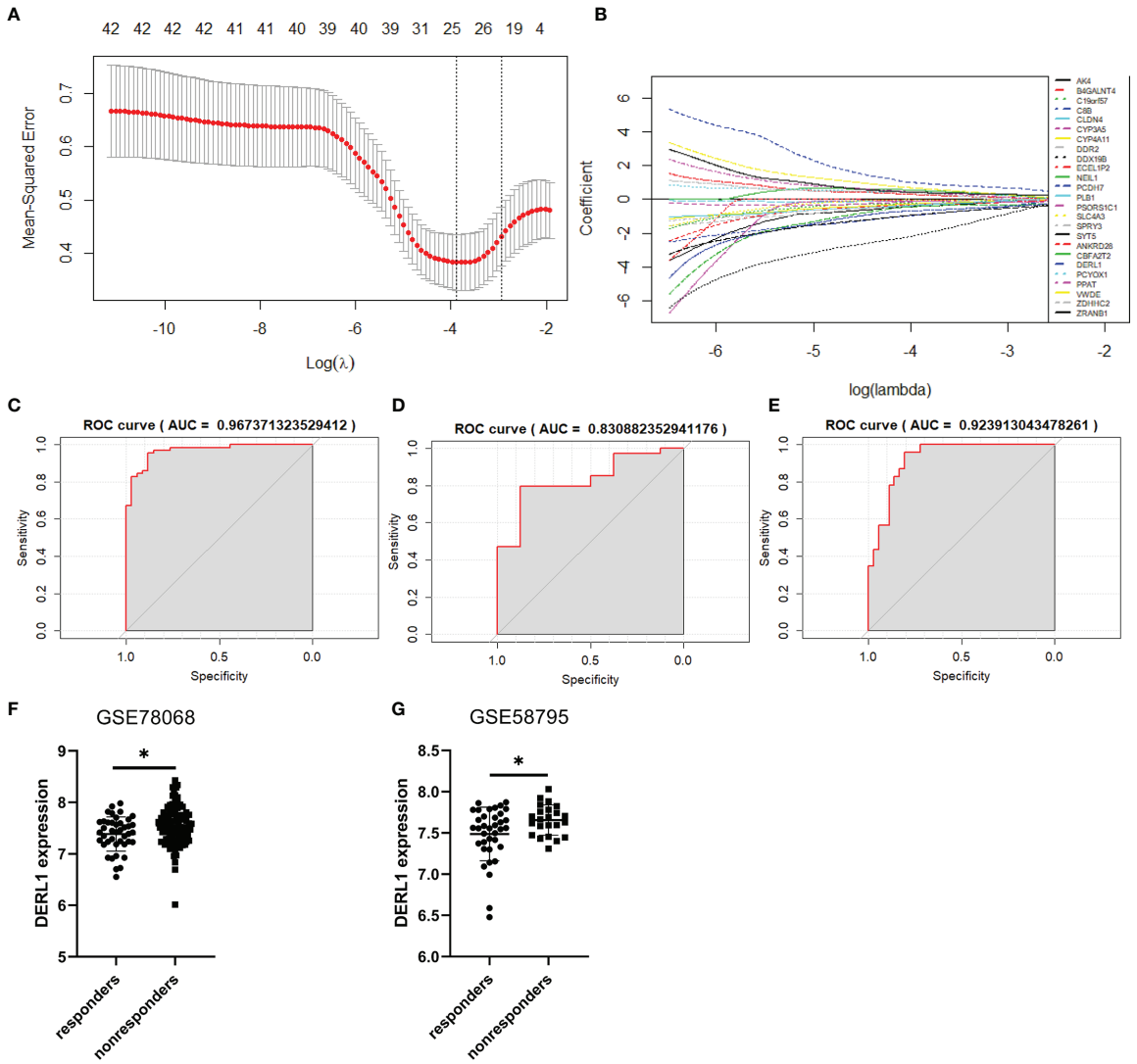



**FIGURE 5** | Construction of the prediction model and identification of the hub genes associated with nonresponse to IFX therapy for RA. **(A)** Cross-validation for the gene signature selection of optimal lambda value in least absolute shrink and selection operator (LASSO) model. **(B)** LASSO coefficient profiles of the 25 prediction genes. **(C)** The receiver operating characteristic (ROC) curves of the 25-gene signature in training set of GSE78068 dataset. **(D)** The ROC curves of the 25-gene signature in internal validation set of GSE78068 dataset. **(E)** The ROC curves of the 25-gene signature in external validation set of GSE58795 dataset. **(F, G)** Differential expression of DERL1 between responders and nonresponders in GSE78068 and GSE58795 datasets. Student's-t test was used for the data analyses in **(F)**. Student's-t test with Welch's correction was used for the data analyses in **(G)**. *$P < 0.05$.

DERL1 expression and autophagy score. The scatter plot results revealed that DERL1 expression was positively correlated with the autophagy score (**Figure 6E**).

## Experimental Validation of the Relationship Between DERL1 and Autophagy

Chronic inflammatory hyperplasia of the synovium was the most characteristic pathological change in RA. Studies have found that hyperplastic synoviocytes are mainly RA-FLSs, which play a key role in the pathological mechanism of RA. To further validate the expression of DERL1 in RA synovial tissue, we performed real-time PCR and immunohistochemistry. Compared with OA synovial tissue, DERL1 expression was increased in RA synovial tissue (**Figures 7A, B**). We also analyzed DERL1 expression in GSE77298, GSE55457, and GSE89408 dataset, and found that DERL1 expression was increased in RA synovial tissue compared with healthy synovial tissue (**Figures 7C–E**). Furthermore, we explored the effect of DERL1 on the autophagy of RA-FLSs. We used DERL1-siRNA to knockdown DERL1 expression and detected the autophagy-related proteins LC3B. After transfection with DERL1-siRNA, the expression of DERL1 and LC3B-II were decreased significantly (**Figures 8A–C**). The decrease in LC3-II reflects only the decrease of autophagosomes formed, no

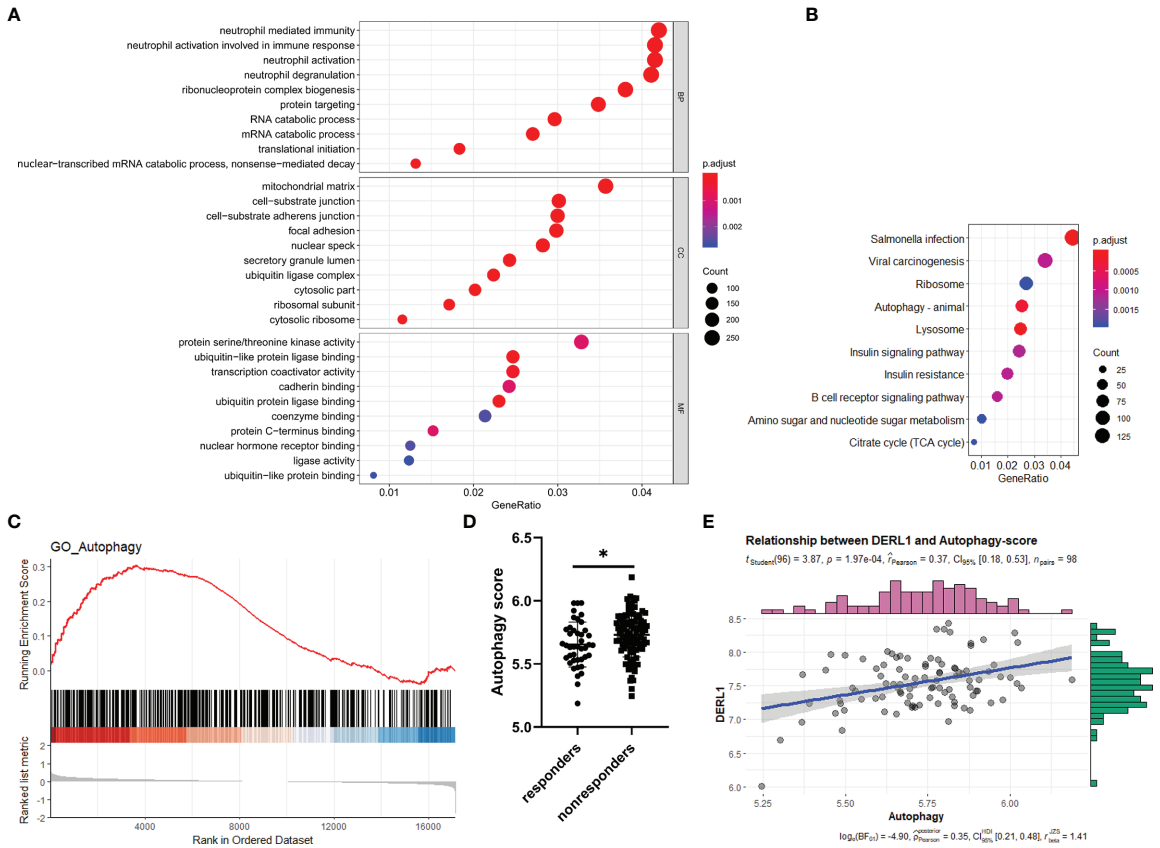



**FIGURE 6** | Potential role of DERL1 in nonresponse to IFX therapy for RA. **(A, B)** The GO and KEGG analysis of the genes screened out through a batch correlation analysis between DERL1 and other genes in nonresponders of GSE78068 dataset. **(C)** Gene set enrichment analysis (GSEA) for comparing autophagy gene term between responders and nonresponders in GSE78068 dataset. **(D)** Differential autophagy score between responders and nonresponders in GSE78068 dataset. **(E)** Scatter plot of the relationship between DERL1 expression and autophagy score in nonresponders of GSE78068 dataset. Student's-t test was used for the data analyses in **(D)**. *$P < 0.05$.

information about the autophagic flux is provided. Therefore, we transfected DERL1-siRNA in the presence and absence of the lysosomal inhibitor Baf-A1 to block the autophagic pathway at a late stage (31). The results showed that DERL1-siRNA inhibited autophagy formation in RA-FLSs, as demonstrated by an decreased amount of LC3B-II in the presence of Baf-A1 (**Figure 8D**). We also detected the expression of autophagic flux-related protein P62, but no significant changes were found (**Supplementary Figure 1**). The effect of DERL1-siRNA on autophagic flux was further assessed by using mCherry-GFP-LC3B adenovirus. Upon infection of mCherry-GFP-LC3B adenovirus, autophagosomes are labeled with orange or yellow signals, due to both GFP and mCherry fluorescence. However, once autophagosome and lysosome are fused, the autolysosomes will labeled with red puncta, due to GFP fluorescence will be rapidly quenched under low PH of lysosome. As shown in **Figure 8E**, there were fewer orange puncta in the DERL1-siRNA group than in the NC-siRNA group, indicating that there were fewer autophagosomes in the DERL1-siRNA group. When the autophagic flux was blocked by Baf-A1, more orange or yellow puncta were observed in both NC-siRNA and DERL1-siRNA group, and the number of orange or yellow puncta in NC-siRNA group was significantly higher than that in DERL1-siRNA group, indicating that DERL1-siRNA inhibited autophagosomes formation. Based on these results, we speculated that DERL1-siRNA partially inhibited the formation of autophagosomes in RA-FLSs.

## DISCUSSION

Clinical studies have shown that IFX is particularly important for controlling the condition of RA, especially in the early stage. However, the clinical application has found that there are still some RA patients who do not respond to IFX, and the patients have to increase the dose or change the medication in the future. Who would benefit from IFX treatment is still unclear. Therefore, prediction of efficacy through biomarkers before IFX treatment for RA is helpful for individualized treatment of RA. In the present study, we identified a 25-gene signature using the LASSO model and AUCs, and the signature had good predictive ability in both the internal validation set and external validation set. Moreover, *DERL1* was identified as the hub gene, which may be a biomarker to predict the efficacy of IFX in RA treatment. Functional analysis revealed that

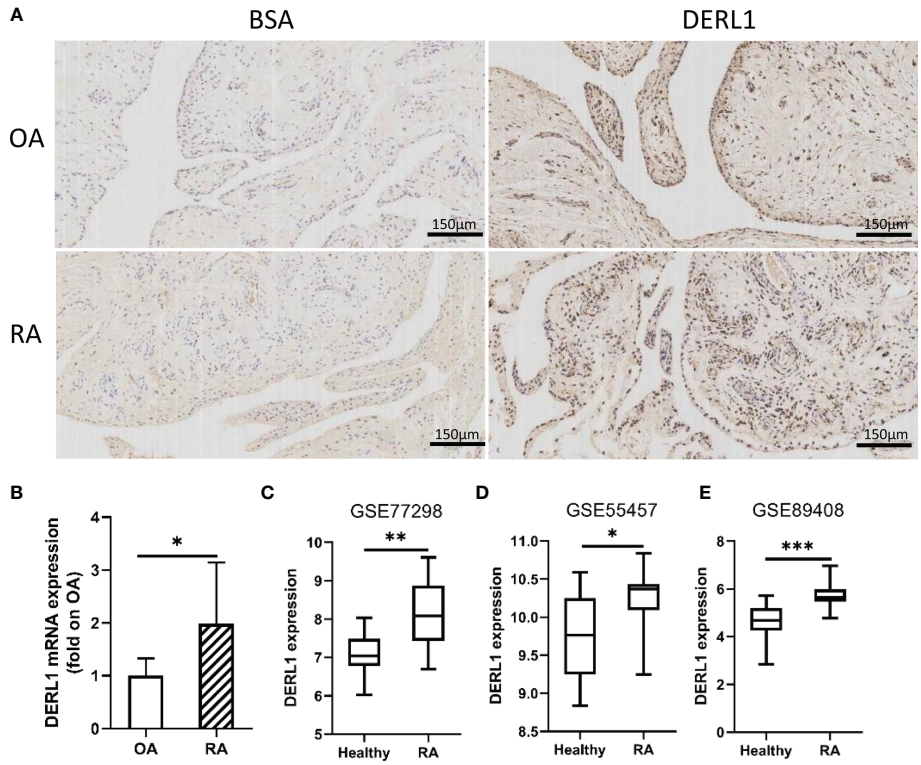



**FIGURE 7** | The expression of DERL1 in OA and RA synovial tissues. **(A)** The expression of DERL1 in RA and OA synovial tissue was detected with immunohistochemistry tests. **(B)** The mRNA of DERL1 in RA and OA synovial tissue was detected with real-time PCR. **(C–E)** The expression of DERL1 in GSE77298 dataset, GSE55457 dataset, and GSE89408 dataset. Student's-t test with Welch's correction was used for the data analyses in **(B)**. Student's-t test was used for the data analyses in **(C, D)**. The Mann-Whitney test was used for the data analyses in **(E)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

there was a close relationship between DERL1 and autophagy, and the autophagy score in nonresponders was also increased compared with that in responders to IFX treatment for RA. Finally, the experimental results validated that DERL1 was increased in RA synovial tissues compared with OA synovial tissues and that DERL1-siRNA partially inhibited autophagosomes formation in RA-FLSs.

GSVA is a gene set enrichment (GSE) method that can be used to detect the differences in pathway activity over a sample population in an unsupervised manner (24). In our study, we used GSVA to explore the different BP and KEGG pathways between responders and nonresponders and found that several BP terms associated with inflammation and immunity or intracellular substance metabolism and transport were highly expressed in nonresponders, such as macrophage inflammatory protein 1 alpha production, regulation of Toll-like receptor-7 signaling pathway, and regulation of endosome to plasma membrane protein transport. For the KEGG pathways, nonresponders were positively correlated with the Toll-like receptor signaling pathway and receptor tyrosine kinase (ERBB) signaling pathway. Consistent with Nakamura's studies (32), they found that inflammasome genes were significantly upregulated with IFX in nonresponders.

Using WGCNA to identify disease-associated modules and explore functional pathways and candidate biomarkers has been proven to be an effective method (25). Twelve modules were identified in the present study, and the yellow-green module was identified as the model most associated with nonresponse to IFX therapy for RA. Consistent with the GSVA results, the enrichment results of the yellow-green module showed that the BP, CC, and MF terms were mainly associated with inflammation and immunity or intracellular substance metabolism and transport. Interestingly, the enriched KEGG pathways were inconsistent with the GSVA results.

We identified a 25-gene signature using a combination of the LASSO model and AUC in our study, and the AUC results suggested that the 25-gene signature was an effective model for the prediction of the efficacy of IFX in RA. Some of the 25 biomarkers, such as phospholipase B1 (PLB1) (33), discoidin receptor 2 (DDR2) (34), and psoriasis susceptibility 1 candidate 1 (PSORS1C1) (35), have been identified as biomarkers and participate in the pathogenesis of RA. PSORS1C1 was also related to the efficacy of TNF-α inhibitors in RA treatment. Ciccacci et al. found that SNPs of PSORS1C1 were associated with a severe disease phenotype and response to TNF-α treatment in RA patients (36, 37).

We further identified the hub genes associated with nonresponse to IFX therapy for RA using a combination of the LASSO model and WGCNA and found that only DERL1 was

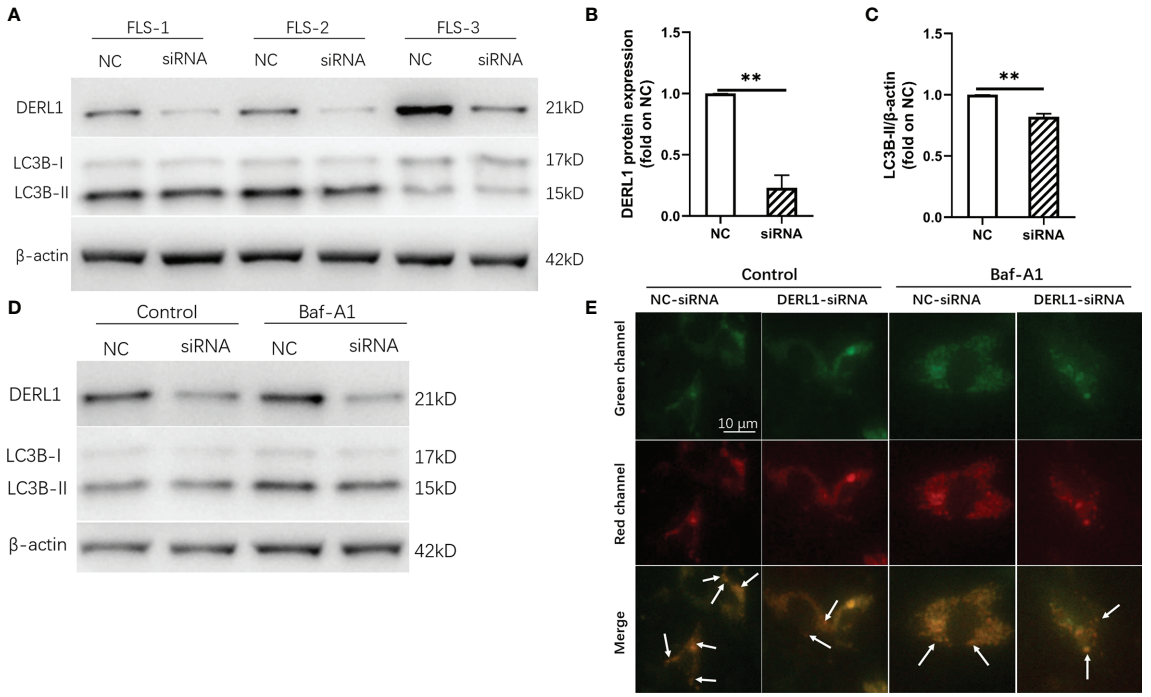



**FIGURE 8** | Experimental validation of the relationship between DERL1 and autophagy. **(A)** RA-FLS were transfected with NC-siRNA (80nM) and DERL1-siRNA (80nM) for 48h, the level of DERL1 and LC3B were detected by western blotting. **(B, C)** Relative densitometric analyses of DERL1 and LC3B in **(A)**. **(D)** Autophagic flux was monitored in RA-FLSs after 48 hours of transfection with DERL1-siRNA or NC-siRNA in the presence or absence of 0.1 μM bafilomycin A1 (Baf-A1). **(E)** RA-FLSs infected with mCherry-GFP-LC3B adenovirus were transfected with DERL1-siRNA or NC-siRNA for 48 hours in the presence or absence of 0.1 μM Baf-A1, and the autophagic flux was assessed under fluorescence microscope. Student's-t test with Welch's correction was used for the data analyses in **(B, C)**. $^{**}P < 0.01$.

screened out. DERL1, an endoplasmic reticulum (ER)-associated protein, mediates the elimination of misfolded proteins from the ER and retrotranslocation of proteins into the cytosol (38, 39). Studies have reported that the expression of DERL1 is increased in some diseases and it can reduce ER stress-induced apoptosis (40, 41). Dong et al. found that increased DERL1 was correlated with chemoradiotherapy resistance in esophageal squamous cell carcinoma, and the PI3K/AKT/Bcl-2 signaling pathway was involved in this process (42). Interestingly, different results were found in another study (43). Human breast cancer resistance protein (BCRP) could protect cells or tissues from xenobiotic-induced toxicity by facilitating the disposition of endogenous and exogenous harmful xenobiotics, and overexpression of BCRP reduced the intracellular concentration of anticancer drugs. Sugiyama et al. found that DERL1 was involved in the posttranslational regulation of BCRP and was a negative regulator of BCRP expression (43).

To further explore the role of DERL1 in RA, we conducted a batch correlation analysis and ssGSEA in the GSE78068 dataset. The results showed that autophagy-related genes were mainly enriched in nonresponders and that the autophagy score was significantly increased in the nonresponders, which indicated that autophagy might play a role in the nonresponse to IFX therapy for RA and that DERL1 might be involved in autophagy regulation in this process. Studies have found that enhanced autophagy may be associated with the development of drug resistance (44). Our previous study found that the levels of autophagy in RA synovial tissue were increased, MTX stimulated the autophagy response in RA-FLSs, and MTX-induced apoptosis of RA-FLSs was increased after inhibition of autophagy, which indicated that autophagy induction contributed to resistance to MTX treatment in RA-FLSs (45). Xu et al. found that DERL1 expression was elevated in most non-small lung cancer cell lines, and DERL1-siRNA blocked autophagic flux in A549 cells (46). Consistent with the above study, we found that the expression of DERL1 was increased in RA synovial tissue compared with OA synovial tissue. After transfection with DERL1-siRNA, we found that LC3-II expression was decreased, which may be a result of either reduction of autophagosomes formation or increase of autophagic degradation. Therefore, autophagic flux should be detected (31). We found that DERL1-siRNA decreased the expression of LC3-II in the presence of Baf-A1, a lysosomal protease inhibitor, which indicated that DERL1-siRNA might partially inhibit autophagosomes formation in RA-FLSs.

There are still some potential limitations that need to be taken into account when interpreting these findings. First, the two datasets in our study contain different populations of RA; patients in the GSE78068 dataset are Asian, and those in the GSE58795 dataset are European. Second, therapeutic outcomes were defined for the GSE78068 dataset at 6 months after initiation of IFX treatment and the GSE58795 dataset at 14 weeks, which may affect the

interpretation of the results. Third, the two datasets were obtained from public databases, and the sample size was still insufficient. Further research is still needed to support our findings. Fourth, we only detected the expression of DERL1 in RA synovial tissue, and its expression in the peripheral blood of RA patients who do not respond to IFX therapy still needs to be detected. More importantly, all synovial tissues were obtained from RA patients who underwent arthroplasty, which means that all RA patients were in the advanced stage of RA. Last, the detailed role of DERL1 in the pathogenesis of nonresponse to IFX therapy for RA remains to be further verified *in vivo* and *in vitro* experiments.

## CONCLUSION

In conclusion, our 25-gene signature may have potential predictive value for IFX therapy in RA at the beginning of IFX treatment. The results suggest that the autophagy level is increased in nonresponders, which may be involved in nonresponse to IFX therapy for RA. We screened *DERL1* as the hub gene and observed that it was increased in both peripheral blood cells and RA synovial tissue. Moreover, DERL1 is involved in autophagy regulation, and DERL1-siRNA may partially inhibit autophagosomes formation in RA-FLSs. These findings deepen our understanding of the potential molecular mechanism of nonresponse to IFX therapy for RA and provide a direction for future research.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study are available online, and the name of the database and accession number(s) can be found in the article. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The human research ethics committee of Xi'an Hong Hui Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

PX, YC, and KX designed the study. YC, YA, and ZL carried out the bioinformatic analysis. QY, JX, HZ, and MY conducted the experimental validation. BW and YNY drew the figures. YC, KX, and YA drafted the manuscript. YY and PX participated in modifying the manuscript. All authors approved the final submitted manuscript.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.795912/full#supplementary-material

## REFERENCES

1. Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al. Rheumatoid Arthritis. *Nat Rev Dis Primers* (2018) 4:18001. doi: 10.1038/nrdp.2018.1
2. Süß P, Rothe T, Hoffmann A, Schlachetzki JCM, Winkler J. The Joint-Brain Axis: Insights From Rheumatoid Arthritis on the Crosstalk Between Chronic Peripheral Inflammation and the Brain. *Front Immunol* (2020) 11:612104. doi: 10.3389/fimmu.2020.612104
3. Chaudhari K, Rizvi S, Syed BA. Rheumatoid Arthritis: Current and Future Trends. *Nat Rev Drug Discovery* (2016) 15(5):305–6. doi: 10.1038/nrd.2016.21
4. van Schouwenburg PA, Rispens T, Wolbink GJ. Immunogenicity of Anti-TNF Biologic Therapies for Rheumatoid Arthritis. *Nat Rev Rheumatol* (2013) 9(3):164–72. doi: 10.1038/nrrheum.2013.4
5. Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, et al. Randomised Double-Blind Comparison of Chimeric Monoclonal Antibody to Tumour Necrosis Factor Alpha (Ca2) Versus Placebo in Rheumatoid Arthritis. *Lancet (London England)* (1994) 344(8930):1105–10. doi: 10.1016/s0140-6736(94)90628-9
6. Zhou H, Jang H, Fleischmann RM, Bouman-Thio E, Xu Z, Marini JC, et al. Pharmacokinetics and Safety of Golimumab, a Fully Human Anti-TNF-Alpha Monoclonal Antibody, in Subjects With Rheumatoid Arthritis. *J Clin Pharmacol* (2007) 47(3):383–96. doi: 10.1177/0091270006298188
7. Weinblatt ME, Keystone EC, Furst DE, Moreland LW, Weisman MH, Birbara CA, et al. Adalimumab, a Fully Human Anti-Tumor Necrosis Factor Alpha Monoclonal Antibody, for the Treatment of Rheumatoid Arthritis in Patients Taking Concomitant Methotrexate: The ARMADA Trial. *Arthritis Rheumat* (2003) 48(1):35–45. doi: 10.1002/art.10697
8. Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, et al. Treatment of Rheumatoid Arthritis With a Recombinant Human Tumor Necrosis Factor Receptor (P75)-Fc Fusion Protein. *N Engl J Med* (1997) 337(3):141–7. doi: 10.1056/nejm199707173370301
9. Maini SR. Infliximab Treatment of Rheumatoid Arthritis. *Rheumat Dis Clinics North America* (2004) 30(2):329–47, vii. doi: 10.1016/j.rdc.2004.01.009
10. Gisbert JP, Marín AC, McNicholl AG, Chaparro M. Systematic Review With Meta-Analysis: The Efficacy of a Second Anti-TNF in Patients With Inflammatory Bowel Disease Whose Previous Anti-TNF Treatment has Failed. *Aliment Pharmacol Ther* (2015) 41(7):613–23. doi: 10.1111/apt.13083
11. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and Methotrexate in the Treatment of Rheumatoid Arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis With Concomitant Therapy Study Group. *N Engl J Med* (2000) 343(22):1594–602. doi: 10.1056/nejm200011303432202
12. Lu YC, Chuang CH, Chuang KH, Chen IJ, Huang BC, Lee WH, et al. Specific Activation of Pro-Infliximab Enhances Selectivity and Safety of Rheumatoid

Arthritis Therapy. *PloS Biol* (2019) 17(6):e3000286. doi: 10.1371/journal.pbio.3000286
13. Huang QL, Zhou FJ, Wu CB, Xu C, Qian WY, Fan DP, et al. Circulating Biomarkers for Predicting Infliximab Response in Rheumatoid Arthritis: A Systematic Bioinformatics Analysis. *Med Sci Monit: Int Med J Exp Clin Res* (2017) 23:1849–55. doi: 10.12659/msm.900897
14. Baert F, Noman M, Vermeire S, Van Assche G, DH G, Carbonez A, et al. Influence of Immunogenicity on the Long-Term Efficacy of Infliximab in Crohn's Disease. *N Engl J Med* (2003) 348(7):601–8. doi: 10.1056/NEJMoa020888
15. Pascual-Salcedo D, Plasencia C, Ramiro S, Nuño L, Bonilla G, Nagore D, et al. Influence of Immunogenicity on the Efficacy of Long-Term Treatment With Infliximab in Rheumatoid Arthritis. *Rheumatol (Oxford England)* (2011) 50 (8):1445–52. doi: 10.1093/rheumatology/ker124
16. Svenson M, Geborek P, Saxne T, Bendtzen K. Monitoring Patients Treated With Anti-TNF-Alpha Biopharmaceuticals: Assessing Serum Infliximab and Anti-Infliximab Antibodies. *Rheumatol (Oxford England)* (2007) 46 (12):1828–34. doi: 10.1093/rheumatology/kem261
17. Jamnitski A, Bartelds GM, Nurmohamed MT, van Schouwenburg PA, van Schaardenburg D, Stapel SO, et al. The Presence or Absence of Antibodies to Infliximab or Adalimumab Determines the Outcome of Switching to Etanercept. *Ann Rheumat Dis* (2011) 70(2):284–8. doi: 10.1136/ard.2010.135111
18. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy Fights Disease Through Cellular Self-Digestion. *Nature* (2008) 451(7182):1069–75. doi: 10.1038/nature06639
19. Levine B. Cell Biology: Autophagy and Cancer. *Nature* (2007) 446(7137):745–7. doi: 10.1038/446745a
20. Coury F, Ferraro-Peyret C, Le Cam S, Guerin S, Tebib J, Sibilia J, et al. Peripheral Blood Lymphocytes From Patients With Rheumatoid Arthritis Are Differentially Sensitive to Apoptosis Induced by Anti-Tumour Necrosis Factor-Alpha Therapy. *Clin Exp Rheumatol* (2008) 26(2):234–9.
21. Vomero M, Manganelli V, Barbati C, Colasanti T, Capozzi A, Finucci A, et al. Reduction of Autophagy and Increase in Apoptosis Correlates With a Favorable Clinical Outcome in Patients With Rheumatoid Arthritis Treated With Anti-TNF Drugs. *Arthritis Res Ther* (2019) 21(1):39. doi: 10.1186/s13075-019-1818-x
22. Mu N, Gu J, Huang T, Zhang C, Shu Z, Li M, et al. A Novel NF-κb/YY1/microRNA-10a Regulatory Circuit in Fibroblast-Like Synoviocytes Regulates Inflammation in Rheumatoid Arthritis. *Sci Rep* (2016) 6:20059. doi: 10.1038/srep20059
23. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies. *Nucleic Acids Res* (2015) 43(7):e47. doi: 10.1093/nar/gkv007
24. Hänzelmann S, Castelo R, Guinney J. GSVA: Gene Set Variation Analysis for Microarray and RNA-Seq Data. *BMC Bioinf* (2013) 14:7. doi: 10.1186/1471-2105-14-7
25. Langfelder P, Horvath S. WGCNA: An R Package for Weighted Correlation Network Analysis. *BMC Bioinf* (2008) 9:559. doi: 10.1186/1471-2105-9-559
26. Yu G, Wang LG, Han Y, He QY. Clusterprofiler: An R Package for Comparing Biological Themes Among Gene Clusters. *Omics: J Integr Biol* (2012) 16 (5):284–7. doi: 10.1089/omi.2011.0118
27. Walter W, Sánchez-Cabo F, Ricote M. GOplot: An R Package for Visually Combining Expression Data With Functional Analysis. *Bioinf (Oxford England)* (2015) 31(17):2912–4. doi: 10.1093/bioinformatics/btv300
28. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: A Cytoscape Plug-in to Decipher Functionally Grouped Gene Ontology and Pathway Annotation Networks. *Bioinf (Oxford England)* (2009) 25(8):1091–3. doi: 10.1093/bioinformatics/btp101
29. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: An Open-Source Package for R and S+ to Analyze and Compare ROC Curves. *BMC Bioinf* (2011) 12:77. doi: 10.1186/1471-2105-12-77
30. Wang Y, Zhao W, Xiao Z, Guan G, Liu X, Zhuang M. A Risk Signature With Four Autophagy-Related Genes for Predicting Survival of Glioblastoma Multiforme. *J Cell Mol Med* (2020) 24(7):3807–21. doi: 10.1111/jcmm.14938
31. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, Abdellatif M, Abdoli A, Abel S, et al. Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy (4th Edition)(1). *Autophagy* (2021) 17(1):1–382. doi: 10.1080/15548627.2020.1797280
32. Nakamura S, Suzuki K, Iijima H, Hata Y, Lim CR, Ishizawa Y, et al. Identification of Baseline Gene Expression Signatures Predicting Therapeutic Responses to Three Biologic Agents in Rheumatoid Arthritis: A Retrospective Observational Study. *Arthritis Res Ther* (2016) 18:159. doi: 10.1186/s13075-016-1052-8
33. Okada Y, Diogo D, Greenberg JD, Mouassess F, Achkar WA, Fulton RS, et al. Integration of Sequence Data From a Consanguineous Family With Genetic Data From an Outbred Population Identifies PLB1 as a Candidate Rheumatoid Arthritis Risk Gene. *PloS One* (2014) 9(2):e87645. doi: 10.1371/journal.pone.0087645
34. Su J, Yu J, Ren T, Zhang W, Zhang Y, Liu X, et al. Discoidin Domain Receptor 2 Is Associated With the Increased Expression of Matrix Metalloproteinase-13 in Synovial Fibroblasts of Rheumatoid Arthritis. *Mol Cell Biochem* (2009) 330 (1-2):141–52. doi: 10.1007/s11010-009-0127-0
35. Sun H, Xia Y, Wang L, Wang Y, Chang X. PSORS1C1 may be Involved in Rheumatoid Arthritis. *Immunol Lett* (2013) 153(1-2):9–14. doi: 10.1016/j.imlet.2013.06.001
36. Ciccacci C, Conigliaro P, Perricone C, Rufini S, Triggianese P, Politi C, et al. Polymorphisms in STAT-4, IL-10, PSORS1C1, PTPN2 and MIR146A Genes Are Associated Differently With Prognostic Factors in Italian Patients Affected by Rheumatoid Arthritis. *Clin Exp Immunol* (2016) 186(2):157–63. doi: 10.1111/cei.12831
37. Conigliaro P, Ciccacci C, Politi C, Triggianese P, Rufini S, Kroegler B, et al. Polymorphisms in STAT4, PTPN2, PSORS1C1 and TRAF3IP2 Genes Are Associated With the Response to TNF Inhibitors in Patients With Rheumatoid Arthritis. *PloS One* (2017) 12(1):e0169956. doi: 10.1371/journal.pone.0169956
38. Lilley BN, Ploegh HL. A Membrane Protein Required for Dislocation of Misfolded Proteins From the ER. *Nature* (2004) 429(6994):834–40. doi: 10.1038/nature02592
39. Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA. A Membrane Protein Complex Mediates Retro-Translocation From the ER Lumen Into the Cytosol. *Nature* (2004) 429(6994):841–7. doi: 10.1038/nature02656
40. Wang J, Hua H, Ran Y, Zhang H, Liu W, Yang Z, et al. Derlin-1 Is Overexpressed in Human Breast Carcinoma and Protects Cancer Cells From Endoplasmic Reticulum Stress-Induced Apoptosis. *Breast Cancer Res: BCR* (2008) 10(1):R7. doi: 10.1186/bcr1849
41. Mori A, Yamashita S, Uchino K, Suga T, Ikeda T, Takamatsu K, et al. Derlin-1 Overexpression Ameliorates Mutant SOD1-Induced Endoplasmic Reticulum Stress by Reducing Mutant SOD1 Accumulation. *Neurochem Int* (2011) 58 (3):344–53. doi: 10.1016/j.neuint.2010.12.010
42. Dong Q, Fu L, Zhao Y, Liu Y, Li Q, Qiu X, et al. Derlin-1 Is a Target to Improve Radiotherapy Effect of Esophageal Squamous Cell Carcinoma. *Oncotarget* (2017) 8(33):55135–46. doi: 10.18632/oncotarget.19069
43. Sugiyama T, Shuto T, Suzuki S, Sato T, Koga T, Suico MA, et al. Posttranslational Negative Regulation of Glycosylated and Non-Glycosylated BCRP Expression by Derlin-1. *Biochem Biophys Res Commun* (2011) 404(3):853–8. doi: 10.1016/j.bbrc.2010.12.074
44. Mele L, Del Vecchio V, Liccardo D, Prisco C, Schwerdtfeger M, Robinson N, et al. The Role of Autophagy in Resistance to Targeted Therapies. *Cancer Treat Rev* (2020) 88:102043. doi: 10.1016/j.ctrv.2020.102043
45. Xu K, Cai YS, Lu SM, Li XL, Liu L, Li Z, et al. Autophagy Induction Contributes to the Resistance to Methotrexate Treatment in Rheumatoid Arthritis Fibroblast-Like Synovial Cells Through High Mobility Group Box Chromosomal Protein 1. *Arthritis Res Ther* (2015) 17:374. doi: 10.1186/s13075-015-0892-y
46. Xu L, Wang ZH, Xu D, Lin G, Li DR, Wan T, et al. Expression of Derlin-1 and Its Effect on Expression of Autophagy Marker Genes Under Endoplasmic Reticulum Stress in Lung Cancer Cells. *Cancer Cell Int* (2014) 14:50. doi: 10.1186/1475-2867-14-50

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SYSTEMATIC REVIEW
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# The Efficacy of Denosumab in Patients With Rheumatoid Arthritis: A Systematic Review and Pooled Analysis of Randomized or Matched Data

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**Objective:** The purpose of this study was to evaluate the efficacy of denosumab treatment in patients with rheumatoid arthritis (RA).

**Methods:** The Medline, Embase and Cochrane Library databases were searched for relevant clinical studies. Studies that assessed the efficacy of denosumab in patients with RA were identified. The primary endpoints were the percent changes in bone mineral density (BMD), and the changes in modified total Sharp score (mTSS), modified Sharp erosion score and joint space narrowing (JSN) score. Pooled analyses were calculated using random-effect models.

**Results:** After searching the literature and performing further detailed assessments, 10 studies with a total of 1758 patients were included in the quantitative analysis. Pooled analyses showed that denosumab treatment significantly increased the percent changes in lumbar spine BMD [mean difference (MD): 5.12, confidence intervals (CI): 4.15 to 6.09], total hip BMD (MD: 2.72, 95% CI: 1.80 to 3.64) and femoral neck BMD (MD: 2.20, 95% CI: 0.94 to 3.46) compared with controls. Moreover, denosumab treatment significantly decreased the changes in mTSS (MD: -0.63, 95% CI: -0.86 to -0.41) and modified Sharp erosion score (MD: -0.62, 95% CI: -0.88 to -0.35). Subgroup analysis indicated that denosumab was superior to bisphosphonates for the improvement of BMD and the mitigation of joint destruction.

**Conclusion:** Denosumab treatment was associated with increased BMD and alleviated progression of joint destruction in RA patients, even when compared with bisphosphonates.

**Keywords: denosumab, rheumatoid arthritis, bone mineral density, joint destruction, pooled analysis**

## INTRODUCTION

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation of synovial joints, leading to the progression of joint destruction (1, 2). Multijoint destruction increases the risk of fractures in RA patients, and reduces the patient's abilities of daily living and quality of life (3). Although there are various effective pharmacological therapies (such as conventional synthetic and biological disease-modifying anti-rheumatic drugs, DMARDs) that can abate joint inflammation and relieve joint destruction (2, 4), the joint-protective effect of these reagents is not complete. Additionally, infections and serious adverse events caused by the suppression of the immune system often occur (5, 6). Thus, the development of new therapies is essential for the treatment of RA.

Denosumab (AMG-162) is a fully human monoclonal antibody that specifically binds to human receptor activator of nuclear factor kappa B ligand (RANKL), resulting in decreased survival and activity of osteoclasts, thereby inhibiting bone resorption and bone loss (7, 8). It has been confirmed that denosumab is a highly effective and safe antiresorptive agent for the treatment of metastatic cancers and postmenopausal osteoporosis (9, 10). Several initial clinical trials have investigated the effect of denosumab treatment on patients with RA (11–14). The study by Kinoshita et al. showed that denosumab did not significantly suppress the progression of osteoporosis and the disease activity indices (12). Takeuchi et al. reported that denosumab significantly abated joint destruction and increased bone mineral density (BMD) compared with placebo (13). So et al. found that denosumab therapy was not associated with a significant improvement in erosion parameters at 12 months (14). Therefore, the findings of these studies regarding the efficacy of denosumab in RA remain to be further clarified.

In view of the discrepant findings of clinical studies, we conducted a systematic review and meta-analysis to evaluate the therapeutic effect of denosumab on BMD and joint destruction in patients with RA.

## MATERIALS AND METHODS

### Search Methods and Sources

For identification of all published clinical studies that investigated the effects of denosumab on RA, we comprehensively searched the online published literature using the Medline, Embase and Cochrane Library databases (to October 7, 2021). The search strategy employed relevant keywords including the following: denosumab, AMG-162, RANKL inhibition and rheumatoid arthritis. The search scope was limited to English publications. To maximize the search for related studies, the reference lists of identified studies and systematic reviews were manually assessed. This study did not require ethics committee approval.

### Selection Criteria

(i) Randomized controlled trials (RCTs), matched prospective or matched retrospective studies that compared the efficacy of denosumab with controls in patients with RA; (ii) average age of patients ≥ 18 years; and (iii) reported data on the assessment of at least one of desired clinical endpoints: change in bone mineral density (BMD) and joint destruction scores. We tried to contact the corresponding authors to acquire further information when necessary data were not reported in the published articles. Two researchers (H.Q. and Z.X.) independently conducted the literature search, research eligibility assessment and data extraction.

### Quality Assessment

For the RCTs, we used the Cochrane Collaborative Risk of Bias tool (15) to assess the risk of bias in seven areas: allocation concealment; random sequence generation; blinding of research participants, outcome evaluators and medical service providers; incomplete outcome data; selective reports and other potential sources of bias. The quality of the included observational trials was assessed by the Newcastle–Ottawa Quality Assessment Scale (NOS) (16). Eight questions with nine possible points were included in the NOS scale. The data based on the comparability of the groups, the selection of populations, and the exposure/outcome of interest were judged using a star system. The RCTs and studies with NOS ≥ 7 were rated as being of good quality.

### Data Extraction

In the process of preparing this manuscript, the data extraction and presentation followed the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA, **Table S1**) (17) and the PICOS (population, intervention, comparison, outcome, study design, **Table S2**) framework (18). Two reviewers (H.Q. and Z.X.) used predefined standardized protocols and a data collection instrument to independently extract data from the included trials. Disagreements were resolved by consensus or the opinion of a third independent reviewer (L.P.).

### Outcome

The primary endpoints included percent changes from baseline in lumbar spine, total hip and femoral neck bone mineral density (BMD) and the changes from baseline in the modified total Sharp score (mTSS), the modified Sharp erosion score and the joint space narrowing (JSN) score. The mTSS has been used to evaluate the extent of bone erosions for 44 joints and JSN for 42 joints by scoring patient radiographs, with higher scores representing greater damage. This method was demonstrated to be sensitive enough to assess treatment effect over a short time for RA patients, including distal interphalangeal hand, wrist and feet joints (19). Secondary endpoints included American College of Rheumatology (ACR) 20/50/70 response (20), changes in the Health Assessment Questionnaire (HAQ; 0 = no difficulty; 3 = unable to do) (21), 28-joint count disease activity scores (DAS28) (22), serum C-telopeptide of type I collagen (CTX-I), serum N-propeptide of type I collagen (PINP), urine C-telopeptide of type II collagen (CTX-II)/creatinine and the incidence rates of serious adverse events.

### Statistical Analysis

We performed statistical analyses using RevMan software package 5.3 and STATA software 13.0. The analyses of continuous variables used weighted mean differences (MD) with 95% confidence intervals (CI), while the analyses of dichotomous data used

relative risk (RR) with 95% CI. The Q-statistic was used to assess the existence of significant heterogeneity, and the $I^2$ statistic was used to assess the degree of observed heterogeneity. A random effects model was used to calculate the pooled analyses. To detect any publication bias in the primary endpoints, we examined in detail the asymmetry of the funnel plots and further assessed them using the Begg adjusted rank correlation test and the Egger regression asymmetry test. To explore the influence of diverse covariates on the overall effect of denosumab on the primary endpoints of the percent changes in BMD and the changes in mTSS, we further performed sensitivity, subgroup and meta-regression analyses. A P value < 0.05 was considered statistically significant.

# RESULTS

## Search Results and Study Qualities

Our systematic electronic literature search initially identified 881 studies. After reviewing the titles and abstract, 801 (91%) studies were excluded (**Figure 1**). Of the remaining 52 studies, 42 were excluded after a particular assessment of the full text for the following reasons: single-arm trials (n=12), incorrect populations (n=9), reviews (n=7), duplicates (n=5), case reports (n=4), lack of interesting outcomes (n=4) or unmatched case-control studies (n=1). After rigorous evaluation, 10 studies (5 RCTs and 5 matched studies) with a total of 1758 patients met our eligibility criteria for quantitative analysis (11–14, 23–28).

## Study Characteristics

The characteristics of the included studies were summarized in **Table 1**. Of these included patients, 1164 (66.2%) were women, with an average age of 59.6 years. The average disease duration was 6.2 years, and the average follow-up time was 13.0 months. In all studies, baseline characteristics were comparable between the denosumab group and the control group. Of these included studies, five studies were designed as RCTs (11, 13, 14, 23, 26), and 5 studies were designed as retrospective matched studies (12, 24, 25, 27, 28). Five studies compared denosumab with placebo (11, 13, 14, 23) or blank (24), whereas the remaining 5 studies compared denosumab with bisphosphonates (12, 25–28). Patients with RA were assigned to denosumab 60 mg every 6 months (Q6M) in 8 studies (11–14, 23–25, 28), 60 mg every 3 months (Q3M) in 2 studies (13, 23) and other doses in 3 studies (11, 23, 26). Detailed baseline data on the disease activity, severity and drug usage before the intervention were shown in **Table S3**. Except for one included study (27), all other studies were rated as being of good quality (**Table S4**).

## Percent Changes in BMD

Nine studies with a total of 1623 patients reported the endpoint of percent changes in BMD at the lumbar spine (11–14, 23, 25–28). Pooled analyses showed that the percent changes in lumbar spine BMD were significantly higher in the denosumab group than in the control group (MD: 4.28, 95% CI: 3.13 to 5.42, P < 0.001, **Figure 2**). There was significant heterogeneity observed between these studies (P = 0.002, $I^2$ = 67%). Compared with placebo, denosumab significantly increased the percent changes in lumbar spine BMD (MD: 5.12, 95% CI: 4.15 to 6.09, P<0.001; $I^2$ = 63%, P = 0.05, **Figure 2A**). Compared with bisphosphonates, denosumab still increased the percent changes in lumbar spine BMD (MD: 2.71, 95% CI: 0.42 to 4.99, P = 0.02; $I^2$ = 42%, P = 0.14, **Figure 2B**).

Six studies with a total of 879 patients reported the endpoint of percent changes in BMD at the total hip (11, 14, 23, 25, 27, 28). Pooled analyses showed that the percent change in total hip BMD was significantly higher in the denosumab group than in the control group (MD: 2.72, 95% CI: 1.80 to 3.64, P < 0.001, **Figure 3A**). There was mild heterogeneity observed between these studies (P = 0.22, $I^2$ = 28%). Compared with placebo, denosumab significantly increased the percent changes in total hip BMD (MD: 2.82, 95% CI: 1.49 to 4.14, P < 0.001; $I^2$ = 53%, P = 0.12). Compared with bisphosphonates, denosumab was still associated with increased percent changes in total hip BMD (MD: 2.05, 95% CI: 0.38 to 3.71, P = 0.02; $I^2$ = 0, P = 0.61).

Five studies with a total of 592 patients reported the endpoint of percent changes in BMD at the femoral neck (11, 12, 14, 27, 28). Pooled analyses showed that the percent change in femoral neck BMD was significantly higher in the denosumab group than in the control group (MD: 2.20, 95%CI: 0.94 to 3.46, P < 0.001, **Figure 3B**). No heterogeneity was observed between these studies (P = 0.60, $I^2$ = 0%). Compared with bisphosphonates, denosumab was still associated with increased percent changes in BMD at the femoral neck (MD: 2.82, 95% CI: 0.99 to 4.64, P = 0.002; $I^2$ = 0, P = 0.83).

## Changes in the mTSS, the Erosion Score or the JSN Score

The data on these endpoints were available in seven studies with a total of 1559 patients (11, 13, 14, 23, 24, 27, 28). Pooled analyses found that, when compared with the control, denosumab treatment significantly decreased the changes in the mTSS (MD: -0.63, 95% CI: -0.86 to -0.41, P < 0.001; $I^2$ = 0, P = 0.94, **Figure 4A**). Compared with placebo, denosumab significantly decreased the changes in the mTSS (MD: -0.66, 95%CI: -0.93 to -0.40, P < 0.001; $I^2$ = 0, P = 0.88, **Figure 4A**). Compared with bisphosphonates, denosumab was still associated with significant reduction in the mTSS (MD: -0.54, 95%CI: -0.99 to -0.10, P = 0.02; $I^2$ = 0, P = 0.58, **Figure 4A**).

Denosumab treatment also significantly decreased the changes in the modified Sharp erosion score (MD: -0.62, 95% CI: -0.88 to -0.35, P < 0.001; $I^2$ = 0, P = 0.95, **Figure 4B**). Compared with bisphosphonates, denosumab was still associated with significant reduction in the modified Sharp erosion score (MD: -0.57, 95% CI: -1.09 to -0.05, P = 0.03; $I^2$ = 0, P = 0.70, **Figure 4B**). However, denosumab treatment was not associated with significant changes regarding the JSN score in either the placebo-controlled subgroup (MD: -0.04 95% CI: -0.22 to 0.14, P=0.65; $I^2$ = 0, P = 0.91, **Figure 4C**) or the bisphosphonate-controlled subgroup (MD: -0.12, 95% CI: -0.44 to 0.20, P = 0.46; $I^2$ = 58%, P = 0.12, **Figure 4C**).

## Secondary Endpoints

Compared with the control, denosumab treatment did not significantly change the HAQ scores (MD: -0.08, 95% CI: -0.17

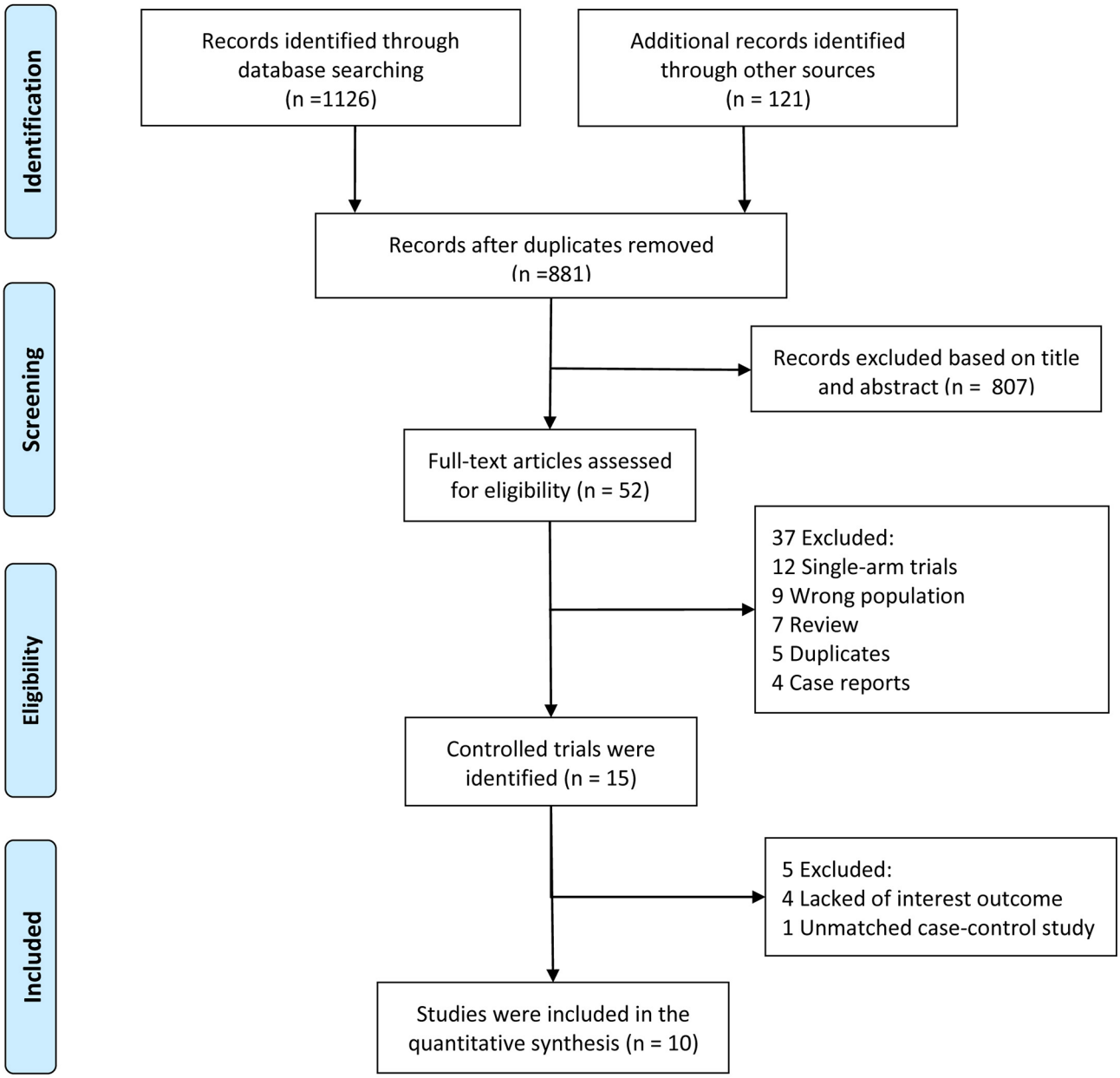


**FIGURE 1** | Flow chart of study selection.

to 0.01, P = 0.09, **Figure S1A**), DAS28 scores (MD: -0.01, 95% CI: -0.05 to 0.02, P = 0.39; **Figure S1B**), ACR20 response (OR: 1.13, 95% CI: 0.87 to 1.47, P = 0.37, **Figure S2A**), ACR50 response (OR: 1.07, 95% CI: 0.67 to 1.73, P = 0.77, **Figure S2B**) and ACR70 response (OR: 1.02, 95%CI: 0.57 to 1.84, P = 0.94, **Figure S2C**). Pooled analyses showed that denosumab treatment substantially suppressed the markers of bone turnover serum CTX-I (MD: -50.69, 95% CI: -64.18 to -37.20, P < 0.001, **Figure S3A**), urine CTX-II/creatinine (MD: -38.59, 95% CI: -57.30 to -19.88, P < 0.001, **Figure S3B**) and PINP (MD: -39.77, 95% CI: -56.10 to -23.43, P < 0.001, **Figure S3C**). Of these included studies, six studies provided the data on adverse events (11, 13, 14, 23, 25, 28), and detailed information were shown in **Table S5**. Pooled analysis indicated that the incidence rates of serious adverse events between the denosumab and control groups were comparable (OR: 0.99, 95% CI: 0.63 to 1.55, P = 0.96; **Figure S4**).

## Sensitivity, Subgroup and Meta-Regression Analyses

Sensitivity analysis (using the single-study-removed method) indicated good stability in the primary endpoints of percent changes from baseline in lumbar spine BMD and changes from

**TABLE 1** | Baseline characteristics of the included studies.

| Study (Ref.) | Year | Study Design | Simple Size, (N) | Women % | Age, Years | Duration, Years | Control | Denosumab, n, Dose | Follow-up, Months |
|---|---|---|---|---|---|---|---|---|---|
| Cohen et al. (11) | 2008 | RCT | 218 | 73.1 | 57.4 | 11.0 | Placebo | 71, 60mg Q6M<br>72, 180mg Q6M | 12 |
| Takeuchi et al-a (23) | 2016 | RCT | 340 | 78.2 | 54.5 | 2.3 | Placebo | 85, 60mg Q6M<br>82, 60mg Q3M<br>85, 60mg Q2M | 12 |
| Hasegawa et al. (24) | 2017 | Retrospective, matched study | 80 | 93.8 | 72.2 | 13.1 | Without denosumab | 40, 60mg Q6M | 12 |
| Kinoshita et al. (12) | 2017 | Retrospective, matched study | 98 | 94.9 | 69.2 | 12.8 | Bisphosphonates | 49,60mg Q6M | 12 |
| Nakamura1 et al. (25) | 2017 | Retrospective, matched study | 52 | 100 | 70.2 | 15.4 | Bisphosphonates | 26, 60mg Q6M | 24 |
| Yue et al. (26) | 2017 | RCT | 40 | 100 | 58.5 | 10.4 | Bisphosphonate (alendronate) | 20, 60mg Q1W | 6 |
| Ebina et al. (27) | 2018 | Retrospective, matched study | 60 | 100 | 68.0 | 18.0 | Bisphosphonates | 30, NR | 12 |
| Takeuchi et al-b (13) | 2019 | RCT | 654 | 74.8 | 57.4 | 2.2 | Placebo | 217, 60mg Q6M<br>219, 60mg Q3M | 12 |
| Mori1 et al. (28) | 2021 | Retrospective, matched study | 106 | 100 | 69.4 | 10.5 | Bisphosphonates | 56, 60mg Q6M | 12 |
| So et al. (14) | 2021 | RCT | 110 | 80.0 | 56.8 | 5.4 | Placebo | 55, 60mg Q6M | 24 |

*RCT, randomized controlled trial; Q6M, every 6 months; Q3M, every 3 months; Q2M, every 2 months; Q1W, every 1 week.*

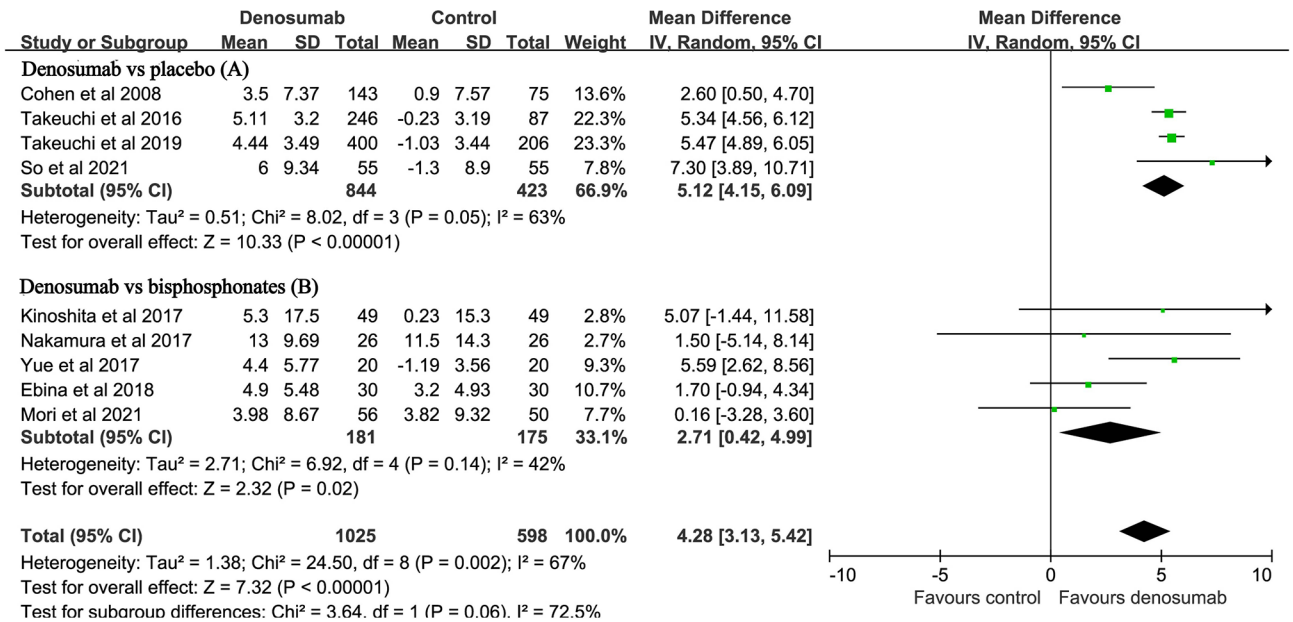


**FIGURE 2** | Forest plot for the effect of denosumab on the percent changes in lumbar spine BMD compared with placebo **(A)** and bisphosphonates **(B)**.

baseline in the mTSS (**Figure S5**). In the subgroup analysis, denosumab treatment was still associated with increased percent changes in lumbar spine BMD and decreased changes in the mTSS, except for the case–control subgroup in lumbar spine BMD (**Table 2**). For the endpoint of percent changes in lumbar spine BMD, meta-regression indicated that the duration of RA was the major source of heterogeneity of denosumab treatment ($P < 0.01$, **Table S6**). This result indicated that the effect of denosumab on percent changes in lumbar spine BMD may be negatively correlated with the duration of RA (**Figure 5**). The sex, glucocorticoids use, baseline lumbar spine BMD, positive rheumatoid factor and denosumab dose were not significantly correlated with the major source of heterogeneity (**Table S6**).

## Publication Bias

For the primary endpoints of percent changes from the baseline in BMD and the changes in the mTSS, funnel plots of all included studies displayed symmetry, showing a low risk of publication

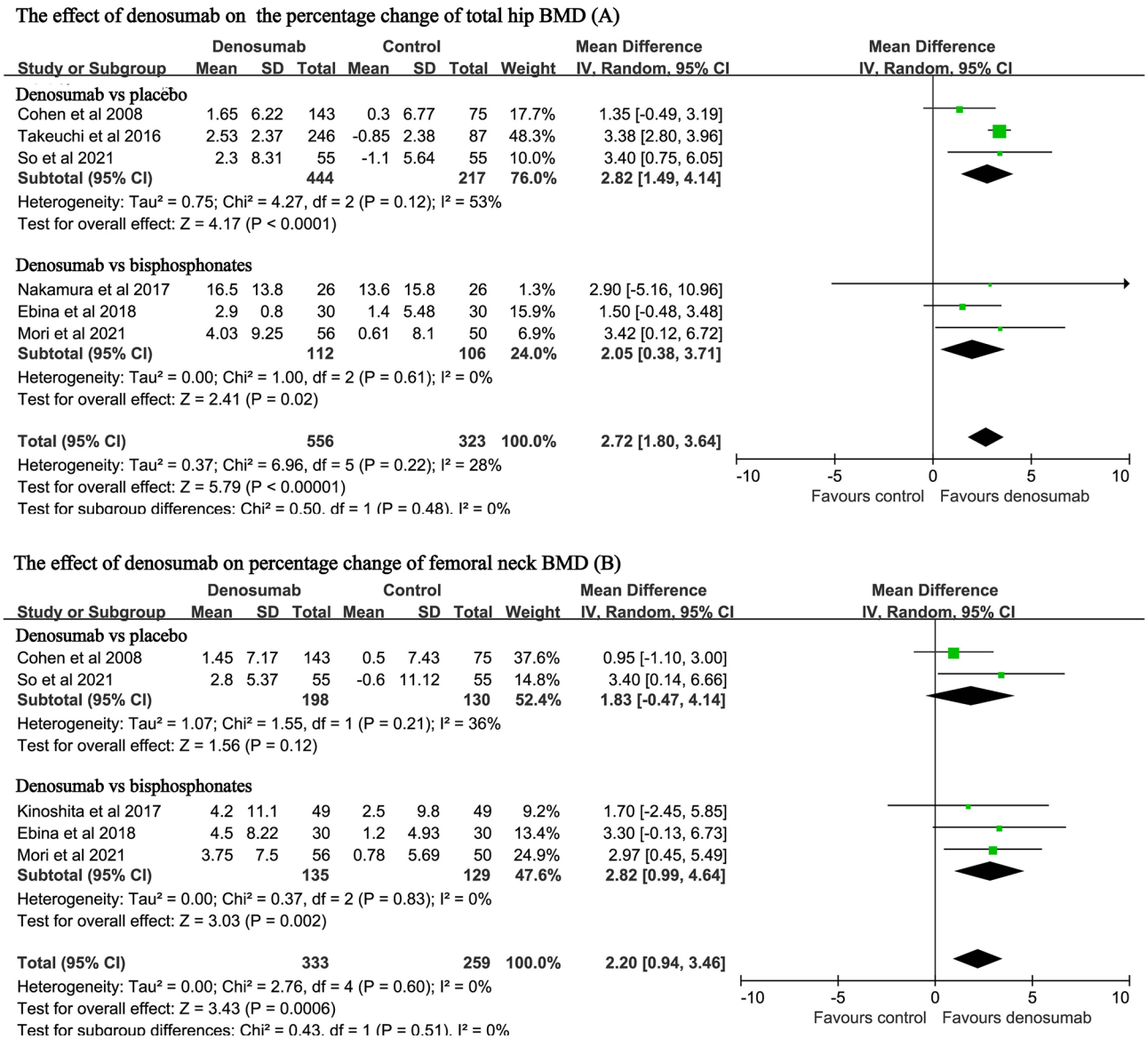


**FIGURE 3** | Forest plot for the effect of denosumab on the percent changes in total hip BMD **(A)** and femoral neck BMD **(B)**.

bias (**Figure S6**). For Begg's and Egger's tests, potentially significant publication bias did not exist for each primary endpoint ($P_{begg}$=0.13 and $P_{egger}$=0.14 for percent changes in BMD; $P_{begg}$=0.25 and $P_{egger}$=0.12 for changes in the mTSS).

## DISCUSSION

To our knowledge, this is the first systematic review and pooled-analysis to analyze the efficacy of denosumab in patients with RA. On the grounds of the available published evidence, we found that denosumab treatment significantly increased lumbar spine, total hip and femoral neck BMD in RA patients. Denosumab treatment significantly decreased the changes in the mTSS and the modified Sharp erosion score. Although denosumab treatment did not significantly change the HAQ scores, DAS28 scores and ACR20/50/70 responses, it suppressed serum CTX-I, PINP and urine CTX-II/creatinine level. Additionally, denosumab treatment was not associated with an increased risk of serious adverse events.

Patients with RA have significantly increased risks of bone loss and fractures (29). In this study, we found that denosumab increased lumbar spine BMD by 5.12% (4.15 to 6.09) and total hip BMD by 2.82% (1.49 to 4.14) in RA patients compared with placebo. Takeuchi et al. reported that regardless of whether the patient was taking glucocorticoids or suffering from osteoporosis, an increase (5.47%) in lumbar spine BMD was observed during denosumab treatment in RA (13, 23), which was consistent with

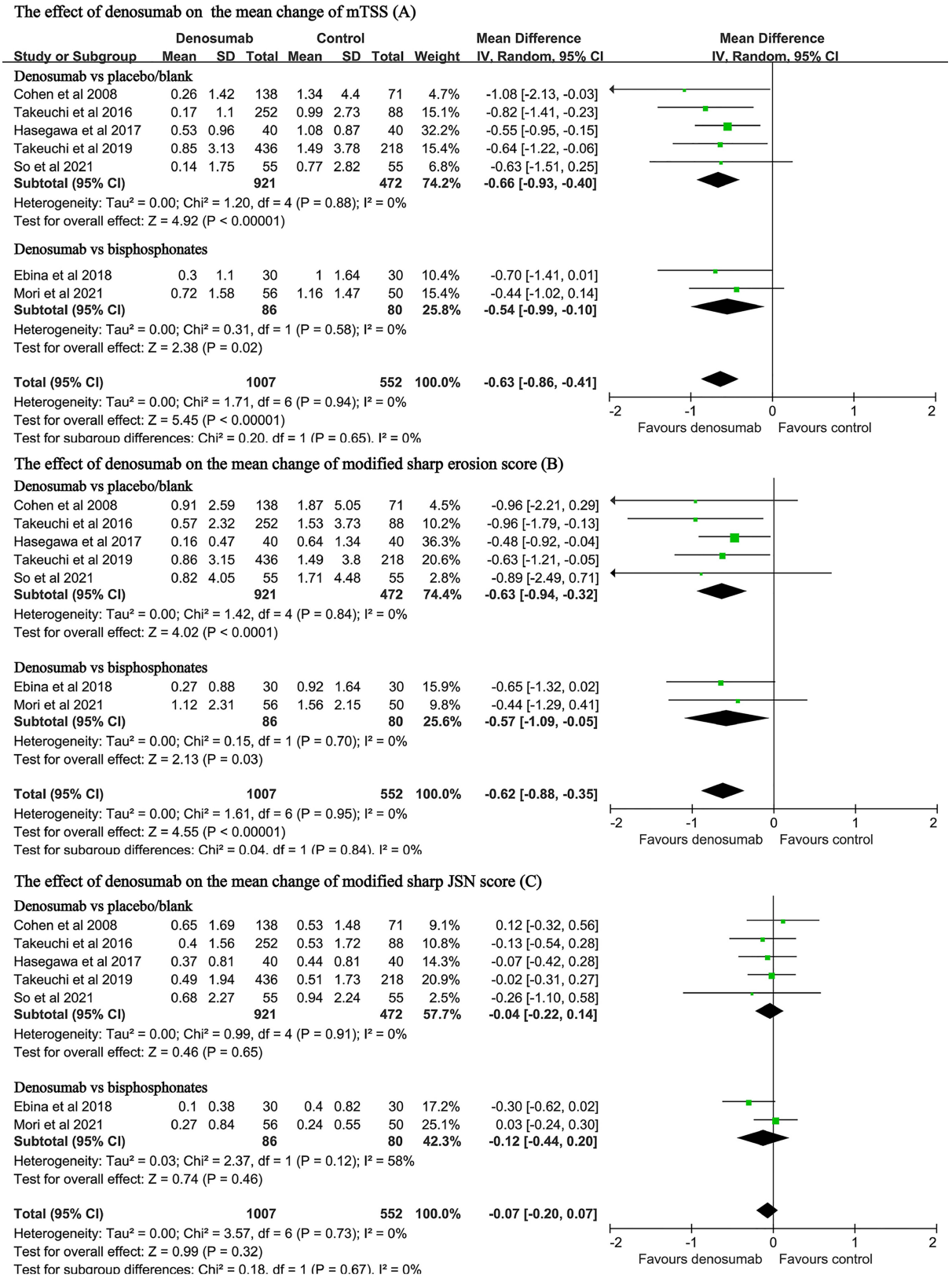


**FIGURE 4** | Forest plot for the effect of denosumab on the changes in mTSS **(A)**, modified sharp erosion score **(B)** and modified sharp JSN score **(C)**.

**TABLE 2** | Outcomes of subgroup analysis.

| Endpoint | Subgroup | No. of Studies (N) | MD (95% CI) | *P*-value | $I^2$ (%) |
|---|---|---|---|---|---|
| LS-BMD | RCT* | 5 (1307) | 5.18 (4.33 – 6.03) | <0.001 | 50 |
| | Case-control# | 4 (316) | 1.50 (-0.41 – 3.41) | 0.12 | 0 |
| | Duration < 10 y | 3 (1049) | 5.46 (5.00 – 5.92) | <0.001 | 0 |
| | Duration ≥ 10 y | 6 (574) | 2.67 (1.07 – 4.27) | 0.001 | 28 |
| | 60mg, Q6M | 7 (1087) | 3.97 (2.67 – 5.27) | <0.001 | 67 |
| | 60mg, Q3M | 2 (575) | 5.70 (5.11 – 6.28) | <0.001 | 9 |
| | Other dose | 3 (357) | 5.03 (2.89 – 7.17) | <0.001 | 75 |
| mTSS | RCT* | 4 (1313) | -0.75 (-1.10 – -0.40) | <0.001 | 0 |
| | Case-control# | 3 (246) | -0.55 (-0.85 – -0.25) | <0.001 | 0 |
| | Duration < 10 y | 3 (1104) | -0.71 (-1.08 – -0.34) | <0.001 | 0 |
| | Duration ≥ 10 y | 4 (455) | -0.59 (-0.88 – -0.30) | <0.001 | 0 |
| | 60mg, Q6M | 6 (1044) | -0.58 (-0.83 – -0.33) | <0.001 | 0 |
| | 60mg, Q3M | 2 (607) | -0.81 (-1.22 – -0.40) | <0.001 | 0 |
| | Other dose | 2 (313) | -0.97 (-1.53 – -0.42) | <0.001 | 0 |

*LS-BMD, lumbar spine-bone mineral density; mTSS, modified total Sharp score; MD, mean differences; Q3M, 60 mg every 3 months; Q6M, 60 mg every 6 months; RCT, randomized controlled trials; *age < 65 years; #age ≥ 65 years.*

our findings. Patients with rheumatic diseases were at high risk of systemic bone loss and osteoporotic fractures and were suggested to be treated with bisphosphonates (30, 31). Our data indicated that denosumab appeared to have a better effect on increasing lumbar spine, total hip and femoral neck BMD than bisphosphonates, for a difference of 2.71%, 2.05% and 2.82% respectively. These discrepant effects between denosumab and bisphosphonates may be explained by their distributions and mechanisms of action. Although bisphosphonates have an antiresorption effect by acting on osteoclasts, denosumab directly targets the production of osteoclasts through its specific effect on the RANKL pathway (32).

Subgroup analysis indicated that denosumab treatment appeared to be more effective to increase lumbar spine BMD in RCTs than in observational studies. The probable reason was that most of these RCTs had a large sample size and were compared with placebo. Although meta-regression analysis indicated that the effect of denosumab on the percent change in lumbar spine BMD was negatively correlated with the duration of RA, we could not rule out the interaction between diverse variables, especially age and RA severity. Whether denosumab should be administered in the early stage of RA still needs further study. A statistically significant dose-response curve had not been observed in meta-regression analysis. The possible reason was that most of the RA patients included in our data administered denosumab with Q6M, while fewer patients administered denosumab with other dosage.

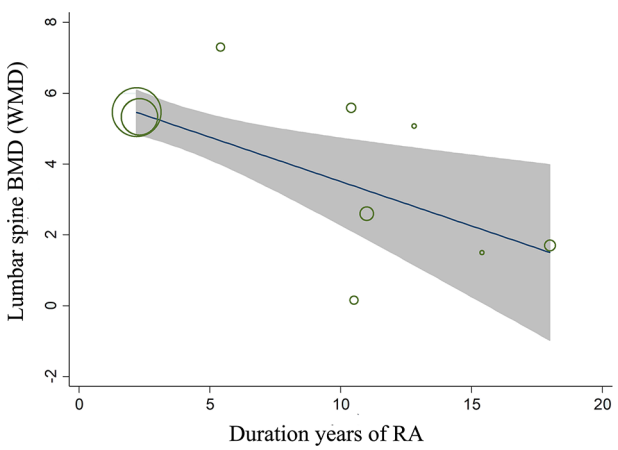


**FIGURE 5** | Meta-regression analysis between the duration of RA and the effect of denosumab on the percent changes in lumbar spine BMD.

RA is a systemic autoimmune inflammatory disease, which leads to osteoporosis and joint destruction by activating osteoclasts (2, 33). The joint damage of RA is irreversible and is closely related to clinical outcomes (3, 34). Consequently, the prevention of joint destruction is vital to alleviate the progression of RA. Our data showed that denosumab treatment was associated with significantly smaller changes in the mTSS (-0.66, -0.93 to -0.40) and the modified Sharp erosion score (-0.63, -0.94 to -0.32) compared to placebo, which were consistent with the results (-0.70 to -0.54) of several previous single-arm studies (35, 36). Furthermore, when compared with bisphosphonates, the therapeutic effect of denosumab on joint destruction was not significantly weakened. Subgroup analysis showed that denosumab treatment effectively reduced the mTSS in different study designs (RCTs or observational studies), dosage (Q6M, Q3M or other) and disease duration. The study by So et al. found that significant radiological changes in the mTSS and the modified Sharp erosion score could not be detected in the RA patients treated with denosumab (14), which may be caused by the small sample size. Although denosumab suppressed joint margin erosion, it did not block the changes in JSN. This may be related to the mechanism of denosumab, suggesting that denosumab may have no inhibitory effect on cartilage destruction (13, 37).

For the changes in DAS28 scores, HAQ scores and any component of ACR response, no clinically meaningful differences were observed between the denosumab and control groups. These findings showed that denosumab might have no effect on the activity of RA disease, which was consistent with previous reports (28, 38). Additionally, the mean 13-month

follow-up may be too short to achieve low disease activity or remission in patients with RA. More long-term studies need to further clarify the efficacy of denosumab on functional disability.

In the present study, we found that bone turnover markers (CTX-I and PINP) and cartilage markers (urine CTX-II/Cre) were suppressed by denosumab treatment. The suppression of urine CTX-II/Cre suggested the possibility that the use of denosumab to prevent bone destruction might lead to secondary inhibition of cartilage destruction. However, Takeuchi et al. and So et al. found that denosumab did not affect the cartilage turnover marker serum cartilage oligomeric protein (COMP) (13, 14), indicating that denosumab might have no anti-inflammatory effect on RA patients. Our data found that denosumab was not associated with increased risk of serious adverse events. However, recently, several studies reported that the rapid bone loss and the rebound fractures may occur when treatment is stopped (39, 40), which needs to be considered when choosing this agent.

Our study had several limitations: First, the mean 13-month follow-up might be too short to fully clarify the effect of denosumab on the healing of erosions and its effect on functional disability. Long-term follow up studies (for example, 5 years) are still needed. Second, five of these included studies were retrospective, which led to possible biases in our results. Although we tried to overcome this limitation by performing multiple-sensitivity, subgroup and meta-regression analyses, potential bias could still not be ruled out. Third, only five studies with a total of 356 patients compared the efficacy of denosumab with bisphosphonates. Whether denosumab treatment is superior to bisphosphonates in patients with RA requires confirmation in a larger RCT. Finally, the patients included in this study were mainly from Japan, and potential racial bias cannot be ruled out.

In conclusion, data from our meta-analysis indicated that denosumab treatment was associated with increased lumbar spine and total hip BMD in patients with RA. Denosumab treatment decreased the changes in the mTSS and the modified Sharp erosion score. Additionally, denosumab may be superior to bisphosphonates for the prevention of osteoporosis and bone erosion.

## 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.

## AUTHOR CONTRIBUTIONS

All authors participated in the drafting or critical revision of this article and contributed important intellectual content. All authors read and approved the final version of the manuscript. QH is responsible for the completeness of the data and the accuracy of data analysis. QH, XZ and PL commented on the study concept and design. QH and XZ carried out the acquisition of data. QH, HT and PL performed the analysis and interpretation of data. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.799575/full#supplementary-material

## REFERENCES

1. Fraenkel L, Bathon JM, England BR, St Clair EW, Arayssi T, Carandang K, et al. 2021 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. *Arthritis Rheumatol* (2021) 73(7):1108–23. doi: 10.1002/art.41752
2. Sparks JA. Rheumatoid Arthritis. *Ann Intern Med* (2019) 170(1):ITC1–ITC16. doi: 10.7326/AITC201901010
3. Meissner Y, Fischer-Betz R, Andreoli L, Costedoat-Chalumeau N, De Cock D, Dolhain RJEM, et al. EULAR Recommendations for a Core Data Set for Pregnancy Registries in Rheumatology. *Ann Rheum Dis* (2021) 80(1):49–56. doi: 10.1136/annrheumdis-2020-218356
4. Burmester GR, Pope JE. Novel Treatment Strategies in Rheumatoid Arthritis. *Lancet* (2017) 389:2338–48. doi: 10.1016/S0140-6736(17)31491-5
5. George MD, Baker JF, Winthrop K, Alemao E, Chen L, Connolly S, et al. Risk of Biologics and Glucocorticoids in Patients With Rheumatoid Arthritis Undergoing Arthroplasty: A Cohort Study. *Ann Intern Med* (2019) 170 (12):825–36. doi: 10.7326/M18-2217
6. Lortholary O, Fernandez-Ruiz M, Baddley JW, Manuel O, Mariette X, Winthrop KL. Infectious Complications of Rheumatoid Arthritis and Psoriatic Arthritis During Targeted and Biological Therapies: A Viewpoint in 2020. *Ann Rheum Dis* (2020) 79(12):1532–43. doi: 10.1136/annrheumdis-2020-217092
7. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, et al. Osteoclast Differentiation Factor Is a Ligand for Osteoprotegerin/ Osteoclastogenesis-Inhibitory Factor and Is Identical to TRANCE/ RANKL. *Proc Natl Acad Sci USA* (1998) 95:3597–602. doi: 10.1073/pnas.95.7.3597
8. Tanaka S, Tanaka Y. RANKL as a Therapeutic Target of Rheumatoid Arthritis. *J Bone Miner Metab* (2021) 39(1):106–12. doi: 10.1007/s00774-020-01159-1
9. Bone HG, Wagman RB, Brandi ML, Brown JP, Chapurlat R, Cummings SR, et al. 10 Years of Denosumab Treatment in Postmenopausal Women With Osteoporosis: Results From the Phase 3 Randomised FREEDOM Trial and Open-Label Extension. *Lancet Diabetes Endocrinol* (2017) 5(7):513–23. doi: 10.1016/S2213-8587(17)30138-9
10. Jakob T, Tesfamariam YM, Macherey S, Kuhr K, Adams A, Monsef I, et al. Bisphosphonates or RANK-Ligand-Inhibitors for Men With Prostate Cancer and Bone Metastases: A Network Meta-Analysis. *Cochrane Database Syst Rev* (2020) 12(12):CD013020. doi: 10.1002/14651858.CD013020.pub2
11. Cohen SB, Dore RK, Lane NE, Ory PA, Peterfy CG, Sharp JT, et al. Denosumab Treatment Effects on Structural Damage, Bone Mineral Density, and Bone Turnover in Rheumatoid Arthritis: A Twelve-Month, Multicenter, Randomized, Double-Blind, Placebo-Controlled, Phase II Clinical Trial. *Arthritis Rheum* (2008) 58(5):1299–309. doi: 10.1002/art.23417
12. Kinoshita H, Miyakoshi N, Kashiwagura T, Kasukawa Y, Sugimura Y, Shimada Y. Comparison of the Efficacy of Denosumab and Bisphosphonates for Treating

Secondary Osteoporosis in Patients With Rheumatoid Arthritis. *Mod Rheumatol* (2017) 27(4):582–6. doi: 10.1080/14397595.2016.1232776
13. Takeuchi T, Tanaka Y, Soen S, Yamanaka H, Yoneda T, Tanaka S, et al. Effects of the Anti-RANKL Antibody Denosumab on Joint Structural Damage in Patients With Rheumatoid Arthritis Treated With Conventional Synthetic Disease-Modifying Antirheumatic Drugs (DESIRABLE Study): A Randomised, Double-Blind, Placebo-Controlled Phase 3 Trial. *Ann Rheum Dis* (2019) 78(7):899–907. doi: 10.1136/annrheumdis-2018-214827
14. So H, Cheng IT, Lau SL, Chow E, Lam T, Hung VW, et al. Effects of RANKL Inhibition on Promoting Healing of Bone Erosion in Rheumatoid Arthritis Using HR-pQCT: A 2-Year, Randomised, Double-Blind, Placebo-Controlled Trial. *Ann Rheum Dis* (2021). doi: 10.1136/annrheumdis-2021-219846
15. Higgins JP, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, et al. The Cochrane Collaboration's Tool for Assessing Risk of Bias in Randomised Trials. *BMJ* (2011) 343:d5928. doi: 10.1136/bmj.d5928
16. Wells GA, Shea B, O'Connell D. *The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomized Studies in Meta-Analyses*. Ottawa Health Research Institute. Available at: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.
17. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, et al. The PRISMA Statement for Reporting Systematic Reviews and Meta-Analyses of Studies That Evaluate Health Care Interventions: Explanation and Elaboration. *PloS Med* (2009) 339:b2700. doi: 10.1136/bmj.b2700
18. O'Connor D, Green S, Higgins JP. Defining the Review Question and Developing Criteria for Including Studies. In: *Cochrane Handbook for Systematic Reviews of Interventions*. Hoboken, NJ, USA: Wiley (2008). p. pp.81–94.
19. Van der Heijde D, Simon L, Smolen J, Strand V, Sharp J, Boers M, et al. How to Report Radiographic Data in Randomized Clinical Trials in Rheumatoid Arthritis: Guidelines From a Roundtable Discussion. *Arthritis Rheum* (2002) 47(2):215–8. doi: 10.1002/art.10181
20. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, et al. American College of Rheumatology. Preliminary Definition of Improvement in Rheumatoid Arthritis. *Arthritis Rheum* (1995) 38(6):727–35. doi: 10.1002/art.1780380602
21. Fries JF, Spitz P, Kraines RG, Holman HR. Measurement of Patient Outcome in Arthritis. *Arthritis Rheum* (1980) 23(2):137–45. doi: 10.1002/art.1780230202
22. Prevoo ML, van Gestel AM, van T Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Remission in a Prospective Study of Patients With Rheumatoid Arthritis. American Rheumatism Association Preliminary Remission Criteria in Relation to the Disease Activity Score. *Br J Rheumatol* (1996) 35(11):1101–5. doi: 10.1093/rheumatology/35.11.1101
23. Takeuchi T, Tanaka Y, Ishiguro N, Yamanaka H, Yoneda T, Ohira T, et al. Effect of Denosumab on Japanese Patients With Rheumatoid Arthritis: A Dose-Response Study of AMG 162 (Denosumab) in Patients With RheumatoId Arthritis on Methotrexate to Validate Inhibitory Effect on Bone Erosion (DRIVE)-A 12-Month, Multicentre, Randomised, Double-Blind, Placebo-Controlled, Phase II Clinical Trial. *Ann Rheum Dis* (2016) 75(6):983–90. doi: 10.1136/annrheumdis-2015-208052
24. Hasegawa T, Kaneko Y, Izumi K, Takeuchi T. Efficacy of Denosumab Combined With bDMARDs on Radiographic Progression in Rheumatoid Arthritis. *Joint Bone Spine* (2017) 84(3):379–80. doi: 10.1016/j.jbspin.2016.05.010
25. Nakamura Y, Suzuki T, Kato H. Denosumab Significantly Improves Bone Mineral Density With or Without Bisphosphonate Pre-Treatment in Osteoporosis With Rheumatoid Arthritis : Denosumab Improves Bone Mineral Density in Osteoporosis With Rheumatoid Arthritis. *Arch Osteoporos* (2017) 12(1):80. doi: 10.1007/s11657-017-0371-y
26. Yue J, Griffith JF, Xiao F, Shi L, Wang D, Shen J, et al. Repair of Bone Erosion in Rheumatoid Arthritis by Denosumab: A High-Resolution Peripheral Quantitative Computed Tomography Study. *Arthritis Care Res (Hoboken)* (2017) 69(8):1156–63. doi: 10.1002/acr.23133
27. Ebina K, Hirao M, Hashimoto J, Matsuoka H, Iwahashi T, Chijimatsu R, et al. Impact of Switching Oral Bisphosphonates to Denosumab or Daily Teriparatide on the Progression of Radiographic Joint Destruction in Patients With Biologic-Naïve Rheumatoid Arthritis. *Osteoporos Int* (2018) 29(7):1627–36. doi: 10.1007/s00198-018-4492-y
28. Mori Y, Izumiyama T, Kurishima H, Kamimura M, Baba K, Mori N, et al. Effect of Denosumab Switched From Bisphosphonates in Preventing Joint Destruction in Postmenopausal Rheumatoid Arthritis Patients With Anti-Cyclic Citrullinated Peptide Antibodies. *J Orthop Surg Res* (2021) 16(1):107. doi: 10.1186/s13018-021-02271-2
29. Wysham KD, Baker JF, Shoback DM. Osteoporosis and Fractures in Rheumatoid Arthritis. *Curr Opin Rheumatol* (2021) 33(3):270–6. doi: 10.1097/BOR.0000000000000789
30. Saag KG, Emkey R, Schnitzer TJ, Brown JP, Hawkins F, Goemaere S, et al. Alendronate for the Prevention and Treatment of Glucocorticoid-Induced Osteoporosis. Glucocorticoid-Induced Osteoporosis Intervention Study Group. *N Engl J Med* (1998) 339:292–9. doi: 10.1056/NEJM199807303390502
31. Saag KG, Pannacciulli N, Geusens P, Adachi JD, Messina OD, Morales-Torres J, et al. Denosumab Versus Risedronate in Glucocorticoid-Induced Osteoporosis: Final Results of a Twenty-Four-Month Randomized, Double-Blind, Double-Dummy Trial. *Arthritis Rheumatol* (2019) 71:1174–84. doi: 10.1002/art.40874
32. Baron R, Ferrari S, Russell RG. Denosumab and Bisphosphonates: Different Mechanisms of Action and Effects. *Bone* (2011) 48(4):677–92. doi: 10.1016/j.bone.2010.11.020
33. Steffen U, Schett G, Bozec A. How Autoantibodies Regulate Osteoclast Induced Bone Loss in Rheumatoid Arthritis. *Front Immunol* (2019) 10:1483. doi: 10.3389/fimmu.2019.01483
34. Katz P, Andonian BJ, Huffman KM. Benefits and Promotion of Physical Activity in Rheumatoid Arthritis. *Curr Opin Rheumatol* (2020) 32(3):307–14. doi: 10.1097/BOR.0000000000000696
35. Mochizuki T, Yano K, Ikari K, Kawakami K, Hiroshima R, Koenuma N, et al. Effects of Denosumab Treatment on Bone Mineral Density and Joint Destruction in Patients With Rheumatoid Arthritis. *J Bone Miner Metab* (2018) 36(4):431–8. doi: 10.1007/s00774-017-0848-1
36. Deodhar A, Dore RK, Mandel D, Schechtman J, Shergy W, Trapp R, et al. Denosumab-Mediated Increase in Hand Bone Mineral Density Associated With Decreased Progression of Bone Erosion in Rheumatoid Arthritis Patients. *Arthritis Care Res (Hoboken)* (2010) 62(4):569–74. doi: 10.1002/acr.20004
37. Kostenuik PJ, Nguyen HQ, McCabe J, Warmington KS, Kurahara C, Sun N, et al. Denosumab, a Fully Human Monoclonal Antibody to RANKL, Inhibits Bone Resorption and Increases BMD in Knock-in Mice That Express Chimeric (Murine/Human) RANKL. *J Bone Miner Res* (2009) 24:182–95. doi: 10.1359/jbmr.081112
38. Boleto G, Dramé M, Lambrecht I, Eschard JP, Salmon JH. Disease-Modifying Anti-Rheumatic Drug Effect of Denosumab on Radiographic Progression in Rheumatoid Arthritis: A Systematic Review of the Literature. *Clin Rheumatol* (2017) 36(8):1699–706. doi: 10.1007/s10067-017-3722-6
39. Meier ME, Clerkx SN, Winter EM, Pereira AM, van de Ven AC, van de Sande MAJ, et al. Safety of Therapy With and Withdrawal From Denosumab in Fibrous Dysplasia and McCune-Albright Syndrome: An Observational Study. *J Bone Miner Res* (2021) 36(9):1729–38. doi: 10.1002/jbmr.4380
40. Jähn-Rickert K, Wölfel EM, Jobke B, Riedel C, Hellmich M, Werner M, et al. Elevated Bone Hardness Under Denosumab Treatment, With Persisting Lower Osteocyte Viability During Discontinuation. *Front Endocrinol (Lausanne)* (2020) 11:250. doi: 10.3389/fendo.2020.00250

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# Kinetics of the B- and T-Cell Immune Responses After 6 Months From SARS-CoV-2 mRNA Vaccination in Patients With Rheumatoid Arthritis

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**Objective:** To assess the kinetics of the humoral and cell-mediated responses after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination in rheumatoid arthritis (RA) patients treated with different immunosuppressive therapies.

**Methods:** Following vaccine completed schedule, health care workers (HCWs, n = 49) and RA patients (n = 35) were enrolled at 5 weeks (T1) and 6 months (T6) after the first dose of BNT162b2-mRNA vaccination. Serological response was assessed by quantifying anti-receptor-binding domain (RBD)-specific immunoglobulin G (IgG) and SARS-CoV-2 neutralizing antibodies, while cell-mediated response was assessed by a whole-blood test quantifying the interferon (IFN)-γ response to spike peptides. B-cell phenotype and IFN-γ-specific T-cell responses were evaluated by flow cytometry.

**Results:** After 6 months, anti-RBD antibodies were still detectable in 91.4% of RA patients, although we observed a significant reduction of the titer in patients under Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4)-Ig [median: 16.4 binding antibody units (BAU)/ml, interquartile range (IQR): 11.3–44.3, $p < 0.0001$] or tumor necrosis factor (TNF)-α inhibitors (median: 26.5 BAU/ml, IQR: 14.9–108.8, $p = 0.0034$) compared to controls (median: 152.7 BAU/ml, IQR: 89.3–260.3). All peripheral memory B-cell (MBC) subpopulations, in particular, the switched $IgG^+$ MBCs ($CD19^+CD27^+IgD^-IgM^-IgG^+$), were significantly reduced in RA subjects under CTLA-4-Ig compared to those in HCWs ($p = 0.0012$). In RA patients, a significantly reduced anti-RBD IgG titer was

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observed at T6 vs. T1, mainly in those treated with CTLA-4-Ig (p = 0.002), interleukin (IL)-6 inhibitors (p = 0.015), and disease-modifying antirheumatic drugs (DMARDs) ± corticosteroids (CCSs) (p = 0.015). In contrast, a weak nonsignificant reduction of the T-cell response was reported at T6 vs. T1. T-cell response was found in 65.7% of the RA patients at T6, with lower significant magnitude in patients under CTLA-4-Ig compared to HCWs (p < 0.0001). The SARS-CoV-2 IFN-γ-S-specific T-cell response was mainly detected in the $CD4^+$ T-cell compartment.

**Conclusions:** In this study, in RA patients after 6 months from COVID-19 vaccination, we show the kinetics, waning, and impairment of the humoral and, to a less extent, of the T-cell response. Similarly, a reduction of the specific response was also observed in the controls. Therefore, based on these results, a booster dose of the vaccine is crucial to increase the specific immune response regardless of the immunosuppressive therapy.

**Keywords: COVID-19, mRNA vaccine, rheumatoid arthritis, whole blood, T-cell response, antibody response, DMARD (disease-modifying antirheumatic drug), biological therapy**

## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerges in 2019, causing the actual COronaVIrus Disease 2019 (COVID-19) pandemic. The clinical manifestations are wide from asymptomatic to mild infection of the upper respiratory tract until the severe respiratory failure that requires intensive care unit hospitalization (1–6).

A program of global vaccination is the most successful strategy to control the COVID-19 pandemic. Evidence is currently available about the efficacy of mRNA vaccines, such as BNT162b2 and mRNA-1273 vaccines, in eliciting strong antibody and cell-mediated immune responses in healthy subjects (7–9).

Recently, promising data, mainly at 1 month from the completion of the vaccine schedule, have demonstrated the immunogenicity and safety of BNT162b2 mRNA vaccine in rheumatoid arthritis (RA) patients (10–12).

Altogether, these studies show that the humoral response to BNT162b2 vaccine is immunogenic in the majority of RA patients, although the response is delayed and decreased compared to controls (10). In particular, we showed that RA patients have a lower quantitative immune response (both antibody- and T cell-specific responses) to BNT162b2 vaccine compared to a control group of health care workers (HCWs), even if the vaccine is qualitatively immunogenic for most RA patients (13).

Recently, it has been shown that the COVID-19 vaccine-induced immunity decreases over time (14–17), and these results promoted a booster vaccination to the population to restore the vaccine efficiency (14, 15). It is unclear if these results are confirmed in RA patients and whether the immune-modulating treatments are associated with an impairment of the B- or T-cell response to vaccine evaluated over time.

Therefore, the aim of the present prospective study was to evaluate the kinetics of both humoral and cell-mediated responses after 5 weeks (T1) and 6 months (T6) from the first dose of SARS-CoV-2 vaccination in RA patients treated with different immunosuppressive therapies who completed the vaccine schedule.

## METHODS

### Study Population

HCWs were prospectively enrolled at the National Institute for Infectious Diseases (INMI) Lazzaro Spallanzani-IRCCS (Approval number 297/2021). Patients with a diagnosis of RA according to the 2010 criteria of the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) (18) were enrolled at Sant'Andrea University Hospital in Rome (Approval number 318/2021). Participants were recruited according to the following criteria: having received two doses of BNT162b2 mRNA vaccine and be longitudinally sampled after 5 weeks (T1) and 6 months (T6) from the first dose (**Figure 1**). The enrolled RA patients were under treatment with a biological drug with or without methotrexate (MTX) or other disease-modifying antirheumatic drugs (DMARDs; i.e., sulfasalazine and leflunomide), with only DMARDs, or low dosage of corticosteroids (CCSs) (prednisone <7.5 mg/day or equivalent). RA subjects treated with anti-CD20 or anti-Janus kinase (JAK) were excluded due to the small number of recruited individuals. Additional exclusion criterion was recent or remote SARS-CoV-2 infection. For the enrollment, both HCWs and RA patients signed a written informed consent.

### Study Design

Demographic and clinical data were collected. We assessed the RA disease activity through the Disease Activity Score based on C-reactive protein (DAS28crp). Blood was collected in heparin tubes; the presence of any clinical adverse events was registered. RA patients were divided into four groups according to drug treatment: TNF-α inhibitors with or without DMARDs (hereafter referred to as TNF-α inhibitors), interleukin (IL)-6

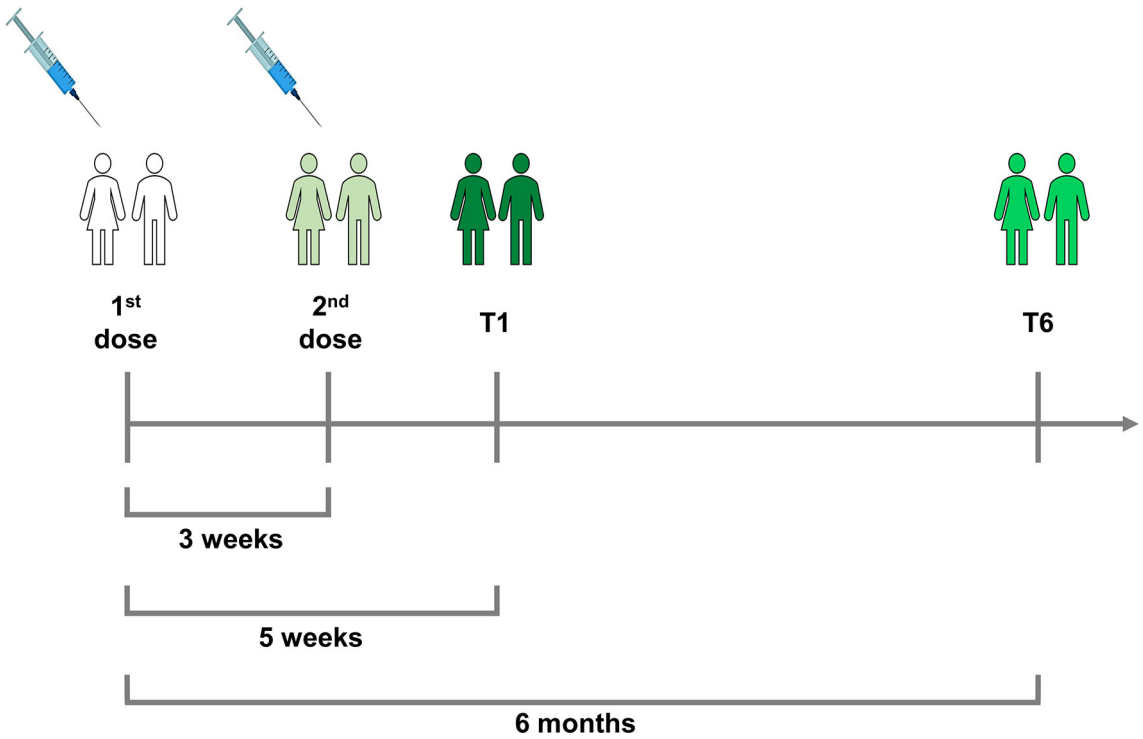


**FIGURE 1** | Timeline of the two-dose schedule of BNT162b2 mRNA vaccine and of the enrollment of rheumatoid arthritis (RA) and health care worker (HCW) individuals. All subjects received the second vaccine dose after 3 weeks from the first one. They were enrolled after 5 weeks (T1) and after 6 months from the first dose of vaccine (T6). At T1 and T6, blood samples were collected from all the participants.

inhibitors with or without DMARDs/CCSs (hereafter referred to as IL-6 inhibitors), CTLA-4-Ig with or without DMARDs/CCSs (hereafter referred to as CTLA-4-Ig), and DMARDs with or without CCSs (hereafter referred to as DMARDs). Moreover, within a week from blood collection, the lymphocyte count of the RA patients was performed. As previously described (19), the ongoing therapeutic regimen was modified during the vaccination period according to the ACR indications (18). In particular, MTX was interrupted for 1 week after the first and second doses, whereas abatacept (CTLA-4-Ig) was stopped 1 week before and after the first dose only.

## Anti-SARS-CoV-2-Specific IgG Evaluation

The humoral response to vaccination was measured as previously reported (20). The assays detect both the anti-nucleoprotein-immunoglobulin G (Anti-N-IgG) and the anti-RBD-IgG (Architect® i2000sr Abbott Diagnostics, Chicago, IL, USA). Anti-N-IgG is indicated as index value (S/CO) that was considered positive if ≥1.4, while anti-RBD-IgG was expressed as binding antibody units (BAU)/ml and indicated as positive when ≥7.1.

## Micro-Neutralization Assay

To evaluate the levels of neutralizing antibodies to SARS-CoV-2, a micro-neutralization assay (MNA) was performed as described (21) using the live SARS-CoV-2 virus (strain 2019-nCoV/Italy-INMI1; GISAID accession ID: EPI_ISL_412974). The assay evaluates the cytopathic effect (CPE) at 48 h post infection through the inhibition of Vero E6 cell infection by serum dilution curves. Microplates were observed by light microscope for the presence of CPE and, then stained with crystal violet solution containing 2% formaldehyde. Cell viability was measured by photometer at 595 nm (Synergy™ HTX Multi-Mode Microplate Reader, Agilent Biotek, Milan, Italy). Neutralization titer was expressed as the reciprocal of serum dilution ($MNA_{90}$) and corresponds to the highest serum dilution that inhibited at least 90% of the CPE.

## B-Cell Phenotype

B-cell evaluation was performed using the B-cell Tubes (22) according to the manufacturer's procedure (BD Biosciences, San Jose, California (CA), USA) (see **Supplementary Table S1** for antibodies and reagents). Briefly, freshly collected whole blood (300 µl) (HCWs, n = 7; RA, n = 13) was washed three times in a 15-ml tube with 10 ml of FACS Buffer (BD Biosciences, San Jose, USA) to eliminate the IgG present in the serum. Supernatant was removed carefully, and 200 µl of blood was transferred in the B-cell Tube, where lyophilized antibodies were present (CD45, CD19, CD24, CD27, CD38, IgD, IgM, IgG). Each tube was mixed gently for 3–5 s and then incubated for 20 min at room temperature (RT) in the dark. Afterward, 4 ml of BD Lysing solution (BD Biosciences, San Jose, USA) were added to lyse red

blood cells for 15 min at RT. Finally, cells were spinned down for 5 min at 200 × g and resuspended in 300 µl of FACS Buffer. Samples were acquired on a BD Lyric cytometer (BD Biosciences, San Jose, USA), and FlowJo software (version 10.8.1, Tree Star) was used to analyze the data (see **Supplementary Figure S1** for the gating strategy).

## IFN-γ Whole-Blood Assay

To evaluate the specific IFN-γ-Spike (S)-specific T-cell response, a whole-blood platform was used (20, 23–29). We stimulated the whole blood with a pool of peptides that covered the SARS-CoV-2-S protein sequence (SARS-CoV-2 PepTivator® Prot_S1, Prot_S, and Prot_S+, cat. 130-127-048, cat. 130-126-701, and cat. 130-127-312, respectively, Miltenyi Biotec, Germany), consisting of 15-amino acid length with an 11-amino acid overlap.

Briefly, 600 µl of whole blood were *in vitro* stimulated for 24 h at 37°C (5% $CO_2$) with SARS-CoV-2 PepTivator® Peptide Pools in a 48-well flat-bottom plate, as described (23). Whole blood was also stimulated with staphylococcal enterotoxin B (SEB) (Merck Life Science Cat. S4881) at 200 ng/ml as positive control. After 20–24 h, plasma was harvested and stored at -80°C until further use. The IFN-γ levels were quantified by an automatic ELISA (ELLA, protein simple, R&D Systems, Minneapolis, MN, USA, cat. SPCKB-PS-002574), and the values obtained were subtracted from the unstimulated control. The detection limit of the assay was 0.17 pg/ml. IFN-γ values ≥16 pg/ml were considered positive based on a cutoff found by Receiver Operating Characteristic (ROC) analysis comparing the response in COVID-19 patients vs. no-COVID individuals (article in preparation).

Samples used for the intracellular evaluation of the IFN-γ production were also costimulated with α-CD28 and α-CD49d monoclonal antibodies (BD Biosciences, San Jose, USA), as described below.

## Functional Analysis by Intracellular Staining and Flow Cytometry

To assess if the T-cell subpopulations CD4 and CD8 mount a SARS-CoV-2-S-specific response, we analyzed the IFN-γ-S-specific T-cell frequency by flow cytometry. To this aim, cells from whole blood were stimulated with the spike peptide pool and then fixed before the flow cytometry analysis. Briefly, spike peptide pool (0.1 µg/ml) was used to stimulate whole blood from RA and HCWs for 24 h with α-CD28 together with α-CD49 (1 µg/ml each). After collecting plasma, whole blood was cultured for another 5 h in the presence of brefeldin A (1 µg/ml) (cat. B7450, Life Technologies, Monza, Italy) to inhibit cytokine secretion. Then, blood was harvested and stained with Fixable Viability stain 700 (BD Biosciences, San Jose, USA) for 10 min at RT protected from light. Red blood cells were lysed with BD Lysing Solution (BD Biosciences, San Jose, USA) 1X+ 4% of formaldehyde for 10 min at RT, then cells were washed with 1 ml of Phosphate Buffered Saline (PBS) and centrifuged at 600 × g for 5 min. Cells were fixed with 4% formaldehyde for 5 min, washed again with 1 ml of PBS, and centrifuged at 600 × g for 5 min. At the end of the procedure, cells were frozen in Fetal Calf Serum (FCS) + 10% Dimethyl Sulfoxide (DMSO) until further analysis (see **Supplementary Table S1** for the complete list of reagents and stimuli used for flow cytometry analysis) (30, 31). Stimulated and fixed cells were thawed at 37°C, washed twice with PBS 1X at 600 × g for 5 min and transferred to a 96-well round plate (COSTAR, Sigma Aldrich, Milan, Italy) to proceed with the staining procedures. Here, 100 µl/sample of Perm/Wash 1X (BD Biosciences, San Jose, USA) were added for 10 min at RT to permeabilize cells. Subsequently, cells were washed in PBS 1X for 5 min at 600 × g. Then, cells were stained for the surface and intracellular markers, prepared in Brilliant Stain Buffer (BD Biosciences, San Jose, USA) (see **Supplementary Table S1** for the complete list of antibodies and reagents). After 1 h at 4°C, samples were washed twice in Perm/Wash 1X (30, 31). Samples were acquired on a BD Lyric cytometer (BD Biosciences, San Jose, USA), and the analyses were performed with FlowJo software (version 10.8.1, Tree Star) and stratified according to the drug treatment (see **Supplementary Figures S2A, B** for the gating strategy). We considered as positive an IFN-γ-S-specific T-cell response if the percentage of the SARS-CoV-2 peptide-stimulated cells was at least 2-fold higher compared to that of the unstimulated control and if a minimum of 10 events were present within the cytokine gate (32).

## Statistical Analysis

GraphPad software (GraphPad Prism 8 XML ProjecT) was used to analyze the results. The continuous variables IFN-γ levels and anti-RBD and $MNA_{90}$ titers were reported as median and interquartile range (IQR), while categorical variables were stated as count and proportion. The following non-parametric statistical inference tests were used: the Kruskal–Wallis test for comparisons among groups; the Mann–Whitney U-test and Wilcoxon test for pairwise comparisons (for unpaired and paired data, respectively). Bonferroni correction was applied when appropriate. Categorical variables were analyzed by the chi-square test. Correlations between assays were evaluated by non-parametric Spearman's rank test. A Spearman's rho >0.7 indicated a high correlation, 0.7 > rho > 0.5 indicated a moderate one, and rho <0.5 indicated a low correlation. Two-tailed p-values were considered significant if <0.05, except for subgroup analyses of RA patients, where a Bonferroni correction was applied with a significant two-tailed p-value threshold of 0.0125 (α/4) or 0.025 for the B-cell analysis (α/2).

# RESULTS

## Demographic and Clinical Characteristics of the Enrolled Subjects

We prospectively enrolled 84 vaccinated individuals: 35 RA patients and 49 HCWs. Significant differences were found for age ($p < 0.0001$) but not for sex or origin between the two groups (**Table 1**).

The RA enrolled cohort was stratified according to the drug treatment: 5 patients were under TNF-α inhibitors, 8 were under

**TABLE 1** | Demographical and clinical characteristics of the 84 enrolled subjects at T6.

| Characteristics | | RA patients | Health care workers | p-value |
|---|---|---|---|---|
| N (%) | | 35 (41.7) | 49 (58.3) | |
| Age median (IQR) | | 59 (55–66) | 51 (45–56) | <0.0001* |
| Female N (%) | | 28 (80) | 39 (79.6) | 0.963§ |
| Origin N (%) | West Europe | 31 (88.6) | 48 (98) | 0.288§ |
| | East Europe | 2 (5.8) | 1 (2) | |
| | Africa | 1 (2.8) | 0 (0) | |
| | Sud America | 1 (2.8) | 0 (0) | |
| Rheumatologic Treatment N (%) | TNF-α inhibitors ± DMARD | 5 (14.3) | – | |
| | IL-6 inhibitors ± DMARD/CCS | 8 (22.9) | – | |
| | CTLA-4-Ig ± DMARD/CCS | 11 (31.4) | – | |
| | DMARD ± CCS | 11 (31.4) | – | |
| Disease activity median (IQR) | DAS28crp T6 | 3.3 (2.4–4.0) | – | |
| Therapy | Years | 5.4 (2.5–10.3) | – | |
| Lymphocytes count N (%) | | 32 (91.4) | 0 (0) | |
| Lymphocytes count N (%) Median $\times 10^3$/μl (IQR) | TNF-α inhibitors ± DMARD | 5 (15.6) | – | 0.276# |
| | | 2.5 (2.2–3.8) | | |
| | IL-6 inhibitors ± DMARD/CCS | 8 (25) | – | |
| | | 1.9 (1.5–2.5) | | |
| | CTLA-4-Ig ± DMARD/CCS | 11 (34.4) | – | |
| | | 2.4 (1.8–2.9) | | |
| | DMARD ± CCS | 8 (25) | – | |
| | | 2.2 (1.6–2.5) | | |

*DMARDs, disease-modifying antirheumatic drugs; CCS, corticosteroid; RA, rheumatoid arthritis; DAS28, Disease Activity Score 28; N, number; IQR, interquartile range.*
**Mann–Whitney U-statistic test.*
*§Chi-square test.*
*#Kruskal–Wallis test.*

IL-6 inhibitors, 11 were under CTLA-4-Ig, and 11 were under DMARDs only. At vaccination, the median treatment duration for TNF-α inhibitors was 3.3 years (IQR: 2.0–13.8), for IL-6 inhibitors 6.4 years (IQR: 5.3–9.8), for CTLA-4-Ig 6.3 years (IQR: 2.2–10.3), and for DMARDs 5.3 years (IQR: 2.6–8.3). Non-serious adverse events to vaccine were reported by either RA patients or HCWs, such as pain at the injection site, headache, or mild fever. Moreover, no serious adverse events (i.e., requiring hospitalization, resulting in death, or life threatening) were reported in vaccinated individuals.

## Humoral Response Persists 6 Months After SARS-CoV-2 Vaccination in Health Care Workers and Rheumatoid Arthritis Patients

We evaluated the humoral response by assessing the anti-RBD IgG and neutralizing antibodies. All enrolled individuals were negative for anti-N antibodies, confirming that the cohort was naive for SARS-CoV-2 (data not shown). All HCWs (49, 100%) and most RA subjects (32/35, 91.4%) showed a detectable anti-RBD-IgG response after 6 months from vaccination (**Figure 2A** and **Table 2**). However, RA patients under TNF-α inhibitors and CTLA-4-Ig showed a significant lower anti-RBD IgG median titer compared to HCWs ($p = 0.0034$ and $p < 0.0001$, respectively) (**Figure 2B**). By contrast, we did not find significant differences for the anti-RBD antibody response in IL-6 inhibitors or DMARD-treated individuals (**Table 2** and **Figure 2B**). We also evaluated the neutralization activity in the sera of all RA patients and in 42/49 HCWs (**Figure 2C**). We found that the majority of HCWs (37/42, 88%), but only 15/35 (42.8%) RA patients, showed detectable neutralizing antibodies. In particular, a neutralizing activity was present in 2/5 (40%) patients treated with TNF-α inhibitors, 5/8 (62.5%) patients under IL-6 inhibitors, 1/11 (9.1%) under CTLA-4-Ig, and 7/11 (63.6%) DMARD-treated patients. Moreover, a significant lower neutralizing titer was observed in patients treated with TNF-α inhibitors or CTLA-4-Ig compared to HCWs ($p = 0.017$ and $p < 0.0001$, respectively) (**Figure 2D**). A strong significant correlation was observed between the neutralizing antibody and anti-RBD IgG titers in both HCWs (rho = 0.700, $p < 0.0001$) and RA patients (rho = 0.870, $p < 0.0001$) (**Figures 3A, B**), while there was no correlation between the number of lymphocytes and the anti-RBD antibody titer (rho = -0.234, $p = 0.198$) (data not shown).

## Decay of the Antibody Response From T1 to T6 in Health Care Workers and Rheumatoid Arthritis Patients

To assess the decay of the humoral response to BNT162b2-mRNA vaccine, 42 (85.7%) HCWs and 29 (82.8%) RA patients were longitudinally sampled at T1 and T6. Among the RA patients, 5 subjects under treatment with TNF-α inhibitors, 7 under IL-6 inhibitors, 10 under CTLA-4-Ig, and 7 under DMARDs were followed up. The anti-RBD IgG titer was reduced at T6 compared to T1 in both HCWs (T1 median: 1,891 BAU/ml, IQR: 1,314–3,794 vs. T6 median: 149.4 BAU/ml, IQR: 82.35–226.8, $p < 0.0001$) (**Figure 4A**) and RA patients (T1 median: 784.7 BAU/ml, IQR: 448.8–2,006 vs. T6 median: 44.9 BAU/ml, IQR: 19.5–115.2, $p < 0.0001$) (**Figure 4B**). Among RA patients, a significantly reduced titer was observed mainly in

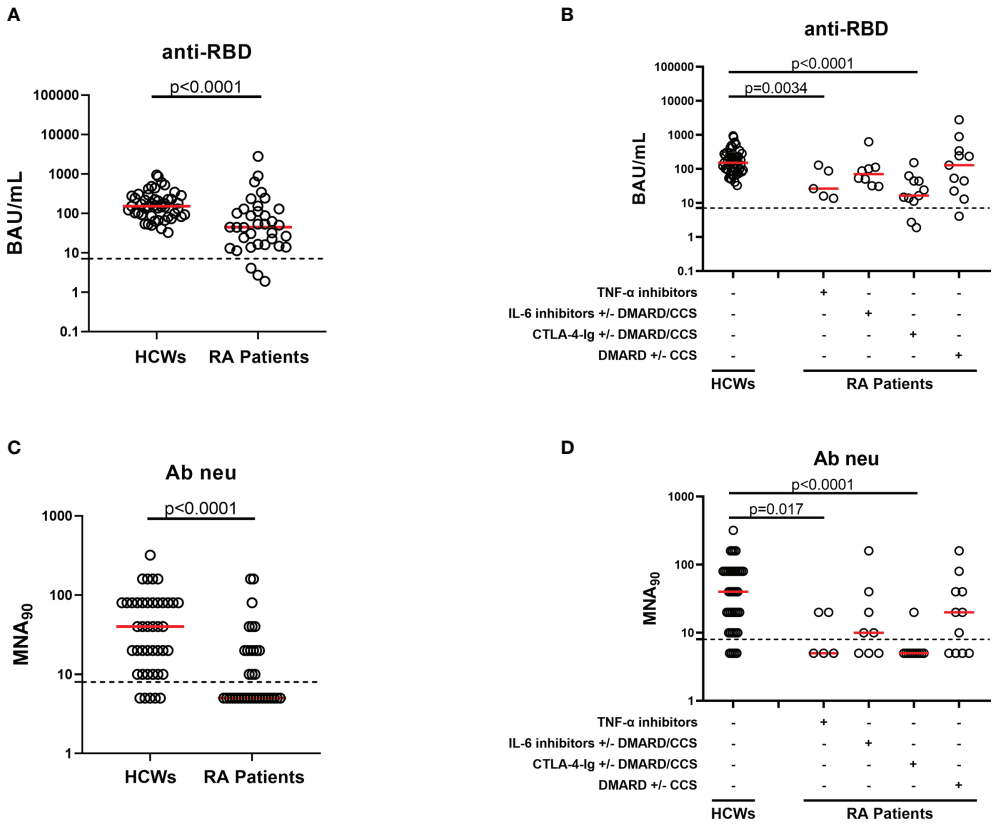


**FIGURE 2** | Humoral response elicited by SARS-CoV-2 vaccination after 6 months in HCWs and RA patients. Evaluation of SARS-CoV-2-specific anti-RBD **(A, B)** and neutralizing **(C, D)** antibodies 6 months after vaccination in the total of HCWs (n = 49) and RA patients (n = 35) analyzed. **(B, D)** RA patients were divided according to drug treatment into four groups: TNF-α inhibitors (n = 5), IL-6 inhibitors with or without DMARD/CCS (n = 8), CTLA-4-Ig with or without DMARD/CCS (n = 11), and DMARD with or without CCS (n = 11). Anti-RBD **(A, B)** and neutralizing **(C, D)** antibodies were quantified in serum samples and expressed as binding antibody units (BAU)/ml and reciprocal of dilution ($MNA_{90}$), respectively. Medians were indicated by red horizontal lines, and dashed lines represent the cutoff of each test (anti-RBD: 7.1 BAU/ml and $MNA_{90}$: 8). Mann–Whitney U-test with Bonferroni correction ($p \leq 0.0125$) was used for the statistical analysis. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CCS, corticosteroid; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis; RBD, receptor-binding domain; HCWs, health care workers.

those treated with IL-6 inhibitors (T1 median: 518.9 BAU/ml, IQR: 441.4–1,016 vs. T6 median: 53.5 BAU/ml, IQR: 32.1–101, p = 0.015), CTLA-4-Ig (T1 median: 507.3 BAU/ml, IQR: 212.3–986.9 vs. T6 median: 20.3 BAU/ml, IQR: 9.1–49, p = 0.002), and DMARDs ± CCSs (T1 median: 3,170 BAU/ml, IQR: 942.9–4,797 vs. T6 median: 129.3 BAU/ml, IQR: 44.9–244.7, p = 0.015) (**Figure 4C**). An almost significant decrease of the antibody titer was also observed in RA patients treated with TNF-α inhibitors (T1 median: 1,239 BAU/ml, IQR: 520.1–3,706 vs. T6 median: 26.5 BAU/ml, IQR: 14.9–108.8, p = 0.062).

## Memory B Cells Are Reduced in Rheumatoid Arthritis Patients Compared to Health Care Workers at T6

To assess the B-cell compartment of RA patients likely to explain the reduced antibody response at T6, we evaluated the B-cell subpopulation by flow cytometry. We did not observe significant differences between RA patients and HCWs in terms of percentage of total B cells, naïve B cells, transitional B cells, and plasma blasts (**Supplementary Figures S3A–D**).

Memory B cells (MBCs) ($CD27^+CD38^-$) were significantly reduced in subjects under CTLA-4-Ig compared to those in HCWs (CTLA-4-Ig median: 8.36%, IQR: 7.0–17.1 vs. HCW median: 29.4%, IQR: 20.9–32.6, p = 0.0012) (**Figure 5A**). In detail, a significant reduction in the unswitched MBCs ($CD27^+IgD^+$) (CTLA-4-Ig median: 3.6%, IQR: 1.1–5.8 vs. HCW median: 13.1%, IQR: 10.7–16.5, p = 0.014) (**Figure 5B**) and in the switched MBCs ($CD27^+IgD^-$) (CTLA-4-Ig median: 8.0%, IQR: 4.6–9.7 vs. HCW median: 14.7%, IQR: 11.0–19.2, p = 0.0033) was observed (**Supplementary Figure S3E**). Looking at the switched compartment ($CD27^+IgG^+IgM^-$ or $CD27^+IgG^-IgM^+$ or $CD27^+IgG^-IgM^-$), a significant reduction was observed in the $IgG^+$ switched MBCs of only CTLA-4-Ig-treated patients compared to those of HCWs (CTLA-4-Ig median: 2.9%, IQR: 1.8–3.9 vs. HCW median: 8.0%, IQR: 3.7–8.9, p = 0.0093) (**Figure 5C**, left panel). We did not find significant differences

**TABLE 2** | Serological and T-cell specific response at T6.

| Characteristics | | | | RA patients | Health care workers | p-value | |
|---|---|---|---|---|---|---|---|
| | | | N (%) | 35 (41.7) | 49 (58.3) | | |
| Antibody response | Qualitative response | Anti-RBD Ab responders, N (%) | | 32 (91.4) | 49 (100) | | 0.069§ |
| | | Anti-RBD Ab responders within the subgroups,N (%) | TNF-α inhibitors | 5/5 (100) | – | 0.641§ | >0.999§ |
| | | | IL-6 inhibitors± DMARD/CCS | 8/8 (100) | – | | >0.999§ |
| | | | CTLA-4-Ig± DMARD/CCS | 9/11 (81.8) | – | | 0.031§ |
| | | | DMARD±CCS | 10/11 (90.9) | – | | 0.183§ |
| | Quantitative response | Anti-RBD Abs, BAU/ml Median (IQR) | | 44.9 (16.1–129.3) | 152.7 (89.3–260.3) | | <0.0001* |
| | | | TNF-α inhibitors | 26.5 (14.9–108.8) | – | 0.054# | **0.0034*** |
| | | | IL-6 inhibitors± DMARD/CCS | 70.4 (36.6–109.6) | – | | 0.023* |
| | | | CTLA-4-Ig± DMARD/CCS | 16.4 (11.3–44.3) | – | | **<0.0001*** |
| | | | DMARD±CCS | 129.3 (22.5–342.3) | – | | 0.521* |
| Spike-specific IFN-γ T-cell response | Qualitative response | Anti-S responders, N (%) | | 23 (65.7) | 48 (97.9) | | <0.0001§ |
| | | Anti-S responders within the subgroups, N (%) | TNF-α inhibitors± DMARD | 4/5 (80) | – | 0.007§ | 0.178§ |
| | | | IL-6 inhibitors± DMARD/CCS | 8/8 (100) | – | | >0.999§ |
| | | | CTLA-4-Ig± DMARD/CCS | 3/11 (27.3) | – | | **<0.0001§** |
| | | | DMARD±CCS | 8/11 (72.7) | – | | 0.017§ |
| | Quantitative response | Anti-S IFN-γ, pg/ml Median (IQR) | | 45.3 (6.3–121.4) | 199.5 (81.8–310.8) | | <0.0001* |
| | | | TNF-α inhibitors± DMARD | 83.1 (27.7–268.4) | – | 0.033# | 0.172* |
| | | | IL-6 inhibitors± DMARD/CCS | 133.7 (22.6–241.2) | – | | 0.197* |
| | | | CTLA-4-Ig± DMARD/CCS | 6.3 (1.2–23.6) | – | | **<0.0001*** |
| | | | DMARD±CCS | 87.8 (9.5–121.4) | – | | 0.022* |

*DMARDs, disease-modifying antirheumatic drugs; CCS, corticosteroid; RA, rheumatoid arthritis; N, number; IQR, interquartile range; Abs, antibodies; RBD, receptor-binding-domain; S, spike.*
*§Chi-square test.*
**Mann–Whitney U-statistic test.*
*#Kruskal–Wallis test.*
*In bold are only those values that were significant after multiplicity correction by the Bonferroni method (α/4=0.0125).*

comparing the results from the patients under TNF-α inhibitor-treated subjects with the results from the controls (**Figures 5A–C** and **Supplementary Figures S3A–D**). Regarding the B-cell subpopulations and memory cell compartment, we did not find significant differences analyzing the results from patients treated with IL-6 inhibitors or DMARDs compared to those from the HCWs (data not shown).

## SARS-CoV-2-Spike-Specific T-Cell Response at T6 in Health Care Workers and Rheumatoid Arthritis

All HCWs, but one, showed an IFN-γ-S-specific response (48/49, 97.9%), whereas in RA patients a significantly different proportion of responders and a lower quantitative IFN-γ response was observed compared to controls (23/35, 65.7%, $p < 0.0001$) (**Table 2** and **Figure 6A**). In particular, patients under CTLA-4-Ig showed the lowest number of responders (3/11, 27.3%) (**Table 2**) associated with significantly lower IFN-γ-S-specific levels compared to HCWs ($p < 0.0001$) (**Figure 6B**). Differently, no significant differences were found between the IFN-γ-S-specific response of RA subjects under TNF-α inhibitors, IL-6 inhibitors, or DMARDs compared to that of HCWs ($p = 0.172$, $p = 0.197$, and $p = 0.022$, respectively). Most RA patients showed a positive response to SEB stimulus, used as positive control. However, the magnitude of the response was significantly lower compared to that of HCWs (RA median: 3,163 pg/ml, IQR: 1,598–6,698 vs. HCW median: 6,172 pg/ml, IQR: 3,422–9,447, $p = 0.0027$) (**Supplementary Figure S4A**). In detail, patients under TNF-α inhibitors and DMARDs showed the lowest response to SEB stimulus (TNF-α median: 1,170 pg/ml, IQR: 889–4,380, $p = 0.011$ vs. DMARD median: 2,918 pg/ml, IQR: 1,598–4,301, $p = 0.006$) (**Supplementary Figure S4B**).

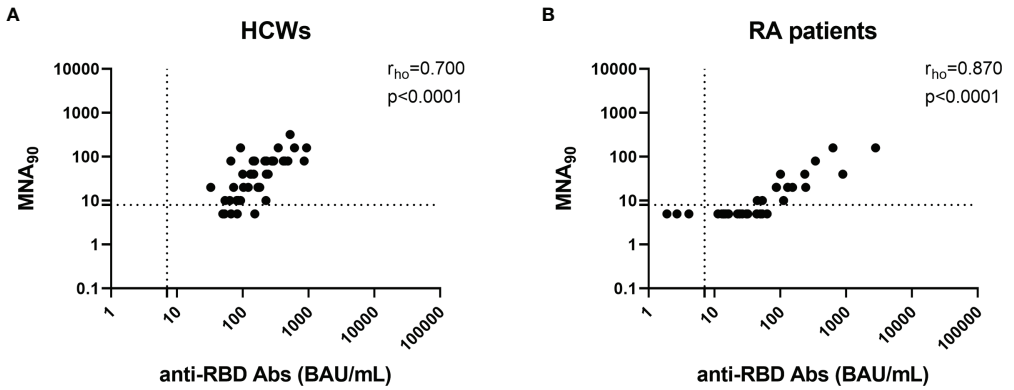


**FIGURE 3** | Anti-RBD and neutralizing antibodies correlate with each other in both HCWs and RA patients. Correlation between anti-RBD-IgG titers and neutralizing antibodies in 42 HCWs **(A)** and 35 RA patients **(B)**. Anti-RBD and neutralizing antibodies were quantified in serum samples and expressed as binding antibody units (BAU)/ml and reciprocal of dilution ($MNA_{90}$), respectively. Dashed lines identify the cutoff of each test (anti-RBD: 7.1 BAU/ml and $MNA_{90}$: 8). Statistical analyses were performed using Mann–Whitney U-test with Bonferroni correction ($p \leq 0.0125$). Correlation between assays was assessed by non-parametric Spearman's rank test, and $p < 0.05$ was considered significant. CCS, corticosteroid; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis; RBD, receptor-binding domain; HCWs, health care workers.

We then evaluated the correlation between the B- and T-cell arms of the immune response. No significant correlations between SARS-CoV-2 IFN-γ-S-specific response and neutralizing or anti-RBD antibody titer were observed in HCWs (rho = 0.102, p = 0.525 and rho = 0.078, p = 0.598, respectively) (**Figures 7A, B**). By contrast, moderate significant correlations were found in RA patients between the spike IFN-γ response and neutralizing antibodies (rho = 0.480, p = 0.0035) or anti-RBD antibodies (rho = 0.565, p = 0.0004) (**Figures 7C, D**). Overall, among RA patients, 14 were full responders (neutralizing activity, anti-RBD antibody and T-cell responses), 3 were not responders, and 18 were partial responders. Among these, 9 scored positive for both spike IFN-γ- and anti-RBD antibody responses, 8 for only anti-RBD antibodies, and 1 subject showed a neutralizing and anti-RBD antibody response (**Figure 7E**). There was no correlation between the number of lymphocytes and the IFN-γ-S-specific response (rho = 0.082, p = 0.654) (data not shown).

Flow cytometry analysis revealed that, after 6 months from the first dose of mRNA vaccine, the IFN-γ-specific response is mainly detectable within the $CD4^+$ T cells more than within the $CD8^+$ T cell compartment, both in HCWs and in RA patients (**Figures 8A, B**). The $CD4^+$ T-cell response was observed in 43% HCWs (3/7) and in 34% of the RA patients (11/32) (**Figure 8A**). No differences were observed also considering the magnitude of the response in both HCWs and RA patients (median $CD4^+IFN\text{-}\gamma^+$ HCWs: 0.001%, IQR 0.00–0.05 vs. median $CD4^+IFN\text{-}\gamma^+$ RA: 0.00%, IQR: 0.00–0.43, p = 0.5). By contrast, we did not detect a $CD8^+$ IFN-γ response in none of the HCWs and only in 12.5% of the RA patients (4/32) (median $CD8^+IFN\text{-}\gamma^+$ HCWs: 0.00%, IQR 0.00–0.00 vs. median $CD8^+IFN\text{-}\gamma^+$ RA: 0.00%, IQR: 0.00–0.00, p = 0.5) (**Figure 8B**). However, both HCWs and RA patients showed an IFN-γ-specific response to the positive control (SEB), providing evidence of the not impaired cytokine production (**Supplementary Figures S5A, B**).

## Decay of the T Cell-Specific Response From T1 to T6

Afterward, we evaluated the kinetics of the cellular response comparing the results from the two time points, T1 and T6. In HCWs, a significant reduction of the magnitude of the SARS-CoV-2 IFN-γ-S-specific response was observed at T6 compared to T1 (T1 median: 282.7 pg/ml, IQR: 136.2–570.4 vs. T6 median: 194.3 pg/ml, IQR: 87.0–331.1, p = 0.0049) (**Figure 9A**). By contrast, no significant differences were reported in the entire RA patient cohort, in which the T-cell response remains more stable over time (T1 median: 39.4 pg/ml, IQR: 5.3–177 vs. T6 median: 51.8 pg/ml, IQR: 8.7–180, p = 0.717) (**Figure 9B**). Also stratifying RA patients according to the drug treatment, no significant differences were found: TNF-α inhibitors (T1 median: 89.6 pg/ml, IQR: 42.5–254.6 vs. T6 median: 83 pg/ml, IQR: 28–268, p > 0.999), IL-6 inhibitors (T1 median: 41.3 pg/ml, IQR: 19.5–80.5 vs. T6 median: 170 pg/ml, IQR: 30.6–258, p = 0.297), CTLA-4-Ig (T1 median: 7.42 pg/ml, IQR: 3.03–35.3 vs. T6 median: 7.10 pg/ml, IQR: 2.4–27.6, p = 0.160) and DMARDs±CCSs (T1 median: 130 pg/ml, IQR: 17.2–364 vs. T6 median: 91.0 pg/ml, IQR: 45.3–268, p = 0.578) (**Figure 9C**). A quantitatively increased T-cell response at T6 compared to T1 was found in 11/42 (26.2%) HCWs (median proportion of increase: 69.7%) and in 12/29 (41.4%) RA patients (median proportion of increase: 185%). A reduction of the quantitative T-cell response at T6 compared to T1 was found in 30/42 (71.4%) HCWs (median proportion of reduction: 61.6%) and in 16/29 (55.2%) RA patients (median proportion of reduction: 43.9%). No significant differences were found comparing these two groups (p = 0.2). One subject for each group maintained a

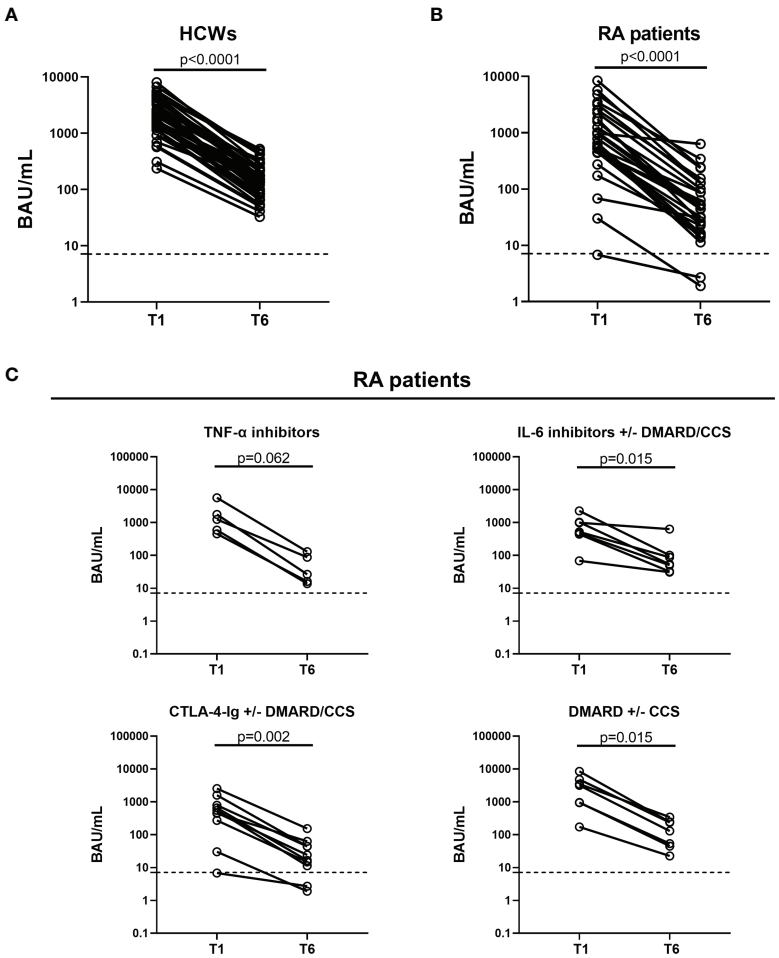


**FIGURE 4** | Kinetics of the humoral response induced by SARS-CoV-2 vaccination in HCWs and RA patients. Evaluation of the humoral response in 42 HCWs **(A)** and 29 RA patients **(B)** who were longitudinally sampled after 5 weeks (T1) and 6 months (T6) from the first vaccine dose. **(C)** RA patients were stratified based on the drug treatment: TNF-α inhibitors (n = 5), IL-6 inhibitors with or without DMARD/CCS (n = 7), CTLA-4-Ig with or without DMARD/CCS (n = 10), and DMARD with or without CCS (n = 7). Anti-RBD antibodies were quantified in serum samples and expressed as binding antibody units (BAU)/ml. Dashed lines indicate the cutoff of the test (anti-RBD: 7.1 BAU/ml). Statistical analysis was performed using Wilcoxon test, and $p < 0.05$ was considered significant. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CCS, corticosteroid; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis; RBD, receptor-binding domain; HCWs, health care workers.

stable T-cell response. Finally, among RA patients, 2 subjects initially negative at T1 scored positive at T6.

## DISCUSSION

Currently, vaccination is the most powerful tool to fight the COVID-19 pandemic in the general population and in particular in the fragile populations, such as RA patients. Although evidence is showing suboptimal humoral and cellular immune responses to COVID-19 vaccination in immunocompromised individuals (10, 20, 33, 34), there is a paucity of data regarding the over-time evaluation of both T- and B-cell responses in RA patients.

In this study, we describe the immune response to BNT162b2 vaccine in RA patients vaccinated following ACR indications (19). Accordingly, patients under MTX have interrupted the therapy 1 week after the first and the second dose of the vaccine, while patients under CTLA-4-Ig have interrupted the drug for 1 week before and after only the first dose. The results indicate that neutralizing and anti-RBD IgG titers after BNT162b2 vaccine significantly decrease over time in both HCWs and RA patients. Importantly, patients under TNF-α inhibitors and CTLA-4-Ig showed a significantly lower anti-RBD IgG median titer at T6 compared to HCWs, and this was associated with a reduction in the MBC compartment. Qualitative and quantitative IFN-γ responses were reduced in RA patients at T6 compared to the controls, as reported for the T1 (20). Among the RA patients, those under CTLA-4-Ig treatment were the most affected at T6. However, surprisingly, within the RA patients, we observed subjects who developed a T-cell-specific response at T6 (2/29)

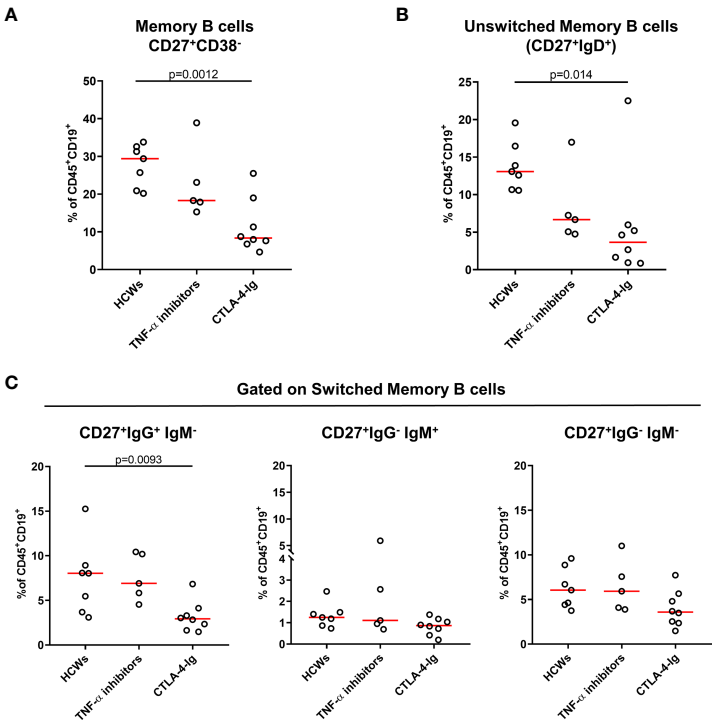


**FIGURE 5** | Evaluation of memory B-cell phenotype of RA patients by flow cytometry. Frequency of memory B cells of HCWs (n = 7) and RA patients (n = 13) was evaluated by flow cytometry. Within RA patients, 5 subjects were treated with TNF-α inhibitors and 8 with CTLA-4-Ig. Frequency of **(A)** memory B cells ($CD27^+CD38^-$), subdivided also as **(B)** unswitched memory B cells ($CD27^+IgD^+$) and **(C)** switched memory B cells, is shown. Within the switched memory B cells, $CD27^+IgG^+IgM^-$, $CD27^+IgG^-IgM^+$, and $CD27^+IgG^-IgM^-$ cells were included. Values are reported as percentage of total $CD19^+CD45^+$ B cells. Each dot represents an individual, and the red horizontal line represents medians. Mann–Whitney U-test with Bonferroni correction ($p \leq 0.025$) was used for the statistical analysis. RA, rheumatoid arthritis; HCWs, health care workers.

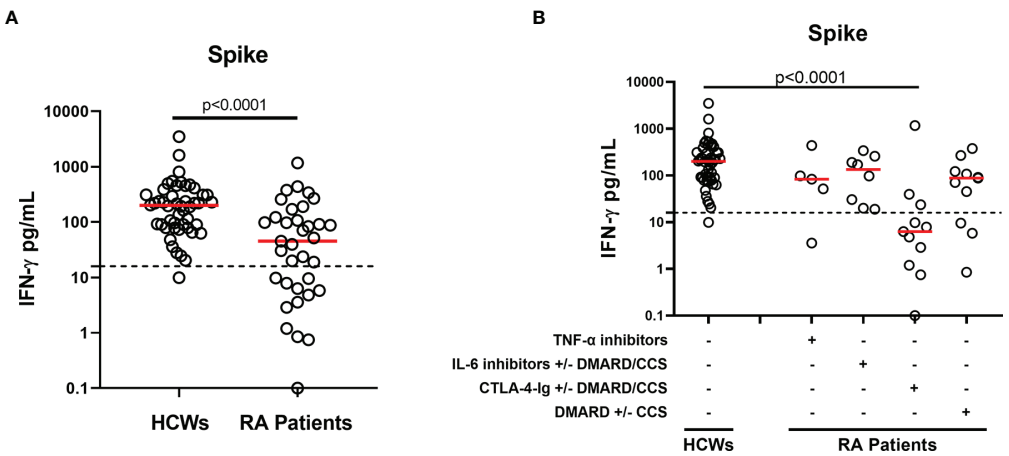


**FIGURE 6** | T-cell response after SARS-CoV-2 vaccination in HCWs and RA patients. **(A)** Evaluation of IFN-γ-spike-specific T-cell response 6 months after vaccination in the total of HCWs (n = 49) and RA patients (n = 35). **(B)** RA patients were stratified based on drug treatment in four groups: TNF-α inhibitors (n = 5), IL-6 inhibitors with or without DMARD/CCS (n = 8), CTLA-4-Ig with or without DMARD/CCS (n = 11), and DMARD with or without CCS (n = 11). T-cell response to spike antigen was assessed by measuring IFN-γ levels in plasma harvested from stimulated whole-blood samples. The reported IFN-γ values were subtracted from the background. Dashed lines identify the cutoff of the test (spike: 16 pg/ml). The red horizontal lines indicate the median. Statistical analyses were performed using Mann–Whitney U-test with Bonferroni correction ($p \leq 0.0125$). SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CCS, corticosteroid; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis; HCWs, health care workers.

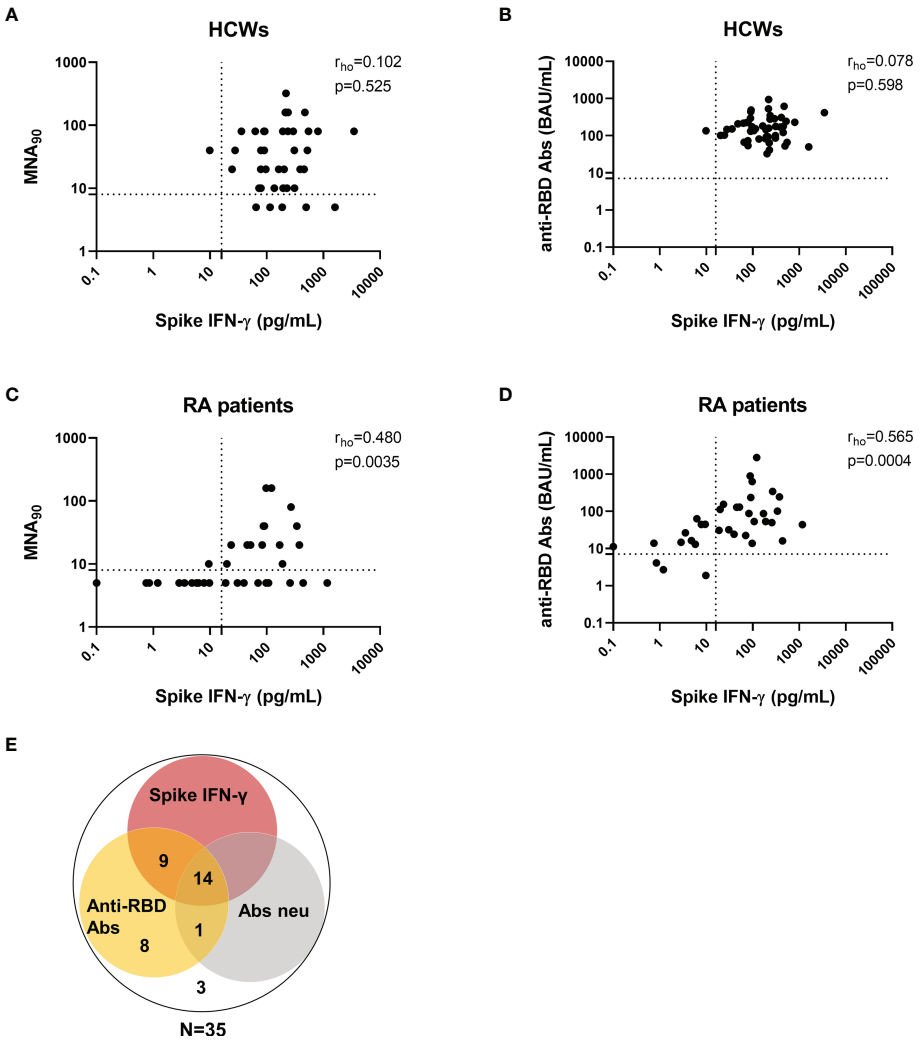


**FIGURE 7** | Correlation between T-cell response and anti-RBD or neutralizing antibodies in HCWs and RA patients. Correlation between neutralizing antibodies and the IFN-γ-spike-specific T-cell response in HCWs **(A)** and RA subjects **(C)**. Correlation between anti-RBD antibodies and the IFN-γ-spike-specific T-cell response in HCWs **(B)** and RA subjects **(D)**. Anti-RBD and neutralizing antibodies were quantified in serum samples and expressed as binding antibody units (BAU)/ml and reciprocal of dilution ($MNA_{90}$), respectively. T-cell response to spike antigen was assessed by measuring IFN-γ levels and reported after subtracting the background. Dashed lines identify the cutoff of each test (anti-RBD: 7.1 BAU/ml; $MNA_{90}$: 8 and spike: 16 pg/ml). Correlation between assays was assessed by non-parametric Spearman's rank test ($p < 0.05$). **(E)** Venn diagram shows positive results with anti-RBD IgG, neutralizing antibodies and IFN-γ T-cell response. RA, rheumatoid arthritis; RBD, receptor-binding domain; HCWs, health care workers.

(initially scored negative at T1). Moreover, comparing the quantitative T-cell response at T6 vs. T1, we observed an increase of this response in 41.4% of RA patients compared to the 26.2% of HCWs. These results may indicate that the fragile population of RA has a delayed T cell-specific response. Further studies are needed to confirm it. Clinically, BNT162b2 vaccine showed a good safety profile during the 6-month follow-up without serious adverse events and disease relapses. Notably, none of the RA patients had a breakthrough infection.

Regarding the vaccine-induced humoral immunogenicity after 6 months from the completed vaccination, we found that the anti-RBD antibody response was still detectable in all HCWs and in the majority of RA patients, although the titer was significantly reduced in those under TNF-α inhibitors and CTLA-4-Ig compared to HCWs. Neutralizing antibodies scored positive in most HCWs (88%), whereas only 42.8% of RA patients showed a detectable neutralizing activity. Moreover, we found that the anti-RBD and neutralizing antibodies significantly correlated in RA patients and in HCWs. In RA subjects, a moderately significant correlation between the anti-RBD antibody titer and SARS-CoV-2 IFN-γ-S-specific T-cell response was found, highlighting the persistence of a good coordination between the two components of the immune system (35). In contrast, the strong correlation between

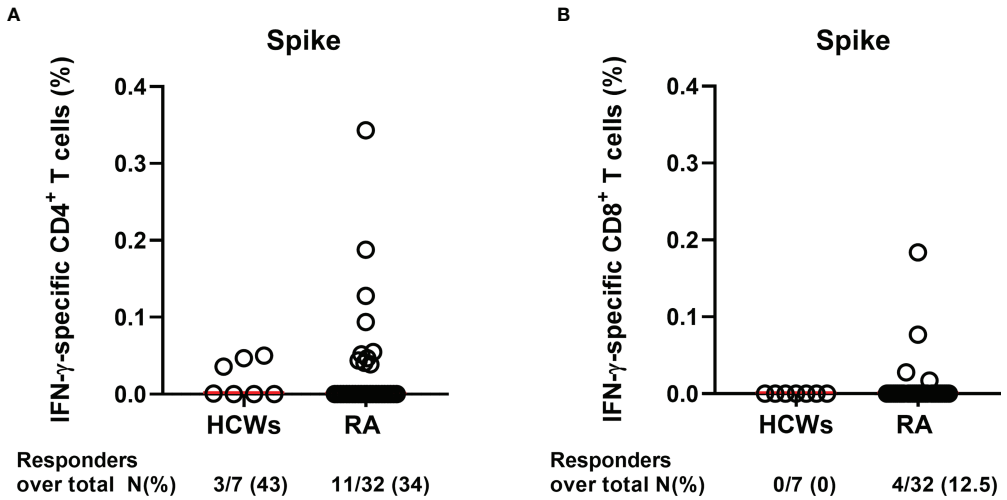


**FIGURE 8** | Evaluation of IFN-γ-S-specific T-cell response by flow cytometry. Cells from whole blood from HCWs (n = 7) and RA patients (n = 32) were stimulated for 24 h with spike peptide pool, and the percentage of IFN-γ-specific T cells was assessed by flow cytometry. Graphs show the frequency of the **(A)** $CD4^+$ and **(B)** $CD8^+$ IFN-γ-specific T-cell response (after subtraction of the unstimulated sample) in HCWs and RA patients. Each dot is a different HCW or RA individual, and red lines indicate the median. Mann–Whitney U-test was performed for the statistical analysis, and the p-value ≤0.05 was considered significant. RA, rheumatoid arthritis; HCWs, health care workers.

humoral and T-cell response observed early after vaccination in HCWs (7) was lost over time probably due to the rapid induction of the cell response at T1 in the healthy population that leads to a fast decay over time. Conversely, RA patients mount a more delayed response that leads to a correlation with the antibody response still present at later time points.

Following vaccination, the immune system is activated and produces short-lived plasma cells together with transient structures called germinal centers (GCs), where antigen-activated B cells differentiate into MBCs and into high-affinity long-lived antibody-producing plasma cells. During recalls, MBCs differentiate into long-lived plasma cells or can enter again in the GC reaction, expanding more and differentiate into plasma cells, as the immunological memory protects individuals from reinfections (36–42). Our results show a significant reduction of total MBCs and switched MBC subpopulations in RA patients treated with the CTLA-4-Ig compared to controls; moreover, the frequency of plasma blasts was lower in both CTLA-4-Ig and TNF-α inhibitor-treated patients even if the difference was not significant.

The reduced antibody response to COVID-19 vaccine is likely due to the well-known effect of the CTLA-4-Ig and TNF-α inhibitors on the reduction of the frequency of MBCs in the human peripheral blood (43–45). TNF-α is essential for lymphoid microarchitecture, and an impairment in B-cell function has been reported in RA patients treated with anti-TNF-α agents (45, 46). On the other hand, abatacept (CTLA-4-Ig) by binding with high-affinity CD80/CD86 molecules can affect T costimulation signals expressed by the antigen-presenting cells (47). In this context, a reduction in the humoral immunogenicity to pneumococcal and influenza vaccine has been shown in CTLA-4-Ig-treated patients (48).

Correlates of protection against SARS-CoV-2 infection are not yet established, and, up to now, we evaluated B- and T-cell parameters altogether (49–51). Therefore, looking at the overall specific B- and T-cell responses, patients may be stratified as full responders (both humoral and cellular), not responders, or partial responders (only humoral or T-cell response). Based on this stratification, in our cohort of RA patients, we found that after 6 months from the first dose of SARS-CoV-2 vaccine, 14/35 (40%) RA patients were full responders, 18/35 (51.4%) were partial responders, and only 3 (8.6%) were not responders. Regarding the antibody response, the full responders present both a good anti-RBD antibody titer and a good neutralizing antibody activity, which is one of the best predictors of *in vivo* vaccine efficacy, although it has not been possible so far to obtain a commercial test suggestive of a good correlate of protection (52). Nevertheless, this stratification may help to prioritize the vaccine schedule for RA patients; indeed, the full responders may follow the vaccination schedule as for the healthy individuals. Differently, the partial and/or not responders are very likely to be at higher risk for COVID-19 despite the complete vaccination, underlining the need for a tailored strategy. Although data on the immunogenicity effect of a third booster dose in the immune-mediated inflammatory disease patients are still lacking, an earlier administration could be hypothesized in not responders or partial responders, as already suggested by others (53).

Recently, we reported that the therapeutic strategy suggested by ACR (19) to interrupt MTX or CTLA-4-Ig (1 week before and after the first dose of the vaccine administration) reduced the negative impact on antibody production previously described (33, 34). Moreover, this strategy was beneficial also for those under CTLA-4-Ig, albeit with a significant lower antibody titer compared to HCWs. Importantly, this temporary therapy

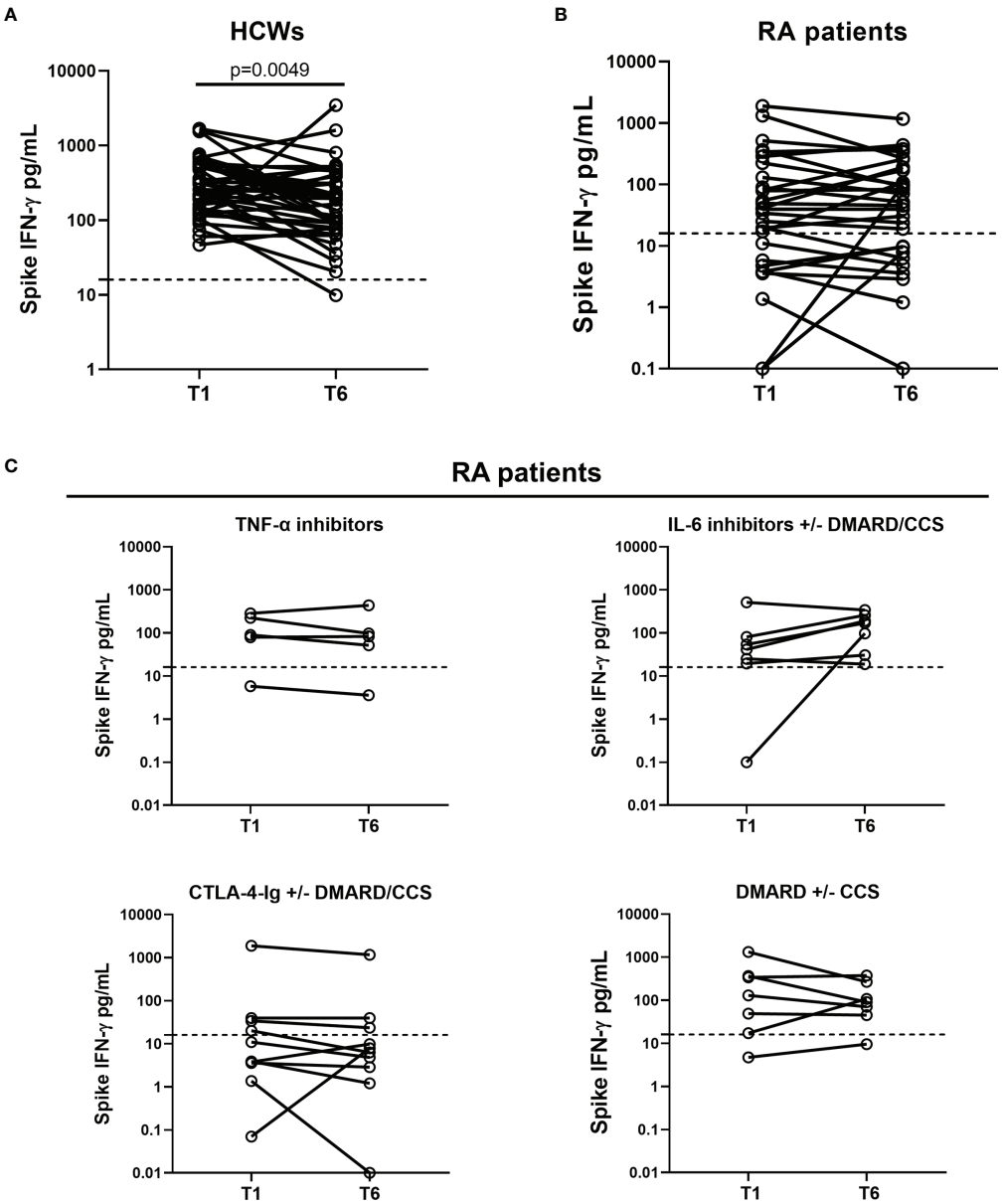


**FIGURE 9** | Kinetics of the cell-mediated response induced by SARS-CoV-2 vaccination in HCWs and RA patients. Evaluation of the spike IFN-γ response in 42 HCWs **(A)** and 29 RA patients **(B)** who were longitudinally sampled after 5 weeks (T1) and 6 months (T6) from the first vaccine dose. **(C)** RA patients were stratified in four groups: TNF-α inhibitors (n = 5), IL-6 inhibitors with or without DMARD/CCS (n = 7), CTLA-4-Ig with or without DMARD/CCS (n = 10), and DMARD with or without CCS (n = 7). T-cell response to spike antigen was assessed by measuring IFN-γ levels in plasma harvested from stimulated whole-blood samples. IFN-γ values were reported as median after subtracting the background. Dashed lines indicate the cutoff (spike: 16 pg/ml). Statistical analysis was performed using the Wilcoxon test, and $p < 0.05$ was considered significant. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CCS, corticosteroid; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis; HCWs, health care workers.

interruption did not affect the RA disease activity, as indicated by the stability of the DAS28crp parameter throughout the vaccination period.

In the effort of increasing the chances of complete protection, additional strategies may be explored such as a longer suspension of immunosuppressive agents during the vaccine administration, as well as the switch to drugs that have mechanisms of action with a reduced impact on vaccine immunogenicity (13). The use of a heterologous vaccination may help in enhancing the immune response, as recently reported by a systematic review in the general population, although the immunogenicity was higher in the population vaccinated with ChAdOx1-S followed by BNT162b2 rather than *vice versa* (54). In patients with a "not responder" or "partial responder" profile, a further clinical

option is the use of monoclonal antibodies against SARS-CoV-2-S glycoprotein as primary or secondary prophylaxis prior to or after any known SARS-CoV-2 exposure. Unpublished data from the "prevent" study are likely to support this indication, confirming robust efficacy and long-term prevention (55). Importantly, in parallel, on December 8, 2021, the Food and Drug Administration authorized the use of monoclonal antibodies for pre-exposure prevention of COVID-19 in high-risk population (56).

The main limitation of this study is the small size of the cohort, which may restrict its power, especially for the comparison of the effects of vaccination among the different RA treatments. However, it is important to underline that the enrolled patients are representative of the RA subjects under different immunosuppressive therapies, and that the patients were well characterized, both clinically and immunologically. Finally, the RA cohort and HCWs significantly differed in age, but we previously showed that the immune impairment is associated with the ongoing immunosuppressive therapies more than with the age (20). The main strength of the study is that, for the first time to our knowledge, we deeply characterized over time both humoral and cellular immune responses to BNT162b2 vaccine in RA patients, providing evidence of the kinetics, waning, and drug-induced impairment.

In conclusion, this study shows a significant reduction of the humoral response after 6 months of completed COVID-19 vaccination in both HCWs and RA patients regardless of the immunosuppressive therapy. Interestingly, the T-cell response was significantly decreased in HCWs, whereas it was mostly stable in RA patients. Considering the importance of a coordinated action of both humoral and cell-mediated responses to gain viral protection, our data support the execution of a booster dose of vaccine and a careful timeline of withdrawing some immunosuppressive agents to protect RA individuals from SARS-CoV-2 infection and hospitalization.

## DATA AVAILABILITY STATEMENT

The raw data generated and/or analyzed within the present study are available in our institutional repository (rawdata.inmi.it), subject to registration. The data can be found by selecting the article of interest from a list of articles ordered by year of publication. No charge for granting access to data is required. In the event of a malfunction of the application, the request can be sent directly by e-mail to the Library (biblioteca@inmi.it).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Institute for Infectious Diseases (INMI) Lazzaro Spallanzani-IRCCS (Approval number 297/2021) and Sant'Andrea University Hospital in Rome (Approval number 318/2021). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

DG and EN wrote the project to be submitted to the ethical committee. DG, EN, APD, BL, CA, and CC conceived and designed the study. Experiments were performed by CF, AA, SM, FC, RC, GG, VV, AS, FR, and AMGA. CF and AA performed the statistical analysis. APD, BL, GC, RR, SS, GN, GS, GM, and AC enrolled patients and collected clinical data. CF, APD, AA, EN, DG, BL, VP, and FV drafted the article or revised it critically. All authors critically analyzed, discussed, and interpreted data and contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.846753/full#supplementary-material

## REFERENCES


1. Montaldo C, Messina F, Abbate I, Antonioli M, Bordoni V, Aiello A, et al. Multi-Omics Approach to COVID-19: A Domain-Based Literature Review. *J Transl Med* (2021) 19:501. doi: 10.1186/s12967-021-03168-8
2. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical Course and Risk Factors for Mortality of Adult Inpatients With COVID-19 in Wuhan, China: A Retrospective Cohort Study. *Lancet Lond Engl* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
3. Nicastri E, Petrosillo N, Ascoli Bartoli T, Lepore L, Mondi A, Palmieri F, et al. National Institute for the Infectious Diseases "L. Spallanzani", IRCCS. Recommendations for COVID-19 Clinical Management. *Infect Dis Rep* (2020) 12:8543. doi: 10.4081/idr.2020.8543
4. Najafi Fard S, Petrone L, Petruccioli E, Alonzi T, Matusali G, Colavita F, et al. *In Vitro* Models for Studying Entry, Tissue Tropism, and Therapeutic Approaches of Highly Pathogenic Coronaviruses. *BioMed Res Int* (2021) 2021:8856018. doi: 10.1155/2021/8856018
5. Goletti D, Cantini F. Baricitinib Therapy in Covid-19 Pneumonia - An Unmet Need Fulfilled. *N Engl J Med* (2021) 384:867–9. doi: 10.1056/NEJMe2034982
6. Cantini F, Goletti D, Petrone L, Najafi Fard S, Niccoli L, Foti R. Immune Therapy, or Antiviral Therapy, or Both for COVID-19: A Systematic Review. *Drugs* (2020) 80:1929–46. doi: 10.1007/s40265-020-01421-w
7. Agrati C, Castilletti C, Goletti D, Meschi S, Sacchi A, Matusali G, et al. Coordinate Induction of Humoral and Spike Specific T-Cell Response in a Cohort of Italian Health Care Workers Receiving BNT162b2 mRNA Vaccine. *Microorganisms* (2021) 9:1315. doi: 10.3390/microorganisms9061315
8. Angyal A, Longet S, Moore SC, Payne RP, Harding A, Tipton T, et al. T-Cell and Antibody Responses to First BNT162b2 Vaccine Dose in Previously Infected and SARS-CoV-2-Naive UK Health-Care Workers: A Multicentre Prospective Cohort Study. *Lancet Microbe* (2021) 8:e21–31. doi: 10.1016/S2666-5247(21)00275-5
9. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med* (2021) 384:403–16. doi: 10.1056/NEJMoa2035389
10. Jena A, Mishra S, Deepak P, Kumar-M P, Sharma A, Patel YI, et al. Response to SARS-CoV-2 Vaccination in Immune Mediated Inflammatory Diseases: Systematic Review and Meta-Analysis. *Autoimmun Rev* (2022) 21:102927. doi: 10.1016/j.autrev.2021.102927
11. Geisen UM, Berner DK, Tran F, Sümbül M, Vullriede L, Ciripoi M, et al. Immunogenicity and Safety of Anti-SARS-CoV-2 mRNA Vaccines in Patients With Chronic Inflammatory Conditions and Immunosuppressive Therapy in a Monocentric Cohort. *Ann Rheum Dis* (2021) 80:1306–11. doi: 10.1136/annrheumdis-2021-220272
12. Simon D, Tascilar K, Fagni F, Krönke G, Kleyer A, Meder C, et al. SARS-CoV-2 Vaccination Responses in Untreated, Conventionally Treated and Anticytokine-Treated Patients With Immune-Mediated Inflammatory Diseases. *Ann Rheum Dis* (2021) 80:1312–6. doi: 10.1136/annrheumdis-2021-220461
13. Picchianti Diamanti A, Rosado MM, Nicastri E, Sesti G, Pioli C, Laganà B. Severe Acute Respiratory Syndrome Coronavirus-2 Infection and Autoimmunity 1 Year Later: The Era of Vaccines. *Front Immunol* (2021) 12:708848. doi: 10.3389/fimmu.2021.708848
14. Levin EG, Lustig Y, Cohen C, Fluss R, Indenbaum V, Amit S, et al. Waning Immune Humoral Response to BNT162b2 Covid-19 Vaccine Over 6 Months. *N Engl J Med* (2021) 385:e84. doi: 10.1056/NEJMoa2114583
15. Bar-On YM, Goldberg Y, Mandel M, Bodenheimer O, Freedman L, Kalkstein N, et al. Protection of BNT162b2 Vaccine Booster Against Covid-19 in Israel. *N Engl J Med* (2021) 385:1393–400. doi: 10.1056/NEJMoa2114255
16. Shrotri M, Navaratnam AMD, Nguyen V, Byrne T, Geismar C, Fragaszy E, et al. Spike-Antibody Waning After Second Dose of BNT162b2 or Chadox1. *Lancet Lond Engl* (2021) 398:385–7. doi: 10.1016/S0140-6736(21)01642-1
17. Guerrera G, Picozza M, D'Orso S, Placido R, Pirronello M, Verdiani A, et al. BNT162b2 Vaccination Induces Durable SARS-CoV-2–Specific T Cells With a Stem Cell Memory Phenotype. *Sci Immunol* (2021) 6:eabl5344. doi: 10.1126/sciimmunol.abl5344
18. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* (2010) 62:2569–81. doi: 10.1002/art.27584
19. Curtis JR, Johnson SR, Anthony DD, Arasaratnam RJ, Baden LR, Bass AR, et al. American College of Rheumatology Guidance for COVID-19 Vaccination in Patients With Rheumatic and Musculoskeletal Diseases: Version 3. *Arthritis Rheumatol Hoboken NJ* (2021) 73:e60–75. doi: 10.1002/art.41928
20. Picchianti-Diamanti A, Aiello A, Laganà B, Agrati C, Castilletti C, Meschi S, et al. ImmunosuppressiveTherapies Differently Modulate Humoral- and T-Cell-Specific Responses to COVID-19 mRNA Vaccine in Rheumatoid Arthritis Patients. *Front Immunol* (2021) 12:740249. doi: 10.3389/fimmu.2021.740249
21. Matusali G, Colavita F, Lapa D, Meschi S, Bordi L, Piselli P, et al. SARS-CoV-2 Serum Neutralization Assay: A Traditional Tool for a Brand-New Virus. *Viruses* (2021) 13:655. doi: 10.3390/v13040655
22. Carsetti R, Zaffina S, Piano Mortari E, Terreri S, Corrente F, Capponi C, et al. Different Innate and Adaptive Immune Responses to SARS-CoV-2 Infection of Asymptomatic, Mild, and Severe Cases. *Front Immunol* (2020) 11:610300. doi: 10.3389/fimmu.2020.610300
23. Aiello A, Najafi Fard S, Petruccioli E, Petrone L, Vanini V, Farroni C, et al. Spike Is the Most Recognized Antigen in the Whole-Blood Platform in Both Acute and Convalescent COVID-19 Patients. *Int J Infect Dis IJID Off Publ Int Soc Infect Dis* (2021) 106:338–47. doi: 10.1016/j.ijid.2021.04.034
24. Tortorella C, Aiello A, Gasperini C, Agrati C, Castilletti C, Ruggieri S, et al. Humoral- and T-Cell-Specific Immune Responses to SARS-CoV-2 mRNA Vaccination in Patients With MS Using Different Disease-Modifying Therapies. *Neurology* (2021) 98:e541–54. doi: 10.1212/WNL.0000000000013108
25. Murugesan K, Jagannathan P, Pham TD, Pandey S, Bonilla HF, Jacobson K, et al. Interferon-γ Release Assay for Accurate Detection of Severe Acute Respiratory Syndrome Coronavirus 2 T-Cell Response. *Clin Infect Dis Off Publ Infect Dis Soc Am* (2021) 73:e3130–2. doi: 10.1093/cid/ciaa1537
26. Riou C, Schäfer G, du Bruyn E, Goliath RT, Stek C, Mou H, et al. Rapid, Simplified Whole Blood-Based Multiparameter Assay to Quantify and Phenotype SARS-CoV-2 Specific T Cells. *Eur Respir J* (2021) 59:2100285. doi: 10.1183/13993003.00285-2021
27. Petruccioli E, Najafi Fard S, Navarra A, Petrone L, Vanini V, Cuzzi G, et al. Exploratory Analysis to Identify the Best Antigen and the Best Immune Biomarkers to Study SARS-CoV-2 Infection. *J Transl Med* (2021) 19:272. doi: 10.1186/s12967-021-02938-8
28. Petrone L, Petruccioli E, Vanini V, Cuzzi G, Najafi Fard S, Alonzi T, et al. A Whole Blood Test to Measure SARS-CoV-2-Specific Response in COVID-19 Patients. *Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis* (2021) 27:286.e7–286.e13. doi: 10.1016/j.cmi.2020.09.051
29. Petrone L, Petruccioli E, Alonzi T, Vanini V, Cuzzi G, Najafi Fard S, et al. *In-Vitro* Evaluation of the Immunomodulatory Effects of Baricitinib: Implication for COVID-19 Therapy. *J Infect* (2021) 82:58–66. doi: 10.1016/j.jinf.2021.02.023
30. Kagina BM, Mansoor N, Kpamegan EP, Penn-Nicholson A, Nemes E, Smit E, et al. Qualification of a Whole Blood Intracellular Cytokine Staining Assay to Measure Mycobacteria-Specific CD4 and CD8 T Cell Immunity by Flow Cytometry. *J Immunol Methods* (2015) 417:22–33. doi: 10.1016/j.jim.2014.12.003
31. Nemes E, Hesseling AC, Tameris M, Mauff K, Downing K, Mulenga H, et al. Safety and Immunogenicity of Newborn MVA85A Vaccination and Selective, Delayed Bacille Calmette-Guerin for Infants of Human Immunodeficiency Virus-Infected Mothers: A Phase 2 Randomized, Controlled Trial. *Clin Infect Dis Off Publ Infect Dis Soc Am* (2018) 66:554–63. doi: 10.1093/cid/cix834
32. Roederer M. How Many Events Is Enough? Are You Positive? *Cytom Part J Int Soc Anal Cytol* (2008) 73:384–5. doi: 10.1002/cyto.a.20549
33. Furer V, Eviatar T, Zisman D, Peleg H, Paran D, Levartovsky D, et al. Immunogenicity and Safety of the BNT162b2 mRNA COVID-19 Vaccine in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases and in the General Population: A Multicentre Study. *Ann Rheum Dis* (2021) 80:1330–8. doi: 10.1136/annrheumdis-2021-220647
34. Haberman RH, Herati R, Simon D, Samanovic M, Blank RB, Tuen M, et al. Methotrexate Hampers Immunogenicity to BNT162b2 mRNA COVID-19

Vaccine in Immune-Mediated Inflammatory Disease. *Ann Rheum Dis* (2021) 80:1339–44. doi: 10.1136/annrheumdis-2021-220597
35. Sette A, Crotty S. Adaptive Immunity to SARS-CoV-2 and COVID-19. *Cell* (2021) 184:861–80. doi: 10.1016/j.cell.2021.01.007
36. Akkaya M, Kwak K, Pierce SK. B Cell Memory: Building Two Walls of Protection Against Pathogens. *Nat Rev Immunol* (2020) 20:229–38. doi: 10.1038/s41577-019-0244-2
37. Grimsholm O, Piano Mortari E, Davydov AN, Shugay M, Obraztsova AS, Bocci C, et al. The Interplay Between CD27dull and CD27bright B Cells Ensures the Flexibility, Stability, and Resilience of Human B Cell Memory. *Cell Rep* (2020) 30:2963–2977.e6. doi: 10.1016/j.celrep.2020.02.022
38. Dunn-Walters DK, Stewart AT, Sinclair EL, Serangeli I. Age-Related Changes in B Cells Relevant to Vaccine Responses. *Interdiscip Top Gerontol Geriatr* (2020) 43:56–72. doi: 10.1159/000504479
39. Farroni C, Marasco E, Marcellini V, Giorda E, Valentini D, Petrini S, et al. Dysregulated miR-155 and miR-125b Are Related to Impaired B-Cell Responses in Down Syndrome. *Front Immunol* (2018) 9:2683. doi: 10.3389/fimmu.2018.02683
40. Valentini D, Marcellini V, Bianchi S, Villani A, Facchini M, Donatelli I, et al. Generation of Switched Memory B Cells in Response to Vaccination in Down Syndrome Children and Their Siblings. *Vaccine* (2015) 33:6689–96. doi: 10.1016/j.vaccine.2015.10.083
41. Tsang JS, Schwartzberg PL, Kotliarov Y, Biancotto A, Xie Z, Germain RN, et al. Global Analyses of Human Immune Variation Reveal Baseline Predictors of Postvaccination Responses. *Cell* (2014) 157:499–513. doi: 10.1016/j.cell.2014.03.031
42. Pulendran B, Ahmed R. Immunological Mechanisms of Vaccination. *Nat Immunol* (2011) 12:509–17. doi: 10.1038/ni.2039
43. Scarsi M, Paolini L, Ricotta D, Pedrini A, Piantoni S, Caimi L, et al. Abatacept Reduces Levels of Switched Memory B Cells, Autoantibodies, and Immunoglobulins in Patients With Rheumatoid Arthritis. *J Rheumatol* (2014) 41:666–72. doi: 10.3899/jrheum.130905
44. Sanz I, Wei C, Lee FE-H, Anolik J. Phenotypic and Functional Heterogeneity of Human Memory B Cells. *Semin Immunol* (2008) 20:67–82. doi: 10.1016/j.smim.2007.12.006
45. Anolik JH, Ravikumar R, Barnard J, Owen T, Almudevar A, Milner ECB, et al. Cutting Edge: Anti-Tumor Necrosis Factor Therapy in Rheumatoid Arthritis Inhibits Memory B Lymphocytes *via* Effects on Lymphoid Germinal Centers and Follicular Dendritic Cell Networks. *J Immunol* (2008) 180:688–92. doi: 10.4049/jimmunol.180.2.688
46. Picchianti Diamanti A, Rosado MM, Scarsella M, Germano V, Giorda E, Cascioli S, et al. Abatacept (Cytotoxic T Lymphocyte Antigen 4-Immunoglobulin) Improves B Cell Function and Regulatory T Cell Inhibitory Capacity in Rheumatoid Arthritis Patients Non-Responding to Anti-Tumour Necrosis Factor-α Agents. *Clin Exp Immunol* (2014) 177:630–40. doi: 10.1111/cei.12367
47. Moreland L, Bate G, Kirkpatrick P. Abatacept. *Nat Rev Drug Discov* (2006) 5:185–6. doi: 10.1038/nrd1989
48. Rondaan C, Furer V, Heijstek MW, Agmon-Levin N, Bijl M, Breedveld FC, et al. Efficacy, Immunogenicity and Safety of Vaccination in Adult Patients With Autoimmune Inflammatory Rheumatic Diseases: A Systematic Literature Review for the 2019 Update of EULAR Recommendations. *RMD Open* (2019) 5:e001035. doi: 10.1136/rmdopen-2019-001035
49. Dan JM, Mateus J, Kato Y, Hastie KM, Yu ED, Faliti CE, et al. Immunological Memory to SARS-CoV-2 Assessed for Up to 8 Months After Infection. *Science* (2021) 371:eabf4063. doi: 10.1126/science.abf4063
50. Goletti D, Petrone L, Manissero D, Bertoletti A, Rao S, Ndunda N, et al. The Potential Clinical Utility of Measuring Severe Acute Respiratory Syndrome Coronavirus 2-Specific T-Cell Responses. *Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis* (2021) 27:1784–9. doi: 10.1016/j.cmi.2021.07.005
51. Ferraccioli G, Gremese E, Goletti D, Petrone L, Cantini F, Ugel S, et al. Immune-Guided Therapy of COVID-19. *Cancer Immunol Res* (2022): canimm.0675.2021. doi: 10.1158/2326-6066.CIR-21-0675
52. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing Antibody Levels Are Highly Predictive of Immune Protection From Symptomatic SARS-CoV-2 Infection. *Nat Med* (2021) 27:1205–11. doi: 10.1038/s41591-021-01377-8
53. Ferri C, Ursini F, Gragnani L, Raimondo V, Giuggioli D, Foti R, et al. Impaired Immunogenicity to COVID-19 Vaccines in Autoimmune Systemic Diseases. High Prevalence of Non-Response in Different Patients' Subgroups. *J Autoimmun* (2021) 125:102744. doi: 10.1016/j.jaut.2021.102744
54. Ho T-C, Chen Y-MA, Chan H-P, Chang C-C, Chuang K-P, Lee C-H, et al. The Effects of Heterologous Immunization With Prime-Boost COVID-19 Vaccination Against SARS-CoV-2. *Vaccines* (2021) 9:1163. doi: 10.3390/vaccines9101163
55. *New Analyses of Two AZD7442 COVID-19 Phase III Trials in High-Risk Populations Confirm Robust Efficacy and Long-Term Prevention*. Available at: https://www.astrazeneca.com/media-centre/press-releases/2021/new-analyses-of-two-azd7442-covid-19-phase-iii-trials-in-high-risk-populations-confirm-robust-efficacy-and-long-term-prevention.html (Accessed December 30, 2021).
56. *Commissioner O of the. Coronavirus (COVID-19) Update: FDA Authorizes New Long-Acting Monoclonal Antibodies for Pre-Exposure Prevention of COVID-19 in Certain Individuals*. FDA (2021). Available at: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-new-long-acting-monoclonal-antibodies-pre-exposure (Accessed December 30, 2021).

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# SARS-COV-2 Infection, Vaccination, and Immune-Mediated Diseases: Results of a Single-Center Retrospective Study

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**Objectives:** The relationship between infections or vaccine antigens and exacerbations or new onset of immune-mediated diseases (IMDs) has long been known. In this observational study, conducted during the COVID-19 pandemic, we evaluated the onset of clinical and laboratory immune manifestations related to COVID-19 or SARS-CoV-2 vaccination.

**Methods:** Four groups of patients were evaluated: A) 584 COVID-19 inpatients hospitalized from March 2020 to June 2020 and from November 2020 to May 2021; B) 135 outpatients with previous SARS-CoV-2 infection, assessed within 6 months of recovery; C) outpatients with IMDs in remission and flared after SARS-COV-2 infection; D) outpatients with symptoms of probable immune-mediated origin after SARS-CoV-2 vaccination.

**Results:** In cohort A we observed n. 28 (4.8%) arthralgia/myalgia, n. 2 (0.3%) arthritis, n. 3 (0.5%) pericarditis, n. 1 (0.2%) myocarditis, n. 11 (1.9%) thrombocytopenia or pancytopenia, and in the follow up cohort B we identified 9 (6.7%) cases of newly diagnosed IMDs after the recovery from COVID-19. In all cases, serological alterations were not observed.In cohort C we observed n.5 flares of pre-existing IMD after SARS-COV2 infection, and in the cohort D n. 13 IMD temporally close with SARS-CoV-2 vaccination in 8 healthy subjects (with clinical classifiable IMD-like presentation) and in 5 patients affected by an anamnestic IMD. Also in these latter cases, except in 2 healthy subjects, there were not found serological alterations specific of a classifiable IMD.

**Conclusions:** This study suggests that the interplay between SARS-CoV-2 and the host may induce complex immune-mediated reactions, probably induced by the anti-spike antibodies, in healthy people and IMD patients without specific serological autoimmunity. Moreover, our data suggest that the anti-SARS-CoV-2 antibodies generated by the vaccination may cause in healthy subjects' clinical manifestations similar to well-definite IMDs. These findings support

the hypothesis that SARS-Cov2 infection in COVID-19 induce an innate and adaptive immune response that may be both responsible of the symptoms correlated with the occurrence of the IMDs described in our study. And, in this context, the IMDs observed in healthy people in close temporal correlation with the vaccination suggest that the anti-Spike antibodies may play a key role in the induction of an abnormal and deregulated immune response.

**Keywords: SARS-CoV2, COVID-19, Immuno-Mediated Reactions, autoimmunity, vaccine**

## INTRODUCTION

Relationships between viral infections, vaccine antigens and new onset or exacerbation of Immune-Mediated Diseases (IMDs) have been studied and acknowledged from long time.

Usually, this phenomenon has been attributed to cross-reactivity, where the neutralizing antibodies produced secondary to an antigenic stimulus (viral or vaccine-induced) react towards the body's self-tissues. Systemic Lupus Erythematosus, Rheumatoid Arthritis, Autoimmune Thrombocytopenia, Multiple Sclerosis, Guillain-Barré Syndrome, and other demyelinating neuropathies can be listed among the most important IMDs associated with autoimmune cross-reactivity mechanisms (1–3).

The pathogen SARS-CoV-2 is no exempt from this mechanism. Since the beginning of the COVID-19 pandemic, SARS-CoV-2 ability to induce auto-antibodies production and IMDs clinical manifestations has been observed in multiple studies, both *in vitro* and *in vivo* (4). Consequences secondary to severe SARS-CoV-2 infection are partly attributable to immune-mediated mechanisms of organ damage too (5, 6).

Furthermore, new and previously unknown autoimmune diseases have been described as a complication of SARS-CoV-2 infection, like the Multisystem Inflammatory Syndrome in Children (MIS-C) (7).

The neutralizing antibodies produced by the human body against SARS-CoV-2 are for the vast majority directed against the Spike protein, responsible for the interaction between the virus and ACE-2 receptor on human respiratory cells. Specifically, the site responsible for binding to the ACE-2 receptor is the Receptor Binding Domain (RBD) region of the Spike protein. Consequently, this region is the target of over 90% of neutralizing antibodies. Vaccines are also based on this mechanism, taking advantage of the production of antibodies against the RBD region (8).

Considering the above, the possibility that anti-Spike antibodies could potentially cross-react and cause autoimmune reactions is at least intriguing.

Besides, patients with IMDs, or with familial or genetic predisposition to autoimmunity, have shown greater susceptibility than the general population to manifest COVID-19 related autoimmune reactions (9).

Furthermore, currently few studies adequately investigated the correlation between vaccination and the development of IMDs or the induction of a flare of a pre-existing disease (10–12). Considering the possible role of anti-Spike antibodies in autoimmune manifestations related to SARS-CoV-2 infection, it is worth hypothesizing that even the vaccine-induced antibodies may seldom cross-react, triggering immunological manifestations (13).

If this hypothesis is confirmed, it should lead to a more careful evaluation of the risks and benefits of vaccination in young patients with a predisposition to develop autoimmune reactions.

## MATERIALS AND METHODS

### Patients

Four groups of patients were evaluated in an observational retrospective analysis as follows:

A) patients affected by COVID-19, and B) patients recovered from COVID-19, in outpatient follow up; C) patients affected by flare of IMDs after COVID-19; and D) flare of IMDs or onset of IMD after vaccination anti-SARS-CoV-2.

The primary objective of this single-center retrospective study, conducted during the COVID-19 pandemic, is to assess the onset of clinical and laboratory immune manifestations related to SARS-CoV-2 infection or vaccination.

The primary endpoint of the study was the occurrence or flare of IMDs:

a) in patients affected by acute COVID-19;
b) in patients recovered and in outpatient follow up after COVID-19;
c) in patients affected by anamnestic IMD after infection by SARS-Cov2;
d) in close temporal correlation with anti-SARS-Cov2 vaccination in patients affected by an anamnestic IMD or healthy subjects.

Secondary endpoints of the study were:

- clinical characterization,
- laboratory findings, and
- clinical outcomes after the therapy,

of the IMDs occurring in the groups listed above.

A schematic representation of the study and the patients' clinical characteristics are shown in **Figure 1** and **Table 2**, respectively.

In the cohort A we enrolled 584 patients with moderate to severe COVID-19 disease, hospitalized in the "COV-4" ward of the hospital "Ospedali Riuniti di Ancona" (AOU), Ancona, Italy,

from March 2020 to June 2020 and from November 2020 to May 2021 (**Table 1**).

Inclusion criteria were the following: *a)* age>18 years (yrs); *b)* acute infection by SARS-Cov2 detected with at least two nasopharyngeal swabs and by real-time reverse transcription polymerase chain reaction method.

Exclusion criteria were the following: absence of the inclusion criteria; trauma; neoplastic diseases; non-organ specific connective tissue diseases.

The clinical spectrum of the patients hospitalized for COVID-19 was achieved following the National Institute of Health of the United States of America (NIH) COVID-19 treatment guidelines as follows: a) Moderate Illness: Individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation (SpO2) ≥94% on room air at sea level; b) Severe Illness: Individuals who have SpO2 <94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) <300 mm Hg, a respiratory rate >30 breaths/min, or lung infiltrates >50%; c) Critical Illness: Individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction (14).

All the patients were assessed with clinical examinations and laboratory test daily and with High-resolution computed tomography (HRCT) in the emergency department and during the hospitalization, depending on the clinical evolution.

In the cohort B we evaluated 135 outpatients from cohort A discharged out of the hospital and evaluated within 6 months of the recovery by COVID-19.

Inclusion criteria in the cohort B were the following: *a)* age>18 yrs; *b)* absence of SARS-Cov2 detected with at least two nasopharyngeal swabs and by real-time reverse transcription polymerase chain reaction method; c) absence of any respiratory symptom and fever for at least one week.

The follow up examinations were scheduled at 3 and 6 months from the discharge and included clinical examinations, laboratory test and HRCT. The scheduled follow up was anticipated in case of the occurrence of any clinical symptom.

The outcome of interest in cohort A and B was the onset of: a) symptoms of rheumatic diseases (arthralgias-myalgias) in absence of fever; b) articular pain and/or joint swelling; c) chest pain and pericarditis/myocarditis confirmed by laboratory test (elevation of erythrocyte sedimentation rate, ESR; C-reactive protein, CRP; creatin-kinase, CK; high-sensitivity troponin, hs-Tpn, electrocardiographic recording, and ultrasound-doppler echocardiography; d) clinical signs of involvement of the central and/or peripheral nervous system; e) hematological alterations, not reported in the clinical history.

The cohorts C and D were constituted by two group of patients evaluated for IMDs symptoms among the whole cohort of 1710 outpatients evaluated in our rheumatologic clinic for rheumatologic symptoms from March 2020 to November 2021.

The cohort C was constituted by patients affected by IMD (among the 849 IMD patients in our center currently in follow up for at least 2 years) and satisfying the following inclusion in criteria: *a)* age >18 yrs; b) IMD in stable remission for at least 6 months.

The cohort D was constituted by subjects reporting IMD symptoms or patients affected by flare of IMDs in temporal close correlation with vaccine administration.

The inclusion criteria in cohort D were the following: *a)* age>18 yrs; b) IMD in stable remission for at least 6 months OR healthy subjects without IMD symptoms before the vaccination; *c)* absence SARS-Cov2 detected with at least two nasopharyngeal swabs and by real-time reverse

**TABLE 1** | Descriptive characteristics of the patients hospitalized for COVID-19.

| Variables | Cohort A (n. 584) | Cohort B (n. 135) | p value |
|---|---|---|---|
| **Age (yrs ± SD)** | 62.42±15.78 | 61.71±15.57 | n.s. |
| **Sex (M/F, %)** | 62/38 | 69/31 | n.s. |
| **Smokers, n (%)** | 372/584 (56) | 69/135 (51) | n.s. |
| **COVID-19 clinical, moderate, n (%)[1]** | 490/584 (83.9) | 64/135 (48) | n.s. |
| **COVID-19 clinical, severe, n (%)** | 82/584 (14.04) | 21/135 (15.55) | n.s |
| **COVID-19 clinical, critical, n (%)** | 12/584 (2.06) | 3/135 (2.22) | n.s. |
| **Hypertension, n (%)** | 362/584 (62) | 69/135 (51) | n.s. |
| **Diabetes, type II, n (%)** | 280/584 (48) | 55/135 (41) | n.s. |
| **COPD, n (%)** | 239/584 (41) | 51/135 (38) | n.s. |
| **Cardiovascular Disease[2], n (%)** | 204/584 (35) | 55/135 (41) | n.s. |
| **Renal Failure, n (%)** | 198/584 (34) | 39/135 (29) | n.s. |
| **Dyslipidemia, n (%)** | 415/584 (71) | 92/135 (68) | n.s. |
| **Connective disease[3], n (%)** | 13/584 (2.3) | 2/135 (1.5) | n.s. |
| **Neoplastic disease[4], n (%)** | 12/584 (2.05) | 0/135 (0) | n.s. |
| **Psychiatric disorder[5], n (%)** | 26/584 (4.5) | 4/135 (3) | n.s. |
| **Neurologic disease[6], n (%)** | 38/584 (6.5) | 9/135 (6.6) | n.s. |

*[1]The clinical spectrum of COVID-19 correspond to the definition of the National Institute of Health of the United States of America (NIH) COVID-19 treatment guidelines (14). [2]Ischemic chronic heart disease, chronic cardiac failure, hypertensive cardiopathy. [3]Patients affected by anamnestic connective disease (n.12 rheumatoid arthritis, n. 1 scleroderma, n. 1 undifferentiated connective disease). [4]Patients affected by anamnestic neoplastic disease (n. 5 lung neoplasia, n. 5 breast carcinoma, n. 2 sarcoma). [5]Patients affected by anamnestic psychiatric disease (n.12 severe depression, n. 14 psychotic disease, n. 4 bipolar disease). [6]Patients affected by anamnestic neurologic disease (n. 35 dementia, n. 12 Alzheimers' disease). Statistic: Categorical data were summarized using absolute frequencies and percentages, while continuous data were summarized using mean ± SD. Comparisons between groups were assessed by means of t test for independent samples or Chi square test, as appropriate. A p < 0.05 was considered significant; if 0.05: p not significant (n.s.). Yrs, years; M/F, males/females; COVID-19, COronaVIrus Disease 2019; COPD, chronic obstructive pulmonary disease.*

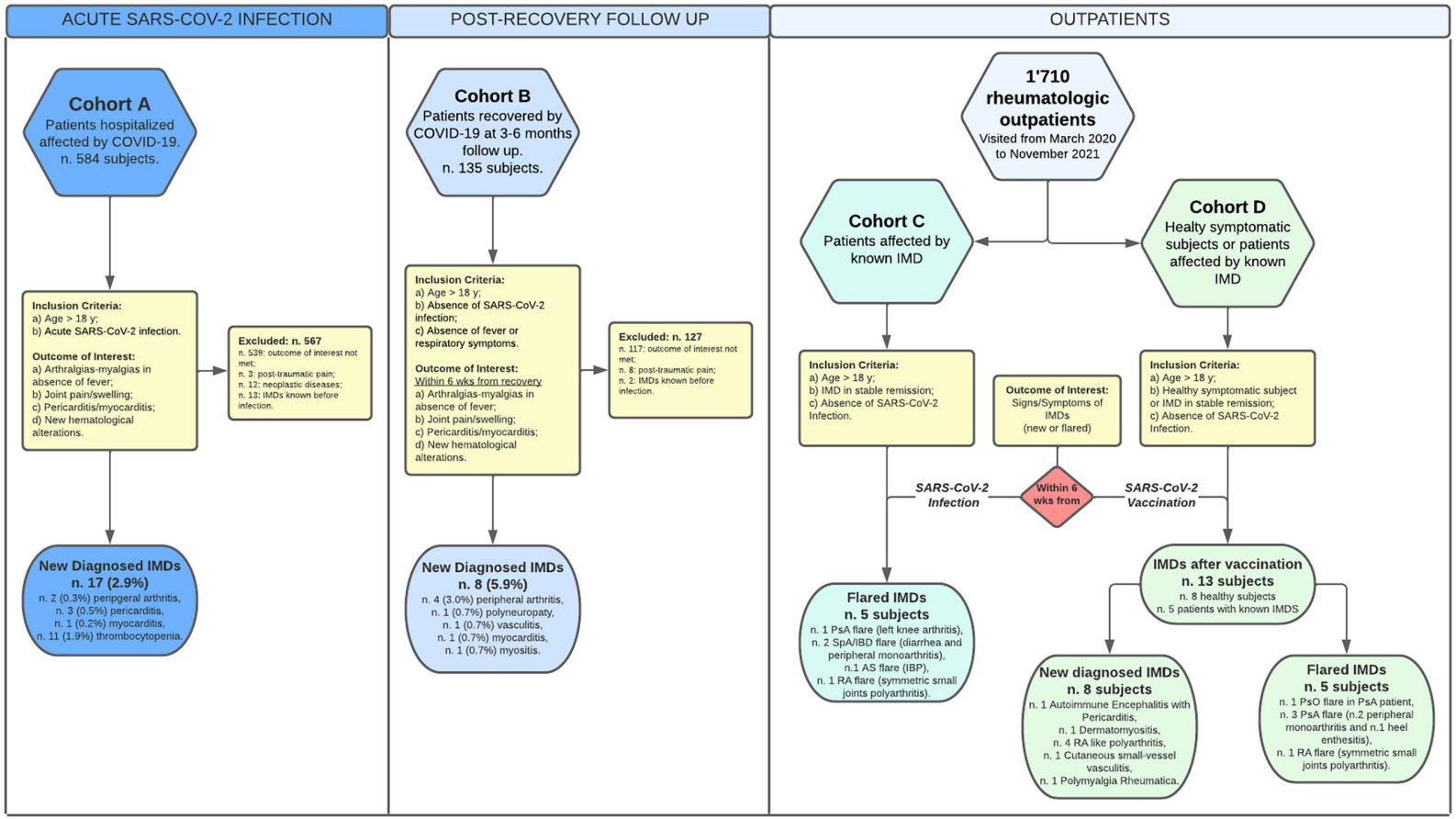


**FIGURE 1** | Schematic representation of the study and patients' groups. In the flow chart are represented the composition of the patients' cohorts/groups, inclusion/exclusion criteria in the study and brief presentations of the clinical outcomes. All the patients who suffered of IMDs symptoms after the vaccination had been vaccinated with two boost doses of the vaccines listed in the **Table 6**. For more details, see the *Materials and Methods* section.

**TABLE 2** | Immune-Mediated Diseases (IMDs) diagnosed in patients hospitalized for COVID-19.

| Parameter | Value [n/tot. number(%)] |
|---|---|
| Sex | |
| Female (52,9%) | 9/584 (1.54%) |
| Male (47,1%) | 8/584 (1.37%) |
| Age (mean) | 60,23 |
| IMDs | |
| Pericarditis | 3/584 (0.5%) |
| Myocarditis | 1/584 (0.2%) |
| Arthritis | 2/584 (0.3%) |
| Thrombocytopenia or Pancytopenia | 11/584 (1.9%) |

transcription polymerase chain reaction method; d) SARS-COV2 vaccination, less than 6 weeks before the onset of the following symptoms: arthralgias-myalgias; articular pain and/or joint swelling; chest pain; dermal lesions or signs of skin inflammation; clinical signs of involvement of the central and/or peripheral nervous system.

The outcome of interest in cohorts C and D was occurrence of IMD flare or the development of new signs/symptoms of IMD. Data were collected by consulting inpatient and outpatient medical records.

The diagnosis of SARS-Cov2 acute infection in all the patients included in the study was made with at least two nasopharyngeal swabs and by real-time reverse transcription polymerase chain reaction method.

The diagnosis of IMDs was carried out by an experienced team of physicians through history, physical examination, and laboratory tests (see the following section).

Immune-Mediated Diseases (IMDs) were considered all the diseases that involve an immune response that is inappropriate or excessive, and is caused, signified, or accompanied by dysregulation of the immune system, with/without the presence of disease-specific autoantibodies (15, 16).

Given that the most common post-infectious or post-vaccinal rheumatological IMD is Reactive Arthritis, and considering that, according to the American College of Rheumatology Diagnostic Criteria (1999), it must occur within 6 weeks of the infectious trigger, we considered relevant only immunological manifestations occurred within 6 weeks from infection or vaccination.

## Laboratory Tests

Apart from clinical manifestations, we also collected data regarding routine laboratory tests including complete blood count, alanine transferase (ALT), aspartate transferase (AST), creatinine, uric acid, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), as available.

If available, we also recorded results of anti-nuclear antibodies (ANAs), anti-cytoplasmic ANCA (c-ANCA) and anti-perinuclear ANCA (p-ANCA) antibodies, rheumatoid factor and anti-citrullinated peptides antibodies (ACPAs), and anti-Ro, anti-La, anti-double strand DNA and anti-Sm autoantibodies.

Any other relevant laboratory or instrumental investigation that had been required for the diagnosis was also recorded.

## Ethical Approval and Data Analysis

The study was carried out in compliance with the Declaration of Helsinki on ethical principles for medical research. Formal approval by the local Ethics Committee (Comitato Etico Regionale delle Marche) was waived due to the retrospective nature of the study.

All data were transferred to an electronic database and analyzed descriptively using the common analysis methods.

Categorical data were summarized using absolute frequencies and percentages, while continuous data were summarized using mean ± SD. Comparisons between groups were assessed by means of t test for independent samples or Chi square test, as appropriate. A $p < 0.05$ was considered significant.

# RESULTS

## New Diagnosis of IMDs in Cohort A

New-onset IMDs were observed in a large group of patients affected by COVID-19 and hospitalized in a COVID ward (cohort A).

Among 584 patients non previously diagnosed with IMDs, 17 (2.9%) received a diagnosis of IMD (9 females, 52.9%; 8 males, 47.1%; mean age 60.23) (**Table 2**).

These reactions were further subclassified as n. 2 (0.3%) peripheral arthritis, n. 3 (0.5%) pericarditis, n. 1 (0.2%) myocarditis (severe), n. 11 (1.9%) thrombocytopenia and/or pancytopenia (**Tables 2**, **3**).

Clinical manifestations were mostly acute and observed during hospitalization, with a mean delay of symptom onset of 11 +/- 4 days. Other confounding factors such as joint trauma, additional concomitant infectious triggers or neoplasms were excluded.

Twenty-eight (4.8%) COVID-19 patients of the cohort A reported non-specific musculoskeletal symptoms (myalgia and arthralgia) but did not fulfill criteria for a defined IMD.

These newly reported IMDs were mostly mild to moderate in severity and resolved within 2-4 weeks with restitutio ab integrum after corticosteroids therapy, except for one case of severe myocarditis and one case of severe thrombocytopenia.

In all the patients, ESR and CRP were elevated but the autoantibodies that were tested resulted negative.

## New Diagnosis of IMDs in Cohort B

Cohort B was constituted by outpatient patients of the cohort A in follow-up after the discharge from the COVID ward, evaluated for suspected IMD occurring within 6 weeks from COVID-19.

Eight patients (5.93%) reported symptoms suspect for IMDs. The following diagnoses were made: n. 4 (3.0%) peripheral arthritis, n. 1 (0.7%) polyneuropathy, n. 1 (0.7%) vasculitis, n. 1 (0.7%) myocarditis, n. 1 (0.7%) myositis (**Table 4**).

The events were mostly mild-to-moderate and were successfully treated with low-dose corticosteroids, non-steroid anti-inflammatory drugs (NSAID), methotrexate and gabapentin (**Table 4**).

All the cases described occurred with a mean delay of days 8.9 ± 12 from the discharge from the hospital.

**TABLE 3 |** Immune-Mediated Diseases (IMDs) diagnosed in patients hospitalized for COVID-19.

| Case | Age | Sex | Clinical Presentation | Laboratory Test[a] | Therapy[b] | Outcome |
|---|---|---|---|---|---|---|
| M.R. | 56 | M | Arthritis of the I MTF | ESR 45 mmh; CRP 6 mg/dl. | Prednisone | Remission in 2 wks |
| M.L.B. | 86 | F | Arthritis of the I MTF | ERS 90 mmh. CRP 6.6 mg/dl. | Prednisone | Remission in 2 wks |
| I.G. | 82 | F | Pericarditis | ERS 120 mmh, CRP 33 mg/dl. | Prednisone | Remission in 4 wks |
| M.O. | 67 | F | Pericarditis | ERS 99 mmh, CRP 8.8 mg/dl. | Prednisone | Remission in 4 wks |
| Y.F. | 35 | F | Pericarditis | ERS 42 mmh. CRP 5.5 mg/dl. | Prednisone | Remission in 4 wks |
| M.P. | 52 | F | Myocarditis, severe. | ESR and CRP above normal values | Prednisone; MMF | Remission in 24 wks |
| M.G. | 58 | M | Thrombocytopenia | ERS 2 mmh, CRP 1.3 mg/dl | Prednisone | Remission in 2-4 wks in all patients |
| D.E. | 45 | | Pancytopenia | ERS 2 mmh, CRP 4.5 mg/dl | | |
| P.M. | 57 | | Pancytopenia | ERS 72 mmh, CRP 1 mg/dl | | |
| M.D. | 63 | | Thrombocytopenia | ERS 65 mmh, CRP 18.6 mg/dl | | |
| F.P. | 62 | | Thrombocytopenia | ERS 23 mmh, CRP 15 mg/dl | | |
| I.M.R. | 47 | | Thrombocytopenia | ERS 19 mmh, CRP 2.8 mg/dl | | |
| C.R. | 74 | | Thrombocytopenia severe | ERS 77 mmh, CRP 10 mg/dl | | |
| S.G. | 42 | F | Pancytopenia | ERS 45 mmh, CRP 8.2 mg/dl | Prednisone | Remission in 2-4 wks in all patients |
| F.E.K | 72 | | Thrombocytopenia | ERS 16 mmh, CRP 8.1 mg/dl | | |
| M.B. | 66 | | Thrombocytopenia | ERS and CRP above normal values | | |
| L.Z. | 60 | | Thrombocytopenia | ERS 16 mmh, CRP 4.8 mg/dl | | |

*Age: years. Sex: M, male, F, female.*

*[a]Laboratory Test: in all the patients the serum levels of anti-nuclear antibodies (ANAs), anti-cytoplasmic ANCA (c-ANCA) and anti-perinuclear ANCA (p-ANCA) antibodies, rheumatoid factor and anti-citrullinated protein antibodies (ACPAs), and anti-Ro, anti-La, anti-dsDNA, anti-Sm autoantibodies were tested and resulted in the normal range.*

*[b]Prednisone was administered at the dose of 25 mg/day, scaling the dose until the stop in 2-4 weeks, in all the patients. In case n. 6 prednisone was administered at the dose of 25 mg/day for one week, scaling the dose in 8 weeks to the minimum dose of 4 mg/day and, because the patient was still symptomatic, mycophenolate mofetil was added at the dose of 1 gr twice a day. Wks, weeks; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MMF, Mycophenolate mofetil;*

## Flare or Onset of New IMDs After SARS-CoV-2 Infection or Vaccination

The flare of an anamnestic IMD or the onset of a new one was evaluated among the whole cohort of 1710 outpatients evaluated in our rheumatologic clinic (n. 849 IMDs in follow up and n. 861 new outpatients evaluated for rheumatologic symptoms).

In the cohort C we evaluated those patients with a pre-existing diagnosis of an immuno-mediated disease and reporting symptoms of the IMDs after SARS-COV-2 infection.

Among this group, 5 patients (all females, mean age 56.8), corresponding to the 0,58% out of the 849 outpatients in follow up in our rheumatologic clinic, reported flares of the IMDs already known but in stable remission of the disease before the SARS-COV2 infection (**Table 5**). The flares presented as follows: acute arthritis of the left knee in n. 1 psoriatic arthritis (PsA); diarrhea and arthritis of the knee or of the right wrist in n. 2 spondyloarthritis associated to inflammatory bowel disease (SpA/IBD), respectively; inflammatory back pain in n. 1 ankylosing spondylitis (AS); and symmetric polyarthritis of the small joints of the hands in n. 1 rheumatoid arthritis (RA). All the patients were successfully treated adding corticosteroid to the ongoing therapy (**Table 5**).

In the cohort D we evaluated n. 13 outpatients with a newly diagnosed or flared IMD in close temporal correlation with SARS-CoV-2 mRNA vaccine administration (**Table 6**). Before the onset of the symptoms, all the patients had received two boost doses of the vaccines types listed in **Table 6**.

There were observed n. 8 new IMDs, corresponding to the 0.93% among the cohort of 861 outpatients evaluated for new rheumatologic symptoms. These patients showed a heterogeneous clinical spectrum (**Table 6**), including two clinical severe presentations: one patient had autoimmune encephalitis with pericarditis and another one dermatomyositis, with the involvement of the peripheral nervous system (**Figure 2**).

In the other patients, n. 4 patients presented a RA-like disease, characterized by symmetric polyarthritis of the wrists and of the small joints of the hands (**Figure 3**), n. 1 had a mild vasculitis of the small vessels of the hands' fingers, and n. 1 was diagnosed with polymyalgia rheumatica (**Table 6**).

In this cohort, serum autoantibodies were found only in the 2 patients: case n. 4 with autoimmune encephalitis (ANA 1/320) and case n. 6 with dermatomyositis (anti-SSA-Ro-antiMDA5, 1/640).

Vaccination induced a flare of an underlying IMD in 5 patients, corresponding to the 0,58% out of the 849 IMDs in follow up in our clinic, as follows: a flare of psoriasis in one patient with PsA; arthritis of the right knee or of the left wrist in two patients with PsA, respectively; enthesitis of the left Achille's tendon in one patient with axial-PsA; and symmetric polyarthritis of the small joints of the hands in one patient with RA.

All the patients of the cohort D were successfully treated with corticosteroids, or plus colchicine or mycophenolate mofetil (case n. 4 and n. 6, respectively), or corticosteroids added to the ongoing therapy with a bDMARD in the 2 patients (cases n. 7 and 8) already affected by an IMD (**Table 6**).

# DISCUSSION

Acute coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 is characterized by variable clinical presentations,

**TABLE 4 |** Immune-Mediated Diseases (IMDs) diagnosed in outpatients previously hospitalized for COVID-19 and in follow up.

| Case | Age | Sex | Timing | Clinical Presentation | Laboratory test[a] | Therapy[b] | Outcome |
|---|---|---|---|---|---|---|---|
| G.V. | 74 | M | 7 | Polineuropaty[c] | ESR 30 mmh. CRP 0,5 mg/dl | Prednisone + Gabapentin | Remission in 6 wks |
| M.R.D. | 49 | F | 10 | Synovitis of left ankle | ESR and CRP above the normal values | Prednisone | Remission in 8 wks |
| M.B. | 52 | M | 2 | Psoriatic arthritis, peripheral[d] | ESR 50 mmh, CRP 0,2 mg/dl | Prednisone + MTX | Remission in 12 wks |
| C.G. | 39 | M | 38 | Urticaria[e] | ESR 70 mmh, CRP 3 mg/dl. | Prednisone | Remission in 16 wks |
| O.M.C. | 54 | F | 2 | Vasculitis[f] | ESR 23 mmh, CRP 1,7 mg/dl | Prednisone | Remission in 12 wks |
| M.P. | 47 | F | 4 | Myocarditis | ESR and CRP above the normal values | Prednisone | Remission in 3 wks |
| F.M. | 82 | F | 5 | Myositis | ESR 39 mmh, CRP 1,0 mg/dl, CK 3539 U/l | Prednisone | Remission in 8 wks |
| L.R. | 56 | F | 3 | Polyarthritis, symmetric, of the small joints | ESR and CRP normal ANA 1/160 | Prednisone | Remission in 4 wks |

*Age: years. Sex: M, male; F, female. Timing: clinical presentation in days.*

*[a]Laboratory Test. Serum levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-nuclear antibodies (ANAs), anti-cytoplasmic ANCA (c-ANCA) and anti-perinuclear ANCA (p-ANCA) antibodies, rheumatoid factor and anti-citrullinated protein antibodies (ACPAs), and anti-Ro, anti-La, anti-dsDNA, anti-Sm autoantibodies were tested. There are shown only the test resulted out of the normal value.*

*[b]Prednisone was administered at the dose of 25 mg/day, scaling the dose until the stop in the weeks shown in the table in all the patients. In case n. 3, methotrexate (MTX) was added to prednisone at the dose of 10 mg/week and stopped when patient achieved the clinical remission.*

*[c]Diagnosed with electromyographic examination (EMG).*

*[d]Patient with a familiar history of psoriasis (father), and previous nail disease evaluated as "psoriatic onychopathy".*

*[e]The diagnosis of urticaria vasculitis was made with skin biopsy (vasculitis with infiltration of neutrophils and eosinophils).*

*[f]The diagnosis of vasculitis was made with skin biopsy (result: leukocytoclastic vasculitis). Wks, weeks; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MTX, methotrexate.*

**TABLE 5 |** Immune-Mediated Diseases (IMDs) flared after COVID-19.

| Case | Age | Sex | Timing | Clinical Presentation* | Laboratory Test[a] | Therapy[b] | Outcome |
|---|---|---|---|---|---|---|---|
| L.B. | 54 | F | 3 | Flare of PsA | ESR 45 mmh, CRP 5 mg/dl | Prednisone + bDMARD | Remission in 4 wks |
| R.B. | 53 | F | 4 | Flare of SpA/IBD | ESR and CRP above the normal values | Prednisone + bDMARD | Remission in 3 wks |
| E.G. | 29 | F | 7 | Flare of SpA/IBD | ESR 65 mmh, CRP 8 mg/dl | Prednisone + bDMARD | Remission in 3 wks |
| M.I. | 61 | F | 2 | Flare of Ankylosing Spondylitis | ESR and CRP above the normal values | Prednisone + bDMARD | Remission in 6 wks |
| A.M.T. | 83 | F | 5 | Flare of Rheumatoid Arthritis | ESR 85 mmh, CRP 18 mg/dl | Prednisone + bDMARD | Remission in 6 wks |

*Age: years. Sex: M, male; F, female. Timing: clinical presentation in days. *The clinical flare of each specific disease is reported in the Result section of the text.*

*[a]Laboratory Test. Serum levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-nuclear antibodies (ANAs), anti-cytoplasmic ANCA (c-ANCA) and anti-perinuclear ANCA (p-ANCA) antibodies, rheumatoid factor and anti-citrullinated protein antibodies (ACPAs), and anti-Ro, anti-La, anti-dsDNA, anti-Sm autoantibodies were tested. There are shown only the test resulted out of the normal value.*

*[b]Prednisone was added to the biologic-disease-modifying anti-rheumatic drug (b-DMARD; adalimumab in cases 1-3, secukinumab in case 4) at the dose of 25 mg/day, scaling the dose until the stop in the weeks shown in the table. Wks, weeks; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MMF, Mycophenolate mofetil; PsA, psoriatic arthritis; SpA/IBD, spondyloarthritis associated with inflammatory bowel disease (Crohn disease).*

**TABLE 6** | Immune-Mediated Diseases (IMDs) after vaccination anti-SARS-COV-2.

| Case | Age | Sex | Timing | Vaccine | Clinical Presentation* | Laboratory Test[a] | Therapy[b] | Outcome |
|---|---|---|---|---|---|---|---|---|
| P.F. | 71 | F | 1 | Comirnaty | Polyarthritis, RA-like | ESR and CRP above the normal values | Prednisone | Remission in 4 wks |
| S.F. | 72 | M | 9 | Comirnaty | Enthesitis/tenosynovitis of the shoulder; Polyarthritis, RA-like | ESR 84 mmh, CRP 3 mg/dl. | Prednisone | Remission in 8 wks |
| A.M. | 78 | F | 15 | Comirnaty | Capillaritis of the palmar surface | ESR and CRP above the normal values | Prednisone | Remission in 12 wks |
| S.M. | 38 | F | 1 | Comirnaty | Encephalitis, acute[c] + myocarditis-pericarditis | ESR and CRP above the normal values; ANA 1/320 | Prednisone + Colchicine | Remission in 8 wks |
| R.T. | 61 | M | 1 | Comirnaty | Polyarthritis RA-like | ESR 10 mmh, CRP 27,7 mg/dl | Prednisone | Remission 4 wks |
| L.M. G. | 68 | M | 5 | Comirnaty | Polyarthritis RA-like | ESR 73 mmh, CRP 2.7 mg/dl | Prednisone | Remission 3 wks |
| A.N. | 46 | F | 7 | Comirnaty | Dermatomyositis | ESR and CRP normal<br>Anti-SSA Ro and antiMDA5 positive, CK normal, AST 155 U/l, ALT 117 U/l. | Prednisone + MMF | Remission in 5 wks |
| O.G. | 72 | M | 3 | Comirnaty | Polymyalgia Rheumatica | ESR 68 mmh, CRP 1,6 mg/dl | Prednisone | Remission in 6 wks |
| G.I. | 25 | F | 3 | Comirnaty | Flare of Pso in Ax-PsA | N.S. | Prednisone + bDMARD | Remission in 3 months |
| F.R. | 58 | M | 30 | Comirnaty | Flare of Ax-PsA | ESR 15 mmh, CRP 6,8 mg/dl | Prednisone + bDMARD | Remission in 3wks |
| T.M. | 59 | F | 10 | Comirnaty | Flare of PsA | ESR 26 mm/h, CRP 1,1 mg/dl | Prednisone + bDMARD | Active |
| L.G. | 54 | F | 1 | Comirnaty | Flare of PsA | N.S. | Prednisone + bDMARD | Active |
| A.M.T. | 83 | F | 3 | Comirnaty | Flare of RA | ESR 85 mmh, CRP 18 mg/dl | Prednisone + bDMARD | Remission in 6 wks |
| L.P. | 60 | F | 14 | Comirnaty | Flare of RA | ESR and CRP normal | Prednisone + bDMARD | Remission in 4 weeks |

*Age: years. Sex: M, male; F, female. Timing: clinical presentation in days. *The clinical flare of each specific disease is reported in the Result section of the text. Before the onset of the symptoms, all the patients had received two boost doses of the vaccines types listed in the table.*
*[a]Laboratory Test. Serum levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-nuclear antibodies (ANAs), anti-cytoplasmic ANCA (c-ANCA) and anti-perinuclear ANCA (p-ANCA) antibodies, rheumatoid factor and anti-citrullinated protein antibodies (ACPAs), and anti-Ro, anti-La, anti-dsDNA, anti-Sm autoantibodies were tested. There are shown only the test resulted out of the normal value.*
*[b]Prednisone was used alone or added to the oncourse biologic-disease-modifying anti-rheumatic drug (b-DMARD) at the dose of 25 mg/day, scaling the dose until the stop in the weeks shown in the table.*
*[c]Diagnosed with magnetic resonance imaging. Wks, weeks; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MMF, Mycophenolate mofetil; RA, rheumatoid arthritis; Pso, psoriasis; Ax-PsA, spondyloarthritis associated with psoriasis; PsA, psoriatic arthritis.*

ranging from asymptomatic infection to fatal respiratory failure. Recently, it has become apparent that an exaggerated immune response plays an important role in the pathogenesis of COVID-19, but the intersection of COVID-19 and autoimmunity still needs to be fully elucidated (17).

In fact, it appears that a preexisting autoimmunity may influence, often deleteriously, the course of COVID-19 in certain individuals and, meanwhile, in other patients the virus may contribute to a *de novo* breakdown in immune tolerance, triggering pathogenic immune-mediated clinical manifestations reminiscent of those seen in autoimmune diseases such as antiphospholipid syndrome, inflammatory arthritis, and systemic lupus erythematosus (SLE) (18). In addition, there are numerous case reports of patients developing classifiable autoimmune diseases, such as rheumatoid arthritis, psoriatic arthritis, and type 1 diabetes concomitantly or immediately following SARS-CoV-2 infection (19–22).

As suggested for other viruses (23), the widespread interaction of Coronaviruses with our defense system can trigger autoimmune diseases favored by a molecular similarity between viral and human peptides. Their sporadic transcription and recombination generate a wide number of epitopes that may contribute to elicit autoimmunity trough molecular mimicry, bystander activation, epitope spreading and cytokine storm (24).

In our work, we described a series of cases of IMDs occurring after COVID-19 in a large cohort of patients. We also described a series of cases of well characterized IMDs temporally correlated with anti-SARS-CoV-2 vaccination.

In patients hospitalized for COVID-19, the most observed severe IMDs were diseases of cardiological interest (as pericarditis and myocarditis) and hematological alterations (thrombocytopenia). In the latter case, to improve diagnostic specificity, we considered only cases with platelet counts <100,000/mmc, with a rapid response to steroid therapy and without alternative explanation. According to previous reports (25), thrombocytopenia has been described as an autoimmune manifestation in COVID-19 although without anti-platelet antibodies, likewise in our patients.

In most cases, the hospitalized COVID-19 patients complained mild IMDs symptoms (arthralgia/myalgia) without patent clinical manifestations, whereas a real acute arthritis occurred only in a small percentage of cases.

A subgroup of patients previously affected by COVID-19 and subsequent IMDs were followed-up in the outpatient clinic. Interestingly, these patients reported mainly joint manifestations, more commonly acute exacerbation of seronegative spondyloarthritis (SpA). Acute flares of SpA after COVID-19 have been recently reported by other studies (26).

It is worth noting that all patients described above, although the clinical manifestations of IMDs after COVID-19 closely resembled those of the classifiable disease, did not show the presence of common autoantibodies associated with autoimmune diseases (i.e. SLE or RA), which might have supported the hypothesis of an autoantibody-mediated pathogenesis of the IMDs.

Indeed, we should underline that in these IMDs, putatively correlated with SARS-COV-2 infection, we have not been able to identify, so far, a "disease-tissue"-specific antibody, but it cannot be excluded that in the next future there will be published studies reporting tissue-specific antibodies induced by the SARS-Cov-2 virus.

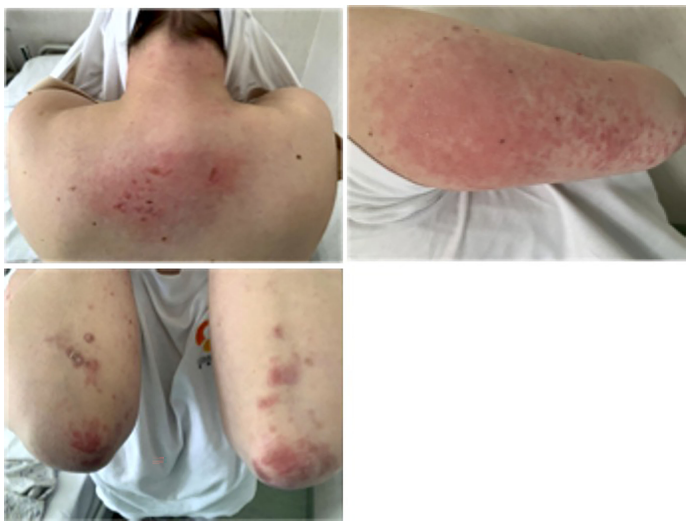

**FIGURE 2** | Dermatomyositis-like clinical presentation closely correlated with anti-SARS-2 vaccination. The figure shows a representative case of a dermatomyositis-like clinical presentation in a patient after SARS-COV2 vaccination.

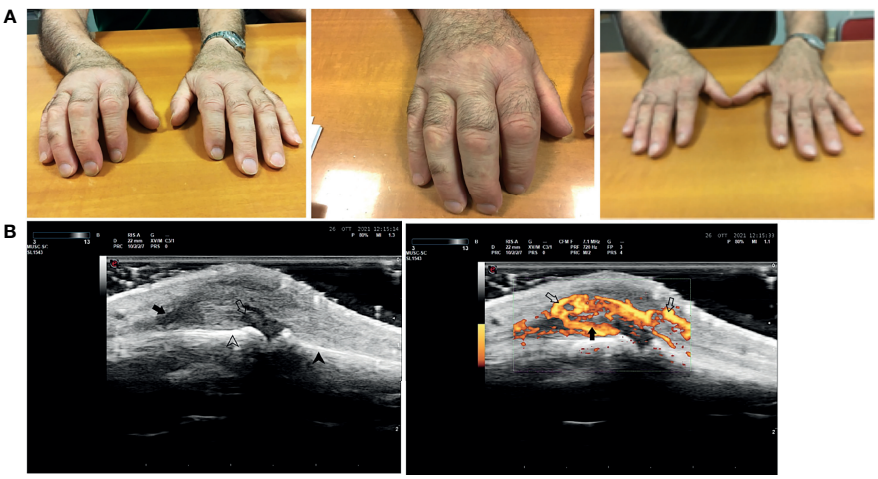


**FIGURE 3** | Rheumatoid arthritis-like clinical presentation closely correlated with anti-SARS-2 vaccination. The figure shows a representative case of a rheumatoid arthritis-like clinical presentation in a patient after SARS-COV2 vaccination. Panel **(A)** From left to right: acute phase of the disease, a particular of the right hand, and clinical examination after 4 weeks of corticosteroid therapy. Panel **(B)** Ultrasound doppler examination of the 3rd metacarpal-phalangeal joint of the right hand (from left to right): Grayscale US, Joint capsule (*arrow*) of MCF III dilated by synovial proliferation (*open arrow*) grade III (gs) according to OMERACT criteria. Below are shown the surfaces of metacarpal bone (arrowhead) and proximal phalanx (open arrowhead); Power Doppler Same scan of **Figure 1** with PD showing severly active synovitis (*arrow*) of grade III (pd) according to OMERACT criteria With PD signal becames evident an associated paratenonitis of third digit extensor tendons (open arrows).

Moreover, it could be hypothesized that the innate immunity may play an important role in the development of the IMDs in the first phases of the COVID-19 and, subsequently, another important pathogenetic role could be played by the adaptive immunity, namely by the production of the anti-SARS-Cov2 antibodies.

The latter hypothesis, in our study, is supported by the occurrence of the IMDs in close temporal correlation with anti-SARS-Cov2 vaccination and a role of the anti-SARS-COV2 spike antibodies or SARS-COV2-recognizing T cells in triggering a prolonged immune-mediated inflammation in these patients might be postulated.

From this point of view, we additionally reported thirteen cases of new IMDs and five flares of pre-existing IMD that we observed in close temporal correlation with SARS-CoV-2 vaccination with a mRNA vaccine.

The possibility that vaccinations could induce a flare of an underlying rheumatic disease is still controversial and vaccines are generally considered to be safe (27).

In our study, the incidence of these IMD putatively correlated with vaccines administration is low, but we should consider that our study has some limitations as well.

The study has been not designed to estimate the incidence and prevalence of IMD onset after COVID-19 or vaccination, but it is a real-life observational study conducted in a single tertiary referral center during a pandemic. Thus, the estimates could be affected by selection bias and the retrospective data collection and analysis.

In a large epidemiological study conducted in 5493 patients with RA, the propensity-scored weighted Poisson regression showed no significant association between arthritis flare and COVID-19 vaccination (28).

Conversely, in two different web-based surveys evaluating systemic rheumatic disease flare incidence post-SARS-CoV-2 vaccine, one study showed about 11.5% of patients and the other n. 66 patients who reported a worsening of the underlying disease closely correlated with the first and/or second dose of different vaccines (10, 29).

The most important finding of the latter case series, like in other reports (11–13), is that all the new IMDs or their exacerbations were mostly mild-to-moderate and, importantly, a short course of corticosteroids was sufficient to control disease manifestations in most cases.

Moreover, although the clinical manifestations were similar to those of the canonical IMDs, we found that conventional or disease-defining serological markers were usually absent.

These considerations open two important questions: how should these vaccine-induced IMDs be managed and for how long? What is their natural history?

These findings in vaccinated subjects potentially support the hypothesis that anti-spike antibodies elicited by vaccination and, likewise, those elicited by COVID-19 (15–19), may participate in triggering downstream signaling pathways common to those encountered in several immune-mediated disorders (13, 30, 31).

Considering the massive campaign of vaccination against SARS-CoV-2, the incidence of vaccine-associated IMDs seems however quite low (https://vaccine-safety-training.org).

In conclusion, this study shows that immunity against SARS-CoV-2 has the potential to stimulate heterogeneous immune-mediated reactions without specific serological autoimmunity. Our findings support the need to investigate on the role that anti-Spike antibodies may play in such cases and, most importantly, reinforce the current understanding that COVID-19 clinical manifestations are often sustained by the host immune system but not by SARS-CoV-2 itself (17, 18). The true incidence of IMDs, both as flares and as new onset manifestations, remains to be established. Larger epidemiological studies should be promoted globally to monitor these potential post-vaccination IMDs and to evaluate their real incidence and clinical significance.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

## 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.

## AUTHOR CONTRIBUTIONS

ML, VP, VM, MG and DB contributed to conception and design of the study and to the clinical examination of the patients. RS, CM, LM organized the database and contributed to the clinical examination of the patients. DB performed the statistical analysis. GM, VP, and DB wrote the first draft of the manuscript. ML, GP, AO, and GM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

## REFERENCES


1. Yahel S, Yehuda S. Vaccine-Induced Autoimmunity: The Role of Molecular Mimicry and Immune Cross Reaction. Cell. *Mol Immunol* (2018) 15:586–94. doi: 10.1038/cmi
2. Perl A. Mechanisms of Viral Pathogenesis in Rheumatic Disease. *Ann Rheumatol Dis* (1999) 58:454–61. doi: 10.1136/ard.58.8.454
3. Bhattacharjee S, Banerjee M. Immune Thrombocytopenia Secondary to COVID-19: A Systematic Review. SN Compr. *Clin Med* (2020) 2:2048–58. doi: 10.1007/s42399-020-00521-8
4. Vojdani A, Kharrazian D. Potential Antigenic Cross-Reactivity Between SARS-Cov-2 and Human Tissue With a Possible Link to an Increase in Autoimmune Diseases. *Clin Immunol* (2020) 217:108480. doi: 10.1016/j.clim.2020.108480
5. Khamsi R. Rogue Antibodies Could be Driving Severe COVID-19. *Nature* (2021) 590:29–31. doi: 10.1038/d41586-021-00149-1
6. Caricchio R, Gallucci M, Dass C, Zhang X, Gallucci S, Fleece D, et al. Preliminary Predictive Criteria for COVID-19 Cytokine Storm. *Ann Rheumatol Dis* (2021) 80:88–95. doi: 10.1136/annrheumdis-2020-218323
7. Dufort EM, Koumans EH, Chow EJ, Rosenthal EM, Muse A, Rowlands J, et al. Multisystem Inflammatory Syndrome in Children in New York State. *N Engl J Med* (2020) 383:347–58. doi: 10.1056/NEJMoa2021756
8. Andreano E, Rappuoli R. SARS-Cov-2 Escaped Natural Immunity, Raising Questions About Vaccines and Therapies. *Nat Med* (2021) 27:759–61. doi: 10.1038/s41591-021-01347-0
9. *Dysregulation of Immunity Predisposing to Severe COVID-19 Infection | Frontiers Research Topic* (2021). Available at: https://www.frontiersin.org/research-topics/24847/dysregulation-of-immunity-predisposing-to-severe-covid-19-infection.
10. Barbhaiya M, Levine JM, Bykerk VP, Jannat-Khah D, Mandl LA. Systemic Rheumatic Disease Flares After SARS-Cov-2 Vaccination Among Rheumatology Outpatients in New York City. *Ann Rheum Dis* (2021) 80:1352–4. doi: 10.1136/annrheumdis-2021-220732
11. Watad A, De Marco G, Mahajna H, Druyan A, Eltity M, Hijaziet N. Immune-Mediated Disease Flares or New-Onset Disease in 27 Subjects Following Mrna/DNA SARS-Cov-2 Vaccination. *Vaccines* (2021) 9:435. doi: 10.3390/vaccines-9050435

12. Velikova T, Georgiev T. SARS-Cov-2 Vaccines and Autoimmune Diseases Amidst the COVID-19 Crisis. *Rheumatol Int* (2021) 41:509–18. doi: 10.1007/s00296-021-04792-9
13. Talotta R. Do COVID-19 RNA-Based Vaccines Put at Risk of Immune-Mediated Diseases? In Reply to "Potential Antigenic Cross-Reactivity Between SARS-Cov-2 and Human Tissue With a Possible Link to an Increase in Autoimmune Diseases. *Clin Immunol* (2021) 224:108665. doi: 10.1016/j.clim.2021.108665
14. COVID-19 Treatment Guidelines Panel. *Coronavirus Disease 2019 (Covid-19) Treatment Guidelines*. National Institutes of Health. Available at: https://www.covid19 treatment guidelines nih.gov/.
15. Williams JP, Meyers JA. Immune-Mediated Inflammatory Disorders (I.M.I.D.s): The Economic and Clinical Costs. *Am J Manage Care* (2002) 8: S664–81.
16. El-Gabalawy H, Guenther LC, Bernstein CN. Epidemiology of Immune-Mediated Inflammatory Diseases: Incidence, Prevalence, Natural History, and Comorbidities. *J Rheumatol* (2010) Suppl. 85:2–10. doi: 10.3899/jrheum.091461
17. Knight JS, Caricchio R, Casanova JL, Combes AJ, Diamond B, Fox SE, et al. The Intersection of COVID-19 and Autoimmunity. *J Clin Invest* (2021) 131 (24):e154886. doi: 10.1172/JCI154886
18. Ishay Y, Kenig A, Tsemach-Toren T, Amer R, Rubin L, Hershkovitz Y, et al. Autoimmune Phenomena Following SARS-Cov-2 Vaccination. *Int Immunopharmacol* (2021) 99:107970. doi: 10.1016/j.intimp.2021.107970
19. Rodríguez Y, Novelli L, Rojasa M, Santis De, Acosta-Ampudia Y, Monsalve DM, et al. Autoinflammatory and Autoimmune Conditions at the Crossroad of COVID19. *J Autoimmun* (2020) 144:102506. doi: 10.1016/j.jaut.2020.102506
20. Talarico R, Stagnaro C, Ferro F, Carli L, Mosca M. Symmetric Peripheral Polyarthritis Developed During SARS-Cov-2 Infection. *Lancet Rheumatol* (2020) :2,e518–e519. doi: 10.1016/S2665-9913(20)30216-2
21. De Stefano L, Rossi S, Montecucco C, Bugatti S. Transient Monoarthritis and Psoriatic Skin Lesions Following COVID-19. *Ann Rheumatol Dis* (2020). doi: 10.1136/annrheumdis-2020-218520
22. Chee YJ, Ng SJH, Yeoh E. Diabetic Ketoacidosis Precipitated by Covid-19 in a Patient With Newly Diagnosed Diabetes Mellitus. *Diabetes Res Clin Pract* (2020) 164:108166. doi: 10.1016/j.diabres.2020.108166
23. Moroncini G, Mori S, Tonnini C, Gabrielli A. Role of Viral Infections in the Etiopathogenesis of Systemic Sclerosis. *Clin Exp Rheumatol* (2013) 31(2 Suppl 76):3–7.
24. Danieli MG, Piga MA, Paladini A, Longhi E, Mezzanotte C, Moroncini G, et al. Intravenous Immunoglobulin as an Important Adjunct in the Prevention and Therapy of Coronavirus 2019 Disease. *Scand J Immunol* (2021) 94: e13101. doi: 10.1111/sji
25. Yazdanpanah N, Rezaei N. Autoimmune Complications of COVID-19. *J Med Virol* (2022) 94:54–62. doi: 10.1002/jmv.27292
26. Zhou Q, Vadakekolathu J, Watad A, Sharif K, Russell T, Rowe H, et al. SARS-Cov-2 Infection Induces Psoriatic Arthritis Flares and Enthesis Resident Plasmacytoid Dendritic Cell Type-1 Interferon Inhibition by JAK Antagonism Offer Novel Spondyloarthritis Pathogenesis Insights. *Front Immunol* (2021) 12:635018:635018. doi: 10.3389/fimmu.2021.635018
27. Murdaca G, Noberasco G, Olobardi D, Lunardi C, Maule M, Delfino L, et al. Current Take on Systemic Sclerosis Patients' Vaccination Recommendations. *Vaccines* (2021) 9:1426. doi: 10.3390/vaccines9121426
28. Li X, Tong X, Yin Yeung WW, Kuan P, Hin Hei Yum S, Ling Chui CS, et al. Two-Dose COVID-19 Vaccination and Possible Arthritis Flare Among Patients With Rheumatoid Arthritis in Hong Kong Ann. *Rheumatol Dis* (2021). doi: 10.1136/annrheumdis-2021-221571
29. Ursini F, Ruscitti P, Raimondo V, De Angelis R, Cacciapaglia F, Pigatto E, et al. Spectrum of Short-Term Inflammatory Musculoskeletal Manifestations After COVID-19 Vaccine Administration: A Report of 66 Cases Ann. *Rheumatol Dis* (2021) 0:1–2. doi: 10.1136/annrheumdis-2021-221587
30. Connolly CM, Ruddy JA, Boyarsky BJ, Barbur I, Werbel WA, Geetha D, et al. Disease Flare and Reactogenicity in Patients With Rheumatic and Musculoskeletal Diseases Following Two-Dose SARS-Cov-2 Messenger RNA Vaccination. *Arthritis Rheumatol* (2022) 74:28–32. doi: 10.1002/art.41924
31. Terracina KA, Tan FK. Flare of Rheumatoid Arthritis After COVID-19 Vaccination. *Lancet Rheumatol* (2021) 3(7):e469–70. doi: 10.1016/S2665-9913(21)00108-9

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BRIEF RESEARCH REPORT
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# Clinical Management of Patients With B-Cell Depletion Agents to Treat or Prevent Prolonged and Severe SARS-COV-2 Infection: Defining a Treatment Pathway

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**Introduction:** Immunocompromised patients with B-cell depletion agents are at risk for persistence and/or severe SARS-COV-2 infection. We describe a case series of 21 COVID-19 patients under B cell depletion therapy, mostly treated with a combined therapy based on intravenous remdesevir (RDV) and steroid associated with SARS-CoV-2 monoclonal antibodies against Spike glycoprotein and/or hyper-immune convalescent plasma.

**Methods:** This is a single-center longitudinal study. We retrospectively enrolled a total number of 21 B-cell depleted consecutive hospitalized patients with COVID-19 at the Lazzaro Spallanzani National Institute for Infectious Diseases, Rome, Italy, from November 2020 to December 2021. Demographic characteristics, medical history, clinical presentation, treatment, adverse drug reactions, and clinical and virological outcome were collected for all patients. In a subgroup, we explore immune T cells activation, T cells specific anti-SARS-COV-2 response, and neutralizing antibodies.

**Results:** Twenty-one inpatients with B-cell depletion and SARS-COV-2 infection were enrolled. A median of 1 B cells/mm$^3$ was detected. Eighteen patients presented hypogammaglobulinemia. All patients presented interstitial pneumonia treated with intravenous RDV and steroids. Sixteen patients were treated with monoclonal antibodies against SARS-CoV-2 Spike protein, four patients were treated with SARS-CoV-2 hyper-immune convalescent plasma infusion, and three patients received both treatments. A variable kinetic of T cell activation returning to normal levels at Day 30 after immunotherapy infusion was observed. All treated patients recovered.

**Conclusion:** In COVID-19 immunosuppressed subjects, it is mandatory to establish a prompt, effective, and combined multi-target therapy including oxygen, antiviral, steroid, and antibody-based therapeutics, tailored to the patient's clinical needs.

**Keywords: immunosuppressed patients, COVID-19, passive immunotherapy, anti-CD20 agent, B-cells depletion, convalescent plasma, anti-SARS-CoV-2 monoclonal antibody**

## INTRODUCTION

Immunocompromised patients with autoimmune, onco-hematologic, and/or neurologic disorders, with B cell depletion and negative serologic evidence against SARS-CoV-2 after both natural infection and/or SARS-CoV-2 vaccination are at higher risk of severe and/or prolonged COVID-19.

Among these patients anti-CD20 antibody-based B cell-depleting strategies such as rituximab are widely used (1). Upon antigen exposure, B cells can form memory cells or differentiate into plasmablasts and plasma cells. Memory B cells are precursors to antibody-secreting cells, and in addition they can function as professional antigen presenting cells, especially in the context of interactions with T cells that recognize the same antigenic target (2). Treatment with rituximab results in complete B cell-depletion within 72 h from administration, with an estimated recovery timing in 6-9 months after the completion of therapy, and with a return to normal levels observed after 9-12 months (3). These long-lasting therapies are associated with an increased risk of infections such as tuberculosis, hepatitis B virus, herpes virus reactivation, and SARS-CoV-2 infection (4).

The ongoing pandemic is a serious issue for patients treated with anti-CD20 monoclonal or similar biological agents with a COVID-19 mortality rate as high as 60% (5). Indeed, immunocompromised patients could have clinical and virological evidence of persistent SARS-CoV-2 infection of more than 21 days' duration and/or more than 2 episodes of acute respiratory syndrome (6, 7). In this setting, it is important to identify and approach these patients from different perspectives in terms of clinical, diagnostic, and therapeutic options. In the last months, scientific evidence on clinical approaches based on passive immunotherapy has been successfully reported (8). Passive immunotherapy such as hyperimmune convalescent plasma and/or monoclonal antibodies (MoAbs) against SARS-CoV-2 infection represent a source of exogenous specific antibodies in immunocompromised patients with primary or secondary humoral disorders (9–11). To date, there are no available robust data to provide evidence-based protocols on the management of immunosuppressed patients. Here, we describe a case series of 21 COVID-19 patients, under B cell depletion therapy, of whom 20 successfully treated with SARS-CoV-2 MoAbs against Spike glycoprotein and/or hyper-immune convalescent plasma.

## METHODS

In this single-center cross-sectional study, we retrospectively enrolled a total number of 21 immunosuppressed patients consecutively hospitalized with prolonged or relapsing COVID-19 (with >21 days' duration and/or >2 episodes of clinical illness) at the Lazzaro Spallanzani National Institute for Infectious Diseases, Rome, Italy (INMI Spallanzani), from November 2020 to December 2021. Demographic characteristics, medical history, clinical presentation, treatment, adverse drug reactions, and clinical outcome (survival/death) at Day 28 post-treatment were collected for all patients from the clinical record.

In a subgroup of 11 patients the expression of CD38 activation marker on CD4 and CD8 T cells was evaluated by flow cytometry at baseline (T0), after 3 (T3), 7 (T7), 14 (T14), and 30 days (T30) of MoAbs and hyperimmune convalescent plasma. The following gating strategy was used to identify T cells population: CD4+ and CD8+ T cells were identified in CD3+ T cells, gated in SSC-A/CD45+ cells, and within SSC-H/SSC-A in FSC singlets gate.

Spike- and Nucleocapside-specific T cells were quantified by IFN-γ Elispot assay, following the kit's instruction. Positive control peripheral blood mononuclear cells (PBMCs) were stimulated with phytohemagglutinin (PHA) (data not shown). As a negative control, the spontaneous interferon (IFN)-γ release by unstimulated PBMC was quantified.

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed according to the laboratory workflow using different platforms: DiaSorin Simplexa® COVID-19 Direct platform, Abbott m2000 RealTime System, and the Cobas® SARS-CoV-2 Test on the fully automated cobas® 6800 Systems. Viral characterization was performed on nasopharyngeal swab (NPS) samples collected, when possible, by Next-Generation Sequencing (NGS) on Ion Torrent Platform using Ion AmpliSeq SARS-CoV-2 Research Panel, following the manufacturer's instructions

SARS-COV-2 serology was performed by an enzyme-linked immunosorbent assay (ELISA) detecting anti-SARS-CoV-2 IgG, IgM, and IgA (ENZY-WELL SARS-CoV-2 ELISA on SkyLab platform; DIESSE), or by two chemiluminescence microparticle assays (CMIA) detecting anti-nucleoprotein (anti-N) IgG and anti-Spike/RBD IgG (ARCHITECT SARS-CoV-2 IgG, and ARCHITECT SARS-CoV-2 IgG II Quantitative, on ARCHITECT® i2000sr; Abbott Laboratories, Wiesbaden, Germany, respectively). According to the manufacturer's instructions, index values S/CO ≥ 1.1 are considered positive for ELISA, while for the two CMIA, Index >1.4 and Binding Antibody Units (BAU)/mL ≥7.1 are considered positive for anti-N and anti-Spike/RBD IgG, respectively.

Neutralizing antibodies were assessed by micro-neutralization assay (MNA) using live SARS-CoV-2. Briefly, seven twofold serial dilutions (starting dilution 1:10) of heat-inactivated serum samples (56°C for 30 min) were titrated in duplicate, mixed with 100 TCID50 SARS-CoV-2 and incubated at 37°C for 30 min. Subsequently, virus-serum mixtures were added to sub-confluent Vero E6 cells seeded in 96-well microplates and incubated at 37°C, 5% $CO_2$. After 48 h, microplates were observed by light microscope for the presence of cytopathic effect (CPE). Neutralization titers were expressed as the reciprocal of the highest serum dilution inhibiting at least 90% (MNA90) of CPE.

## RESULTS

From November 2020 to December 2021, 21 patients with B-cells depletion and SARS-COV-2 infection were enrolled: 12 men

with a median age of 65 (InterQuartile Range – IQR, 54-71.5) years old. **Tables 1**, **2** show patients' clinical and laboratory features (data are reported in chronological order of admission). Among 21 patients, 15 patients had a hematological disorder, 2 patients had a multiple sclerosis, and 4 patients had an autoimmune disease (2 Wegener granulomatosis, 1 psoriatic arthritis, and 1 rheumatoid arthritis). All study population had a history of immunosuppression (median of 1 B cells/mm3, IQR 0-2 cells): 16 patients for a previous treatment with anti-CD20 agents, 2 patients for a previous treatment with anti-CD38 agents, and 2 for previous standard chemotherapy. Residual hypogammaglobulinemia was observed in 18 patients. Of them, 7 subjects were COVID-19 vaccinated. Viral characterization was performed on nasopharyngeal swab samples collected from 4 of the 21 patients. Two patients tested positive for the Alpha variant, 1 patient for a B.1.177.33 lineage (GV clade) and a B.1.177 lineage (BGV), respectively.

Nine patients had a prolonged COVID-19 infection; the remaining 12 patients had a primary infection. All patients presented interstitial pneumonia treated with intravenous remdesivir (RDV) and steroids. Sixteen patients were treated with MoAbs against SARS-CoV-2 Spike glycoprotein (casirivimab/imdevimab in 14 cases and bamlanivimab/etesevimab in 2 cases), 4 patients were treated with SARS-CoV-2 hyper-immune convalescent plasma infusion (1:320 neutralizing Abs against SARS-CoV-2 Spike glycoprotein), and 3 patients received both treatments. One patient died, he refused both treatments. The median time to hospitalization from the first positive nasopharyngeal swab was 11 (IQR 6.25-59) days, the median SARS-CoV-2 viral shedding was 57.5 (IQR, 36.5-90) days, and the median length of hospitalization was 30 days (IQR 16.25-42.5). Stratifying patients according to treatment, a decreasing trend of length of stay (LS), and time to negative SARS-CoV-2 molecular NPS in MoAbs vs. hyperimmune convalescent plasma treated patients was observed (28.6 vs. 39 days for LS and 50.3 vs. 75.2 days for negative NPS, respectively). Oxygen supports varied according to patient clinical severity but no differences in terms of antiviral and MoAb therapies and of clinical outcome were reported. CD4 and CD8 T cell activation ($CD38^+$) was evaluated in 11 patients: 7 patients were treated with MoAbs, 3 patients with plasma, and 1 patient with both regimens. A variable kinetic of T cell activation returning to normal levels at Day 30 after immunotherapy infusion was shown. Interestingly, in the patient sequentially treated first, with plasma infusion and then with MoAb therapy, a significant early reduction of T cell activation was reported. Therefore, all patients showed a positive Spike-specific T cell response that reached a peak between Day 7 and 14 after treatment and then slowed down. In contrast, the patient treated with both therapeutics had a delayed specific T cell response after MoAb infusion only (**Figure 1**).

## DISCUSSION

Patients receiving B-cell lymphocyte depletion agents have been shown to be particularly vulnerable to COVID-19, having four times higher odds of COVID-19-related case fatality rate compared with patients on other immunosuppressive medication (12). Indeed, this group of patients usually has a prolonged viral clearance in the respiratory tract with more than 21 days duration, several episodes of acute respiratory infectious syndrome and no SARS-CoV-2 Spike glycoprotein or N protein seroconversion even at Day 20 from symptom onset (7, 12–15). Here, we described the clinical, virological, and immunological pictures of 20 patients with B-cell depletion and SARS-CoV-2 pneumonia ranging from mild to severe, of them 9 had a prolonged infection.

All subjects were treated with intravenous RDV plus systemic corticosteroid associated with passive immunotherapy with SARS-CoV-2 MoAbs and/or hyperimmune convalescent plasma. This therapeutic approach was adopted in all patients even in those with primary SARS-COV-2 infection because of the higher risk of prolonged or severe COVID-19. Although RDV and MoAb use is recommended in COVID-19 patients in the early phase of SARS-CoV-2 infection, RDV was administered after Day 10 in 65% of cases, and MoAbs after Day 7 in 80% of cases (16).

Randomized controlled clinical trials on hyperimmune convalescent plasma have shown controversial results but clinical benefit has been recently demonstrated when used early within 72 h from symptoms' onset and with high titers of neutralizing antibodies (17). Nevertheless, in our cohort of immune suppressed patients undergoing anti-CD20 biological agents, antivirals and passive immunotherapy have been used later than in the early phase of the infection as they have been studied, suggesting an additional clinical benefit of this combination strategy even in reducing long lasting viral replication. The use of systemic steroids was balanced in all patients, regardless of their immunosuppressed status. In the subgroup of patients with immunological data, T cells activation levels returned to normal levels at Day 30 from immunotherapy, and it was more frequently observed in MoAb-treated than in plasma-treated patients. In one patient first treated with plasma, T cell activation was reduced after MoAb infusion only. Despite the limit of the small sample size of our cohort, a decreasing trend of both length of stay and time to negative molecular NPS in MoAb-treated vs. plasma-treated patients was observed.

Passive immunotherapy (convalescent plasma and/or MoAbs) against SARS-CoV-2 infection represents a main therapeutic option as a source of exogenous specific antibodies in immunocompromised patients with primary or secondary humoral disorders (18). Although to date there is no therapeutic consensus in immunocompromised patients, anecdotal case series of clinical success with combined anti SARS-COV-2 therapy (10, 11) are consistent with our preliminary data. Passive immunotherapy associated with remdesivir, systemic steroid, and oxygen therapy is a successful and safe strategy in our immunosuppressed COVID-19 cohort in terms of reducing viral load, immunoactivation, and case fatality.

Rapid viral clearance, shorter duration of hospitalization, and full recovery were observed in patients treated in 2021 compared

**TABLE 1** | Study population in chronological order of admission: clinical features.

| Pt | Sex | Age | Ongoing IS therapy | Days from last IS administration | Diseases | Days to hospitalization from symptom onset | Days to hospitalization from first NPS | MoAbs | Hyperimmuneplasma | VS | Lenght of stay | Days to PCR negativization | Clinical Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | 54 | Ocrelizumab | 105 | MS | 20 | 21 | CAS +IMD | No | NONE | 51 | 55 | RECOVERY |
| 2 | M | 54 | Rituximab | 163 | NHL | 31 | 17 | CAS +IMD | No | NIV | 32 | 68 | RECOVERY |
| 3 | M | 39 | Rituximab | >12 y | NHL | 54 | 63 | NO | Yes | NIV | 92 | 85 | RECOVERY |
| 4 | M | 66 | Obinutumab | NA | NHL | 52 | 100 | CAS +IMD | Yes | VM | 19 | 168 | RECOVERY |
| 5 | M | 65 | Rituximab | 60 | NHL | 33 | 33 | NO | Yes | VM | 19 | 50 | RECOVERY |
| 6 | M | 67 | Rituximab | 106 | NHL | 3 | 114 | NO | Yes | VM | 30 | 123 | RECOVERY |
| 7 | M | 59 | Rituximab | 150 | NHL | 120 | 6 | NO | Yes | VM | 15 | 43 | RECOVERY |
| 8 | F | 24 | Rituximab | 76 | WG | 78 | 6 | BAM +ETE | No | VM | 11 | 90 | RECOVERY |
| 9 | F | 25 | Ocrelizumab | 13 | MS | 12 | 78 | CAS +IMD | No | VM | 19 | 38 | RECOVERY |
| 10 | F | 66 | Rituximab | 180 | NHL | 7 | 7 | CAS +IMD | No | c-PAP | 26 | 76 | RECOVERY |
| 11 | F | 77 | Rituximab | 217 | NHL | 10 | 8 | CAS +IMD | Yes | NIV | 51 | 90 | RECOVERY |
| 12 | M | 35 | Rituximab | 126 | PS | 46 | 47 | CAS +IMD | No | c-PAP | 62 | 61 | RECOVERY |
| 13 | M | 54 | Rituximab | 11 y | NHL | 120 | 119 | BAM +ETE | Yes | NIV | 6 | 119 | RECOVERY |
| 14 | F | 68 | Rituximab | 66 | CLL | 36 | 36 | CAS +IMD | No | c-PAP | 44 | 60 | RECOVERY |
| 15 | M | 68 | Rituximab | NA | NHL | 13 | 10 | NO | No | VM | 30 | NA | DEATH |
| 16 | M | 73 | Daratumumab | NA | MM | 2 | 11 | CAS +IMD | No | VM | 38 | 36 | RECOVERY |
| 17 | F | 76 | Rituximab | NA | RA | 9 | 9 | CAS +IMD | No | VM | 11 | 37 | RECOVERY |
| 18 | M | 83 | None | >20 y | NHL | 9 | 9 | CAS +IMD | No | VM | 16 | 31 | RECOVERY |
| 19 | M | 63 | None | 2 y | NHL | 41 | 2 | CAS +IMD | No | VM | 35 | 36 | RECOVERY |
| 20 | F | 77 | Daratumumab | 30 | MM | 3 | 2 | CAS +IMD | No | c-PAP | 30 | 39 | RECOVERY |
| 21 | F | 56 | Rituximab | 14 | WG | 4 | 4 | CAS +IMD | No | VM | 17 | 28 | RECOVERY |

*IS, immunosoppressive; NPS, nasopharyngeal swab; MoAbs, monoclonal antibodies; CAS, casirivimab, IMD, imdevimab; BAM, bamlanivimab; ETE, etesevimab; VS, ventilatory support; MS, multiple sclerosis; NHL, non-Hodgkin's lymphoma; PS, psoriatic arthritis; WG, Wegener granulomatosis; MM, multiple myeloma; VM, venturi mask; c-PAP, continuous positive air pressure; NIV, non-invasive ventilation; IOT, orotracheal intubation; NA, not available.*

**TABLE 2** | Immunological features in the study population.

| Pt | Hypogammaglobulinemia | CD20/ mm^3 | SARS-CoV-2 vaccination | Pre-immunotherapy | | | Post-Immunotherapy | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | IgM/IgA/ IgG | Neutralizing Abs | Anti-Spike/RBD IgG | IgM/IgA/ IgG | Neutralizing Abs | Anti-Spike/RBD IgG |
| 1 | Yes | 1 | None | +/-/- | NA | NEG | -/-/+ | >1:640 | 8897.7 |
| 2 | Yes | 1 | None | -/-/- | NA | NEG | -/+/+ | 1:160 | 2961.1 |
| 3 | Yes | 0 | None | -/-/- | <1:10 | NEG | -/-/- | <1:10 | 181 |
| 4 | No | 1 | None | -/-/- | <1:10 | NEG | -/-/+ | 1:80 | 1159 |
| 5 | Yes | 0 | None | -/-/- | <1:10 | NEG | -/-/- | <1:10 | 67 |
| 6 | Yes | 1 | None | -/-/- | <1:10 | NEG | -/-/- | <1:10 | 197 |
| 7 | Yes | 1 | II Doses | -/-/- | <1:10 | NEG | -/+/+ | NA | NA |
| 8 | Yes | 2 | None | +/-/- | <1:10 | NEG | -/-/+ | >1:640 | >11360 |
| 9 | No | 0 | None | -/-/+ | NA | NA | -/-/+ | >1:640 | >11360 |
| 10 | Yes | 2 | II Doses | -/-/- | <1:10 | NEG | -/-/+ | NA | NA |
| 11 | Yes | 2 | I Dose | -/-/- | <1:10 | NEG | -/-/+ | NA | NA |
| 12 | Yes | 1 | None | -/-/- | <1:10 | 13.7 | -/-/+ | NA | NA |
| 13 | Yes | 24 | None | -/-/- | <1:10 | NEG | -/-/+ | 1:20 | 420.9 |
| 14 | Yes | 0 | II Doses | -/-/- | NA | NA | -/-/+ | NA | NA |
| 15 | Yes | 0 | None | NA | NA | NA | NA | NA | NA |
| 16 | Yes | 0 | None | -/-/- | NA | NEG | -/-/+ | NA | >11360 |
| 17 | NA | 0 | II Doses | -/-/- | NA | NEG | -/-/+ | NA | NA |
| 18 | Yes | 3 | II Doses | -/-/- | <1:10 | NEG | -/-/+ | >1:640 | >11360 |
| 19 | Yes | 24 | None | -/-/- | NA | NA | -/-/+ | NA | NA |
| 20 | Yes | 19 | III Doses | -/-/- | <1:10 | NEG | -/-/+ | NA | >11360 |
| 21 | Yes | 2 | None | -/-/- | NA | NA | -/-/+ | NA | NA |

*Ig, immunoglobulins; Abs, antibodies; NA, not available. Anti-Spike/RBD IgG are expressed as Binding Antibody Units (BAU)/mL, values ≥7.1 are considered positive; neutralizing titers >1:10 are considered positive.*

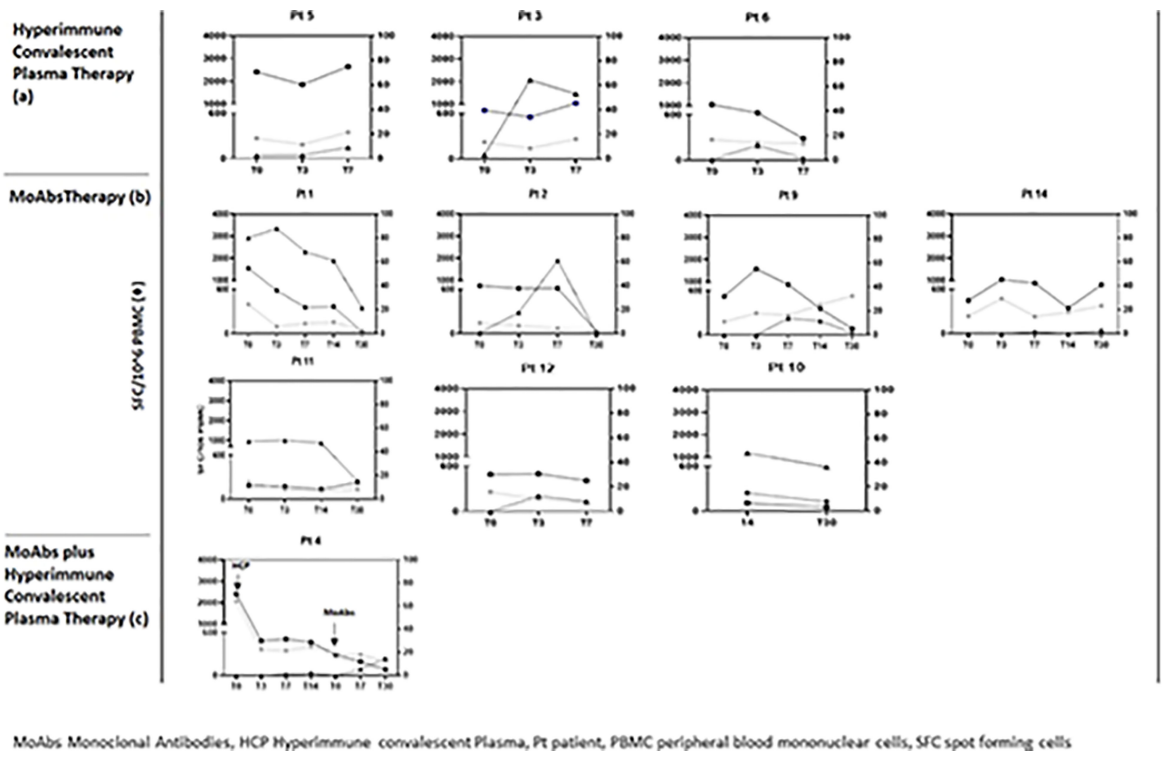


**FIGURE 1** | Impact of hyperimmune convalescent plasma **(A)** anti-SARS-CoV-2 monoclonal antiboby **(B)** and hyperimmune convalescent plasma plus anti-SARS-CoV-2 monoclonal antibody **(C)** therapy on immune parameters and specific T cell response in peripheral blood before and after treatment.

to 2020 when this therapeutic approach was early adopted as a combined drug strategy immediately after hospital admission to prevent SARS-COV-2 persistence.

Limitations to MoAb use are related to its limited availability, currently almost exclusive of patients from high-income countries, to its high cost, to the time required for regulatory approval for routine clinical use, and to drug procurement difficulties during Covid-19 epidemic peaks. Finally, susceptibility to MoAb therapies may be reduced because of changes in circulating variants of concern (19).

A likely alternative to MoAb is the hyperimmune convalescent plasma, which is available in low- and middle-income countries without patent restrictions and at relatively low cost. Hyperimmune convalescent plasma provides a diverse mixture of antibodies with different specificities and functions and should be less vulnerable to the emergence of antibody resistance and viral variants (19). Finally, underlying medical conditions (onco-hematologic malignancies, neurologic and autoimmune disorders), disease activity status, and concomitant/previous treatments that affect immune cell distribution and function are all factors to be considered in the choice of specific treatment for selected categories of COVID-19 patients. In COVID-19 immunosuppressed subjects, it is mandatory to establish a prompt, effective, safe, and combined multi-target therapy including oxygen, antiviral, and steroid and antibody-based therapeutics, tailored to the patient's clinical needs.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

Ethical 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.

## SPALLANZANI COVID-19 CASE INVESTIGATION TEAM

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Conceptualization, AD'A, SV, GM, and EN; Data curation, AB and AC; Funding acquisition, EN; Investigation, AD'A, SV, GM, and ML; Experiments, EC, FC, and CA; Supervision, EN and CA; Validation, EN; Writing—original draft, AD'A, SV, and EN; Writing—review and editing, CA, FC, EC, ML, and EN. All authors contributed to the article and approved the submitted version.

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## REFERENCES

1. Lee DSW, Rojas OL, Gommerman JL. B Cell Depletion Therapies in Autoimmune Disease: Advances and Mechanistic Insights. *Nat Rev Drug Discov* (2020) 20:179–99. doi: 10.1038/s41573-020-00092-2
2. Rodríguez-Pinto D. B Cells as Antigen Presenting Cells. *Cell Immunol* (2005) 238:67–75. doi: 10.1016/j.cellimm.2006.02.005
3. McLaughlin P, Grillo-López AJ, Link BK, Levy R, Czuczman MS, Williams ME, et al. Rituximab Chimeric Anti-CD20 Monoclonal Antibody Therapy for Relapsed Indolent Lymphoma: Half of Patients Respond to a Four-Dose Treatment Program. *JCO* (1998) 16:2825–33. doi: 10.1200/jco.1998.16.8.2825
4. Cantini F, Niccoli L, Capone A, Petrone L, Goletti D. Risk of Tuberculosis Reactivation Associated With Traditional Disease Modifying Anti-Rheumatic Drugs and Non-Anti-Tumor Necrosis Factor Biologics in Patients With Rheumatic Disorders and Suggestion for Clinical Practice. *Expert Opin Drug Saf* (2019) 18:415–25. doi: 10.1080/14740338.2019.1612872
5. Passamonti F, Cattaneo C, Arcaini L, Bruna R, Cavo M, Merli F, et al. Clinical Characteristics and Risk Factors Associated With COVID-19 Severity in Patients With Haematological Malignancies in Italy: A Retrospective, Multicentre, Cohort Study. *Lancet Haematol* (2020) 7:e737–45. doi: 10.1016/s2352-3026(20)30251-9
6. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal Dynamics in Viral Shedding and Transmissibility of COVID-19. *Nat Med* (2020) 26:672–5. doi: 10.1038/s41591-020-0869-5
7. Li N, Wang X, Lv T. Prolonged SARS-CoV-2 RNA Shedding: Not a Rare Phenomenon. *J Med Virol* (2020) 92:2286–7. doi: 10.1002/jmv.25952
8. Franchini M, Corsini F, Focosi D, Cruciani M. Safety and Efficacy of Convalescent Plasma in COVID-19: An Overview of Systematic Reviews. *Diagnostics* (2021) 11:1663. doi: 10.3390/diagnostics11091663
9. Focosi D, Franchini M. Potential Use of Convalescent Plasma for SARS-CoV-2 Prophylaxis and Treatment in Immunocompromised and Vulnerable Populations. *Expert Rev Vaccines* (2021), 1–8. doi: 10.1080/14760584.2021.1932475
10. D'Abramo A, Vita S, Maffongelli G, Mariano A, Agrati C, Castilletti C, et al. Prolonged and Severe SARS-CoV-2 Infection in Patients Under B-Cell-Depleting Drug Successfully Treated: A Tailored Approach. *Int J Infect Dis* (2021) 107:247–50. doi: 10.1016/j.ijid.2021.04.068
11. Brown L-AK, Moran E, Goodman A, Baxendale H, Bermingham W, Buckland M, et al. Treatment of Chronic or Relapsing COVID-19 in Immunodeficiency. *J Allergy Clin Immunol* (2022) 149:557–561.e1. doi: 10.1016/j.jaci.2021.10.031
12. Moor MB, Suter-Riniker F, Horn MP, Aeberli D, Amsler J, Möller B, et al. Humoral and Cellular Responses to mRNA Vaccines Against SARS-CoV-2 in Patients With a History of CD20 B-Cell-Depleting Therapy (RituxiVac): An Investigator-Initiated, Single-Centre, Open-Label Study. *Lancet Rheumatol* (2021) 3:e789–97. doi: 10.1016/s2665-9913(21)00251-4
13. He W, Chen L, Chen L, Yuan G, Fang Y, Chen W, et al. COVID-19 in Persons With Haematological Cancers. *Leukemia* (2020) 34:1637–45. doi: 10.1038/s41375-020-0836-7

14. Mehta P, Porter JC, Chambers RC, Isenberg DA, Reddy V. B-Cell Depletion With Rituximab in the COVID-19 Pandemic: Where do We Stand? *Lancet Rheumatol* (2020) 2:e589–90. doi: 10.1016/s2665-9913(20)30270-8
15. Hueso T, Pouderoux C, Péré H, Beaumont A-L, Raillon L-A, Ader F, et al. Convalescent Plasma Therapy for B-Cell–Depleted Patients With Protracted COVID-19. *Blood* (2020) 136:2290–5. doi: 10.1182/blood.2020008423
16. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the Treatment of Covid-19 — Final Report. *N Engl J Med* (2020) 383:1813–26. doi: 10.1056/nejmoa2007764
17. Sullivan DJ, Gebo KA, Shoham S, Bloch EM, Lau B, Shenoy AG, et al. Early Outpatient Treatment for Covid-19 With Convalescent Plasma. *N Engl J Med* (2022). doi: 10.1056/nejmoa2119657
18. Hammarström L, Abolhassani H, Baldanti F, Marcotte H, Pan-Hammarström Q. Development of Passive Immunity Against SARS-CoV-2 for Management of Immunodeficient Patients—a Perspective. *J Allergy Clin Immunol* (2020) 146:58–60. doi: 10.1016/j.jaci.2020.04.043
19. Focosi D, Maggi F, Franchini M, McConnell S, Casadevall A. Analysis of Immune Escape Variants From Antibody-Based Therapeutics Against COVID-19: A Systematic Review. *IJMS* (2021) 23:29. doi: 10.3390/ijms23010029

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CASE REPORT
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# Case Report: New-Onset Rheumatoid Arthritis Following COVID-19 Vaccination

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Efficient protection against coronavirus disease 2019 (COVID-19) has been achieved by immunization with mRNA-based vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, efficient immune responses against this novel virus by vaccination are accompanied by a wide variety of side effects. Indeed, flares or new-onset of autoimmune disorders have been reported soon after the COVID-19 vaccination. Although pro-inflammatory cytokine responses play pathogenic roles in the development of autoimmunity, cytokines charactering COVID-19 vaccination-related autoimmune responses have been poorly understood. Given that mRNA derived from COVID-19 vaccine is a potent inducer for pro-inflammatory cytokine responses, these cytokines might mediate autoimmune responses after COVID-19 vaccination. Here we report a case with new-onset rheumatoid arthritis (RA) following COVID-19 vaccination. Serum concentrations not only of arthrogenic cytokines, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), but also of type I interferon (IFN) were elevated at the active phase in this case. Induction of remission by methotrexate and tocilizumab was accompanied by a marked reduction in serum concentrations of type I IFN, IL-6, and TNF-α. These results suggest that production of type I IFN, IL-6, and TNF-α induced by COVID-19 vaccination might be involved in this case with new-onset RA.

**Keywords: rheumatoid arthritis, COVID-19 vaccination, type I IFN, IL-6, TNF-alpha**

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## INTRODUCTION

Coronavirus disease 2019 (COVID-19) sometimes causes autoimmunity (1). Molecular mimicry has been considered to be involved in the development of autoimmunity due to cross-reactivity of a COVID-19 spike protein to human antigens (1). Given that BNT162b2 (BioNTech-Pfizer) is a mRNA-based vaccine expressing the spike protein, it is possible that BNT162b2 vaccination can be a trigger for the development of autoimmunity (2–4). In fact, new-onset or flares of autoimmune disorders including rheumatoid arthritis (RA), adult-onset Still's disease, inflammatory bowel diseases, and anti-neutrophil cytoplasmic antibody-associated vasculitis have been observed soon after the COVID-19 vaccination (5–9). Immunopathogenesis underlying COVID-19 vaccination-related autoimmunity has not been fully understood. In this regard, sensing of double-stranded and single-stranded RNA derived from the mRNA-based vaccine by toll-like receptors (TLRs), retinoic

acid inducible gene-I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) can be potent triggers for production of type I interferons (IFN-I) and pro-inflammatory mediators (2–4). Therefore, it is likely that excessive production of IFN-I and pro-inflammatory cytokines is involved in the development of autoimmune responses associated with COVID-19 vaccination. However, cytokines causing autoimmunity have not been identified in such cases. Here, we report a case with new-onset RA following COVID-19 vaccination. In this case, serum and synovial fluid concentrations of interleukin (IL)-6, tumor necrosis factor-α (TNF-α), and IFN-I were elevated, suggesting that pro-inflammatory cytokine responses triggered by COVID-19 vaccination might be involved in the development of *de novo* RA.

## CASE REPORT

A 53-year-old healthy Japanese man received BNT162b2 vaccination twice. His grandmother had RA. Successful immunization was verified by a marked elevation of anti-COVID-19-specific Ab titer (510 U/mL, normal range <15). Four weeks after the final vaccination, his left knee joint became swollen and painful. Soon after, he noticed bilateral omalgia and morning stiffness. Blood examination revealed marked leukocytosis (white blood cell, WBC count; 14,600/μL, normal range; 4,000~9,000) and elevated levels of C-reactive protein (CRP 8.45 mg/dL, normal range; 0~0.60). WBC counts and CRP levels were normal before the second vaccination (**Figure 1A**). His serum levels of anti-cyclic citrullinated peptide antibody and rheumatoid factor were 1,200 U/mL (normal range; 0~4.4) and 51 U/mL (normal range; 0~15), respectively. Magnetic resonance imaging showed diffuse knee effusion. Based on these typical symptoms and serological analyses, he was diagnosed as RA.

Initial treatment with methotrexate (MTX, 3 mg/day) with an escalating dose schedule (1 mg/2 weeks) and prednisolone (5 mg/day) failed to induce remission. PSL was switch to subcutaneous injection of etanercept (50 mg/week), which was again unsuccessful for the induction of remission. MTX (6 mg/day) in combination with subcutaneous injection of tocilizumab (162 mg/week) normalized serum concentration of CRP and WBC count (**Figure 1A**). Around one month after the injection of tocilizumab, induction of remission was achieved and bilateral omalgia, swollen of the left knee, and morning stiffness disappeared at this time point. He was treated with MTX and tocilizumab (162 mg/every two weeks) and maintained complete remission for more than ten months without adverse events.

## METHODS

Concentrations of IFN-α, IFN-β, IL-6, and TNF-α were measured using enzyme-linked immunosorbent assay kits from R&D systems. Serum samples were obtained at the active and remitted phases whereas joint fluid samples were taken at the active phase.

## RESULTS

One major question arising from this case is whether BNT162b2 vaccination promoted the development of RA. Pro-inflammatory

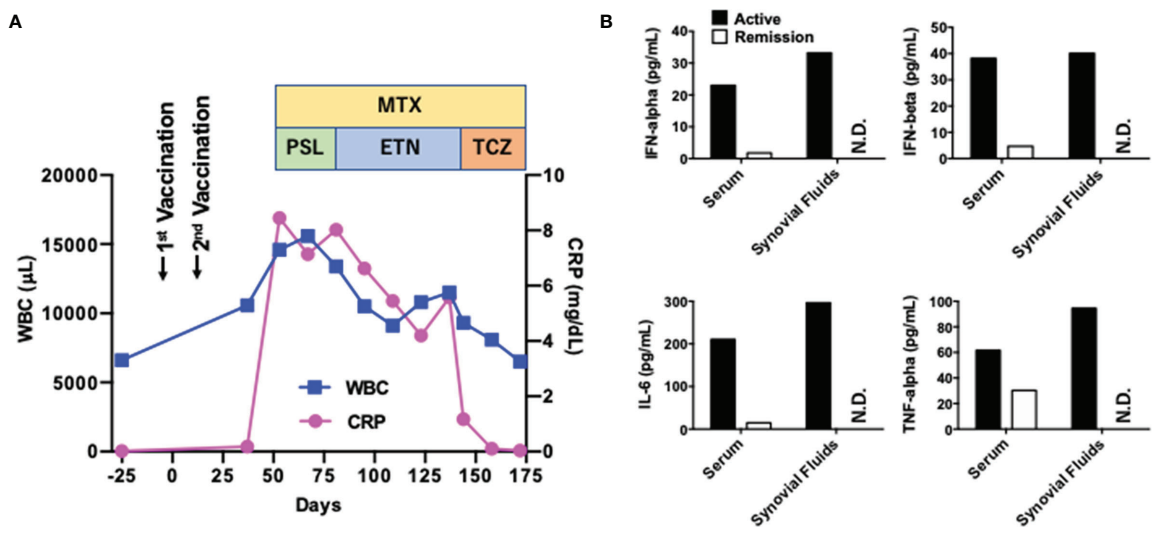


**FIGURE 1** | Clinical course of a patient with rheumatoid arthritis exhibiting systemic pro-inflammatory cytokine responses. **(A)** Clinical course. The first vaccination day was defined as Day 0. WBC, white blood cell; CRP, C-reactive protein; MTX, methotrexate; PSL, prednisolone; ETN, etanercept; TCZ, tocilizumab. **(B)** Serum concentrations of cytokines at the active (Day 53) and remitted (Day 172) phases. Serum concentrations of IL-6, TNF-α, IFN-α, and IFN-β at the active and remitted phases are shown. Synovial fluids were obtained at the active phase alone. N.D., not done.

cytokines such as IL-6 and TNF-α underlie the immunopathogenesis of RA whereas the IFN-I responses are not so prominent in RA (10). mRNA derived from BNT162b2 can cause strong IFN-I responses through sensing by TLRs, RIG-I, and MDA5 (2–4). Therefore, it is possible that not only IL-6 and TNF-α but also IFN-I is involved in the development of RA in this case. As expected, serum concentrations of TNF-α and IL-6 were markedly reduced after treatment with MTX and tocilizumab (**Figure 1B**). Interestingly, serum concentrations of IFN-α and IFN-β were also high before the treatment and declined at the remission phase (**Figure 1B**). In addition, synovial fluid obtained from the left knee before the treatment contained high levels of IFN-I as well as IL-6. Thus, this new-onset RA after BNT162b2 vaccination was characterized by IFN-I as well as IL-6 and TNF-α.

## DISCUSSION

Although mRNA-based COVID-19 vaccines are very effective for the protection against SARS-CoV-2 infection, the induction of efficient immune responses is concomitant with a wide variety of side effects (2–4). New-onset or exacerbation of autoimmune disorders has been reported in patients soon after COVID-19 vaccination (5–9). Given that pro-inflammatory cytokine responses play crucial roles in the development of autoimmune disorders, it is likely that COVID-19 vaccination can be a trigger for production of pro-inflammatory cytokines. Different from conventional vaccines, mRNA-based COVID-19 vaccines do not contain adjuvants with potent immuno-stimulatory functions (2–4). In this regards, recognition of mRNAs derived from COVID-19 vaccine by TLRs, RIG-I, and MDA5 may induce pro-inflammatory cytokines, including IFN-I (2–4). However, molecular mechanisms accounting for the development of autoimmunity following COVID-19 vaccination have not been fully understood. Here we show a case of *de novo* RA following COVID-19 vaccination and characterized by systemic pro-inflammatory cytokine responses including IFN-I, IL-6, and TNF-α. Although new-onset or exacerbation of autoimmune disorders have been reported in patients soon after COVID-19 vaccination (5–9), little information was available regarding pro-inflammatory cytokine responses in such cases.

Although IL-6 and TNF-α are well-established arthrogenic cytokines, roles of IFN-I have been poorly defined. The IFN-I signature is less conspicuous in RA than in the other IFN-I-dependent autoimmunity such as systemic lupus erythematosus (11–13). Thus, this case was atypical in that IFN-I responses as well as prototypical pro-inflammatory cytokine responses were parallel to disease activities. In this regard, we speculate that BNT162b2 vaccination could be a trigger for RA development. This notion is supported by the fact that mRNA derived from BNT162b2 is a potent inducer of IFN-I responses through activation of TLRs, RIG-I, and MDA5 (2, 3). Consistent with high concentrations of IFN-I in the synovial fluid of this case, Lande et al. confirmed the presence of IFN-I in the synovial fluid of patients with RA (14). Taken together, this case suggests that initial IFN-I responses induced by BNT162b2 vaccination might trigger arthrogenic cytokine responses accounting for the development of typical RA *via* induction of IL-6 and TNF-α. This idea is fully supported by the fact that IFN-I can function as upstream cytokines with the ability to induce production of prototypical inflammatory cytokines such as IL-6 and TNF-α (15). However, we cannot exclude the possibility that the timing of RA development with regard to vaccination was coincidental. Moreover, this patient was successfully treated by the blockade of IL-6, which suggests predominant roles of this cytokine. Future studies performing large-scale epidemiological analyses in new-onset or flare RA patients following COVID-19 vaccination are required to establish its link. It would be intriguing to examine whether IFN-I as well as arthrogenic cytokine (IL-6 and TNF-α) responses are elevated in COVID-19 vaccination-associated RA.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kindai University Faculty of Medicine. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

TW and KM wrote the manuscript draft and measured concentrations of cytokines. TW, KM, AH, TY, KK, and MK revised the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES


1. Ehrenfeld M, Tincani A, Andreoli L, Cattalini M, Greenbaum A, Kanduc D, et al. Covid-19 and Autoimmunity. *Autoimmun Rev* (2020) 19(8):102597. doi: 10.1016/j.autrev.2020.102597
2. Teijaro JR, Farber DL. COVID-19 Vaccines: Modes of Immune Activation and Future Challenges. *Nat Rev Immunol* (2021) 21(4):195–7. doi: 10.1038/s41577-021-00526-x
3. Sprent J, King C. COVID-19 Vaccine Side Effects: The Positives About Feeling Bad. *Sci Immunol* (2021) 6(60). doi: 10.1126/sciimmunol.abj9256
4. Sahin U, Kariko K, Tureci O. mRNA-Based Therapeutics–Developing a New Class of Drugs. *Nat Rev Drug Discovery* (2014) 13(10):759–80. doi: 10.1038/nrd4278
5. Hakroush S, Tampe B. Case Report: ANCA-Associated Vasculitis Presenting With Rhabdomyolysis and Pauci-Immune Crescentic Glomerulonephritis After Pfizer-BioNTech COVID-19 mRNA Vaccination. *Front Immunol* (2021) 12:762006. doi: 10.3389/fimmu.2021.762006
6. Leone F, Cerasuolo PG, Bosello SL, Verardi L, Fiori E, Cocciolillo F, et al. Adult-Onset Still's Disease Following COVID-19 Vaccination. *Lancet Rheumatol* (2021) 3(10):e678–80. doi: 10.1016/S2665-9913(21)00218-6
7. Weaver KN, Zhang X, Dai X, Watkins R, Adler J, Dubinsky MC, et al. Impact of SARS-CoV-2 Vaccination on Inflammatory Bowel Disease Activity and Development of Vaccine-Related Adverse Events: Results From PREVENT-COVID. *Inflamm Bowel Dis* (2021). doi: 10.1093/ibd/izab302
8. Sangha M, Roitman I, Sultan K, Swaminath A. SARS-CoV-2 Immunization in Patients With Inflammatory Bowel Disease May Result in Disease Flares. *Am J Gastroenterol* (2021) 116(12):2480–1. doi: 10.14309/ajg.0000000000001416
9. Terracina KA, Tan FK. Flare of Rheumatoid Arthritis After COVID-19 Vaccination. *Lancet Rheumatol* (2021) 3(7):e469–70. doi: 10.1016/S2665-9913(21)00108-9
10. McInnes IB, Schett G. Cytokines in the Pathogenesis of Rheumatoid Arthritis. *Nat Rev Immunol* (2007) 7(6):429–42. doi: 10.1038/nri2094
11. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients With Systemic Lupus Erythematosus, Myositis, Rheumatoid Arthritis and Scleroderma Share Activation of a Common Type I Interferon Pathway. *Ann Rheum Dis* (2011) 70(11):2029–36. doi: 10.1136/ard.2011.150326
12. Watanabe T, Minaga K, Kamata K, Kudo M, Strober W. Mechanistic Insights Into Autoimmune Pancreatitis and IgG4-Related Disease. *Trends Immunol* (2018) 39(11):874–89. doi: 10.1016/j.it.2018.09.005
13. Chasset F, Dayer JM, Chizzolini C. Type I Interferons in Systemic Autoimmune Diseases: Distinguishing Between Afferent and Efferent Functions for Precision Medicine and Individualized Treatment. *Front Pharmacol* (2021) 12:633821. doi: 10.3389/fphar.2021.633821
14. Lande R, Giacomini E, Serafini B, Rosicarelli B, Sebastiani GD, Minisola G, et al. Characterization and Recruitment of Plasmacytoid Dendritic Cells in Synovial Fluid and Tissue of Patients With Chronic Inflammatory Arthritis. *J Immunol* (2004) 173(4):2815–24. doi: 10.4049/jimmunol.173.4.2815
15. Watanabe T, Sadakane Y, Yagama N, Sakurai T, Ezoe H, Kudo M, et al. Nucleotide-Binding Oligomerization Domain 1 Acts in Concert With the Cholecystokinin Receptor Agonist, Cerulein, to Induce IL-33-Dependent Chronic Pancreatitis. *Mucosal Immunol* (2016) 9(5):1234–49. doi: 10.1038/mi.2015.144


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# Assessment of disease activity in patients with rheumatoid arthritis using plasma tumour M2-pyruvate kinase test

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**Background:** Pyruvate kinase M2 (PKM2) is an enzyme that regulates the final process of glycolysis and exists in tetrameric and dimeric forms. The dimeric form of PKM2, also known as tumour M2-PK, increases when aerobic glycolysis is augmented, a feature observed in rheumatoid arthritis (RA). We investigated whether plasma tumour M2-PK is elevated in patients with RA and whether its levels correlate with disease activity.

**Methods:** Plasma levels of tumour M2-PK were measured for patients with RA (n=151), those with osteoarthritis (OA) (n=37), and controls (n=37). We evaluated the association between plasma tumour M2-PK and continuous variables using Pearson's correlation analysis, and multivariate logistic regression analysis to determine the association between plasma tumour M2-PK and disease activity status. Knee synovial tissue blocks from patients with RA and OA were subjected to real-time quantitative PCR (qPCR) using two different primers for PKM2 and tumour M2-PK immunohistochemical (IHC) staining.

**Results:** The tumour M2-PK level significantly correlated with the disease activity score in 28 joints (DAS28)-erythrocyte sedimentation rate (ESR) ($r=0.546$, $p<0.001$) and DAS28-C-reactive protein (CRP) ($r=0.589$, $p<0.001$). Moreover, repeat testing of tumour M2-PK levels in 20 patients revealed a significant decline in tumour M2-PK levels after reduction in inflammation ($p<0.001$). Area under the receiver operating characteristic curve (AUROC) analysis demonstrated that upon incorporation of tumour M2-PK, ESR, and CRP, the area under the curve was 0.962 for distinguishing moderate/high from remission/low disease activity. Adjusted logistic regression also revealed that a tumour M2-PK >43.9 U/mL (OR 3.672, $p=0.042$) independently predicted moderate/high disease activity status. Furthermore, tumour M2-PK levels in patients with RA were significantly higher than in those with OA and controls (all $p<0.001$). However, no differences were found in PKM2 expression in RA and OA synovial tissues as assessed by qPCR, and IHC analysis revealed negligible tumour M2-PK expression in the synovial tissues.

**Conclusion:** Circulating plasma tumour M2-PK levels may be a clinically useful indicator for evaluating disease activity and RA diagnosis.

KEYWORDS

tumour M2-pyruvate kinase, rheumatoid arthritis, disease activity, predictor, biomarker

# Introduction

Pyruvate kinase (PK) regulates the final step of glycolysis and is responsible for the production of adenosine triphosphate, a crucial source of energy for maintaining cellular activity (1). In mammals, PK is encoded by two genes, PKLR and PKM, and four different isoenzymes (M1, M2, L, and R) are known to exist (2). Among these enzymes, isoenzyme M2 (PKM2) is overexpressed in tumour, embryonic, and actively proliferating cells. PKM2 exists in tetrameric and dimeric forms; the tetrameric form of PKM2 exhibits high affinity for its substrate, phosphoenolpyruvate (PEP), possessing a high activity under physiological PEP concentrations. Therefore, the tetrameric form of PKM2 plays a key role in efficiently converting glucose into pyruvate for glucose utilisation. On the other hand, the dimeric form of PKM2 has a low affinity for PEP, and thus, usually remains inactive under physiological conditions (3).

In tumour cells, intermediate products in the early stages of the glycolytic pathway are metabolised through alternative mechanisms to obtain energy to synthesise nucleic acids, phospholipids, and amino acids, which are important components required for cellular function (4). During this process, PKM2 is converted into a dimeric form as a consequence of post-translational modifications by various tumour proteins; thus, the dimeric form of PKM2 is called tumour M2-PK (5, 6). Previous studies have shown that tumour M2-PK increases in the peripheral circulation of patients with various types of cancers (7). In particular, there is evidence indicating that tumour M2-PK is directly related to tumorigenesis, and its inhibition could have therapeutically beneficial effects against cancers (8). The incorporation of blood tests for tumour M2-PK and conventional tumour markers has been suggested to improve the detection of certain types of cancers (9, 10). Furthermore, the clinical utility of assessing tumour M2-PK level is not only for diagnosis, but it may also serve as a prognostic indicator for determining treatment response, predicting survival, and disease recurrence (11–13). The precise cause of the increase in the dimeric form of PKM2 in tumour cells is still unclear. However, unlike normal cells, it is presumed that this phenomenon could be related to a unique feature of tumour cells, which generate energy by aerobic glycolysis instead of oxidative phosphorylation (14).

Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation of the synovial membrane. Uncontrolled chronic inflammation is responsible for the development of irreversible joint deformities (15). In RA, the inflamed synovium contains fibroblast-like synoviocytes (FLSs) and inflammatory cells such as lymphocytes, neutrophils, and macrophages (16). The accumulation and activation of these cells results in the formation of a pannus, which displays a tumour-like phenotype of excessive inflammatory cell accumulation and angiogenesis (17). In fact, a shift toward glycolysis, a characteristic property of cancer cells, has also been identified in FLS from patients with RA and in the synovial tissue of experimental RA (18–20). Therefore, it could be hypothesised that tumour M2-PK, which increases with higher energy requirement, is increased in patients with RA. Based on these shared features, this study was designed to evaluate whether plasma tumour M2-PK level is i) associated with disease activity and blood levels of acute phase reactants such as ESR and CRP, which are the most widely used in clinical care, and ii) higher in patients with RA than in those with osteoarthritis (OA) and controls.

# Materials and methods

## Patients with RA, osteoarthritis, and controls

Plasma samples were collected from 151 patients with RA who had undergone routine laboratory tests in the Department of Rheumatology, Severance Hospital between March 2016 and December 2017. For sample handling, blood collected in EDTA tubes were immediately centrifuged and subsequently stored in a -70°C freezer for enzyme-linked immunosorbent assay (ELISA) experiments. The patients included met the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (21), and none of the patients had malignancies (either solid or haematologic) or active infections at the time of blood collection. Tumour M2-PK levels in the plasma of 37 patients with OA and 37 controls were compared with those of patients with RA. This study was approved by the Severance Hospital's Institutional Review Board (4-2017-0761), and the study was conducted keeping

with the principles of the 1964 Helsinki Declaration and comparable ethical standards.

## Patient and laboratory data

Patient and laboratory data were acquired on the date of obtaining plasma samples from the patients. Patient demographics included age, sex, seropositivity (rheumatoid factor and/or anti-cyclic citrullinated peptide positivity), the presence of interstitial lung disease (ILD), and disease duration. Disease activity measures included the following disease activity scores: disease activity score in 28 joints (DAS28)-erythrocyte sedimentation rate (DAS28-ESR) and DAS28-C-reactive protein (DAS28-CRP) (22, 23); investigated medications that were used for the treatment of RA were glucocorticoids, conventional synthetic (cs) disease-modifying anti-rheumatic drugs (DMARDs) (methotrexate, leflunomide, sulfasalazine, hydroxychloroquine, and tacrolimus), and biologic DMARDs (bDMARDs) (etanercept, adalimumab, golimumab, infliximab, abatacept, and tocilizumab) (24, 25). Laboratory data included white blood cell, platelet, neutrophil, and lymphocyte counts; erythrocyte sedimentation rate (ESR); and C-reactive protein (CRP), alkaline phosphatase (ALP), aspartate aminotransferase, and alanine aminotransferase levels. Those who were newly diagnosed as RA and was treatment naïve were defined as having new-onset disease, and patients with DAS28-ESR $\geq$ 3.2 were considered to have moderate/high disease activity, as previously described (26). We defined patients with DAS28-ESR < 3.2 as having remission/low disease activity.

## ELISA for assessment of tumour M2-PK, TNF-α, and IL-6 levels in patients with RA

A commercial ELISA kit (ScheBo Biotech AG, Giessen, Germany) was used to measure plasma M2-PK levels in stored patient samples. In addition, TNF-α (R&D Systems, Minneapolis, MN, USA) and IL-6 (R&D Systems) were evaluated in identical patient samples. The relevant experimental procedures were performed in accordance with the manufacturer's instructions.

Serial plasma samples of 20 patients with RA who had moderate/high disease activity initially and had remission/low disease activity at follow-up, with a time interval of more than three months, were used to assess changes of tumour M2-PK following the reduction of disease activity.

## Polymerase chain reaction and immunohistochemistry

Total RNA was extracted from 10 knee synovial tissue blocks from patients with RA and OA undergoing total knee replacement surgery using Trizol reagent. Next, a reverse transcription kit (TaKaRa Bio Inc., Kusatsu, Japan) was used for cDNA synthesis, and real-time quantitative PCR (qRT-PCR) analysis was performed using TB Green qPCR Mix (TaKaRa Bio Inc., Kusatsu, Japan) according to the manufacturer's protocol. We used two different primer sequences of PKM2 for qRT-PCR: (i) forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'; and ii) forward: 5'-CCACTTGCAATTATTTGAGGAA-3', reverse 5'-GTGAGCAGACCTGCCAGACT-3). GAPDH was selected as a housekeeping gene (forward: 5'-GGACTGAGGCTCCCACCTTT-3' and reverse: 5'-CCTGCAGCGTACTCCCCACA-3'). The relative PKM2 mRNA expression was calculated using the 2–ΔΔCT method and normalised to that of GAPDH.

Identical tissue samples were subjected to immunohistochemical (IHC) staining using a commercial antibody against tumour M2-PK (MyBioSource Inc., San Diego, CA, USA). Formalin-fixed, paraffin-embedded tissue sections were de-paraffinised and rehydrated, and immunohistochemistry was performed according to the manufacturer's instructions.

## Isolation and culture of FLS from synovial fluid

Synovial fluid was obtained from patients with rheumatoid arthritis (n=3) having knee joint swelling by joint aspiration, and was diluted in PBS (1:9), centrifuged at 1500 rpm for 10 minutes. After removing the supernatant, cell pellet was resuspended to complete Dulbecco's modified Eagle's medium (DMEM) (10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin), placed in T25 flask, and incubated in 5% $CO_2$ incubator at 37°C. When cell confluency was reached 80~90%, adherent cells were separated, neutralized in complete DMEM, and was spin-downed. Cell pellet was then resuspended for the next passage expansion. The media used for cell culture was changed every 3-4 days (27), and cells in the passage of 3-5 were used for all experiments. Cells used for the experiments were confirmed as FLS by flow cytometry, which was determined as positive for fibroblast marker of CD44 and CD90 and negative for macrophage marker of CD14 (all from BD Biosciences, San Diego, CA, USA) (28–30).

## Stimulation of FLS and cytokine measurement

A total of $1.5\times10^5$ cells/well were seeded in a six-well plate, and was cultured with complete DMEM provided with TNF-α, IL-1β (100unit) (Peprotech, Cranbury, NJ, USA), and a PKM2 activator TEPP-46 (10μM) (Cayman Chemical, Ann Arbor, MI,

USA) for 72 hours. TEPP-46 induces the tetramerization of PKM2 and inhibits glycolysis and inflammation (31, 32). Following the end of the culture, the supernatant was collected in 15mL tube and was centrifuged at 1500 rpm for 5 minutes. Cytokines were measured using commercial enzyme-linked immunosorbent assay kits of TNF-α, IL-1β, IFN-γ (Abcam, Cambridge, UK), and IL-6 (Boditech, Chuncheon, South Korea) in the supernatant by duplicate experiments, which was performed according to the manufacturer's instructions.

## Statistical analysis

Continuous data are shown as medians (interquartile ranges) and categorical variables as numbers (percentages). Pearson's correlation analysis was conducted for the analysis of the association between tumour M2-PK levels and continuous variables. Statistical differences between continuous variables were assessed using the Mann–Whitney U test or student's t-test for two groups or by the Kruskal–Wallis test or Analysis of Variance when the groups were more than three, as indicated. Longitudinal changes in plasma tumour M2-PK levels were estimated by a Wilcoxon signed rank test, and derivation of the optimal cut-off of ESR, CRP, and tumour M2-PK was performed using the receiver operating characteristic (ROC) curve analysis. Statistically significant variables in the univariate analysis were subsequently included in the multivariate logistic regression analysis for predicting moderate/high disease activity by applying a forward method. In all statistical analyses, a two-tailed p-value of <0.05 was regarded as significant. Statistical analyses were performed using the MedCalc statistical software version 20.009 (MedCalc Software, Ostend, Belgium).

# Results

## Baseline patient characteristics

The characteristics of the patients are shown in Table 1. The median age of the patients was 57.0 of which 118 (78.1%) were women. Patients with seropositive RA accounted for 92.1% of the patients, and ILD was present in 11 patients (7.3%). The median disease duration, DAS28-ESR, and DAS28-CRP level were 61.7 months, 3.2, and 2.3, respectively. For the treatment of patients, 76 (50.3%) patients were undergoing treatment with glucocorticoids, whereas 115 and 56 patients were undergoing treatment with csDMARDs (76.2%) and bDMARDs (37.1%), respectively. The median ESR and CRP values were 36.0 mm/hr and 2.5 mg/L, respectively; the median TNF-α, IL-6, and tumour M2-PK levels were 10.7, 9.1, and 42.9 U/mL, respectively.

## Association between tumour M2-PK and disease activity or laboratory data

The tumour M2-PK level was significantly associated with the disease activity measures, DAS28-ESR (r=0.546, 95% confidence interval [CI] 0.423–0.649, p<0.001) and DAS28-CRP (r=0.589, 95% CI 0.474–0.684, p<0.001). In addition, the associations between tumour M2-PK and IL-6, ESR, and CRP were significant; however, there was no association between it and TNF-α (Figure 1). Similarly, in patients undergoing DMARD treatment (n=121), a significant relationship was observed between tumour M2-PK level and DAS28-ESR (r=0.539, 95% CI 0.399–0.655, p<0.001) and DAS28-CRP (r=0.590, 95% CI 0.460–0.695, p<0.001) (Supplementary Table 1).

Furthermore, repeat testing of tumour M2-PK levels in 20 patients with decreased disease activity revealed a significant decline in tumour M2-PK levels after the reduction in inflammation (p<0.001) (Figure 2).

## ROC curve of laboratory variables and logistic regression analysis

Patients were divided into moderate/high disease activity (n=75) and remission/low disease activity (n=76) groups to evaluate the performance of tumour M2-PK, ESR, and CRP in differentiating disease status. In particular, the median ESR, CRP, and tumour M2-PK levels were highest in those with high disease activity (Supplementary Table 2). ROC curve analysis revealed that tumour M2-PK, ESR, and CRP could significantly distinguish between disease status (the AUC was 0.877, 0.939, and 0.933 for tumour M2-PK, ESR, and CRP, respectively). The combination of tumour M2-PK, ESR, and CRP in the ROC curve analysis increased the AUC to 0.962 (Figure 3).

In a logistic regression analysis comprised of laboratory results, it was shown that WBC and neutrophil counts; ESR, CRP, ALP, and TNF-α levels; and tumour M2-PK level >43.9 U/mL were significantly associated with moderate/high disease activity. The adjusted analysis revealed that neutrophil count (odds ratio [OR] 1.001, 95% CI 1.000–1.001, p=0.006), ESR (OR 1.108, 95% CI 1.060–1.158, p<0.001), and tumour M2-PK >43.9 U/mL (OR 3.672, 95% CI 1.047–12.878, p=0.042) independently predicted moderate/high disease activity in patients with RA (Table 2).

## Tumour M2-PK levels according to subgroup analysis

To evaluate the difference in tumour M2-PK levels based on disease duration and treatment, patients were classified into

TABLE 1 Baseline characteristics of patients with RA.

| | Values |
|---|---|
| Patient demographics | |
| Age | 57.0 (48.0-64.0) |
| Female sex | 118 (78.1) |
| Seropositive RA | 139 (92.1) |
| Interstitial lung disease | 11 (7.3) |
| Disease duration | 61.7 (9.8-145.2) |
| Disease activity measures | |
| DAS28-ESR | 3.2 (2.4-4.7) |
| DAS28-CRP | 2.3 (1.4-3.8) |
| Concomitant treatment | |
| Glucocorticoid | 76 (50.3) |
| DMARDs | 121 (80.1) |
| csDMARDs | 115 (76.2) |
| bDMARDs | 56 (37.1) |
| Laboratory results | |
| WBC count (/mm$^3$) | 6250.0 (5165.0-8320.0) |
| Platelet count (x 1,000/mm$^3$) | 264.0 (222.3-322.8) |
| Neutrophil count (/mm$^3$) | 3660.0 (2875.0-5472.5) |
| Lymphocyte count (/mm$^3$) | 1900.0 (1512.5-2255.0) |
| ESR (mm/h) | 36.0 (18.0-67.3) |
| CRP (mg/L) | 2.5 (0.6-11.8) |
| Alkaline phosphatase (IU/L) | 67.0 (56.0-79.8) |
| AST (IU/L) | 19.0 (16.0-24.0) |
| ALT (IU/L) | 15.0 (11.0-23.8) |
| TNF-α (pg/mL)¶ | 10.7 (4.5-12.8) |
| IL-6 (pg/mL)† | 9.1 (3.0-27.0) |
| Tumour M2-PK (U/mL) | 42.9 (14.6-104.3) |

¶and †Tested in 148 and 146 patients, respectively.
Data are shown as median (interquartile range) or frequency (percentage), as appropriate. RA, rheumatoid arthritis; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DMARDs, disease-modifying anti-rheumatic drugs; csDMARDs, conventional synthetic disease-modifying anti-rheumatic drugs; bDMARDs, biologic disease-modifying anti-rheumatic drugs; WBC, white blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TNF, tumour necrosis factor; IL, interleukin; Tumour M2-PK, dimeric form of pyruvate kinase M2

those with new-onset (n=24) and those without new-onset disease (n=127). The level of tumour M2-PK was significantly higher in patients with new-onset RA than in those without new-onset disease (p<0.001). Moreover, the tumour M2-PK level was lower in patients who were undergoing any DMARDs treatment or in subjects undergoing bDMARDs treatment (all p<0.001). In contrast, no differences in tumour M2-PK levels were observed with seropositivity, age, and sex (Figure 4).

## Tumour M2-PK levels in osteoarthritis and controls

Comparison of tumour M2-PK levels in patients with RA, patients with OA, and controls showed that tumour M2-PK levels were significantly higher in patients with RA than in those with OA or controls (all p<0.001); in particular, the level of tumour M2-PK in patients with RA with moderate/high disease activity was also higher than those in patients with OA and in controls (Figure 5). The tumour M2-PK levels were comparable between the OA and control groups. ROC analysis revealed that tumour M2-PK could be used to differentiate patients with RA from those with OA or controls (AUC 0.750, p<0.001 for RA vs. OA and AUC 0.707, p<0.001 for RA vs. controls) (Figure 6).

## Expression of PKM2 and tumour M2-PK in synovial tissue

The expression of PKM2 was evaluated in the synovial tissues of patients with RA and OA using two different primer sequences; however, there was no difference in the expression of PKM2 in the tissues of patients with RA and OA (Figure 7A, B). Meanwhile, IHC staining for tumour M2-PK showed that local expression of tumour M2-PK was not evident in both RA and OA synovium (Figure 8).

## Cytokine levels after treatment with TEPP-46

Cultured FLS were stimulated with TNF-α and IL-1β and the supernatant was analyzed to evaluate whether inflammatory cytokines are increased. When stimulation with both cytokines of TNF-α and IL-1β was performed, the level of TNF-α was significantly higher compared to the absence of these cytokines; however, TNF-α levels did not significantly differ even when TEPP-46 was added to TNF-α, IL-1β, and both TNF-α and IL-1β stimulation (p=0.881, p=0.553, and p=0.511, respectively). In addition, there were no difference in the cytokines of IL-1β, IFN-γ, and IL-6 in the supernatant when TEPP-46 was treated in the presence of TNF-α, IL-1β, and both TNF-α and IL-1β stimulation (Figure 9).

# Discussion

In the present study, we investigated whether tumour M2-PK is associated with disease activity in patients with RA and if it could be used to diagnosis RA. First, we found that tumour M2-PK was significantly associated with DAS28-ESR and DAS28-CRP (a composite measure assessing the severity of RA), and ESR and CRP, which decreased after reduction in disease activity. Second, among the laboratory variables included, tumour M2-PK was identified as an independent predictor of moderate/high disease activity in RA. Third, adding tumour M2-PK to ESR and CRP increased the AUC of the ROC curve for differentiating RA with moderate/high from RA with remission/

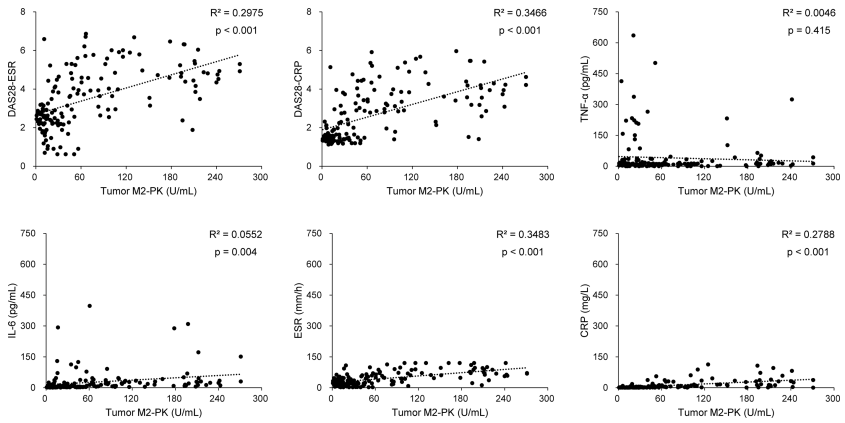


FIGURE 1
Correlation between tumour M2-PK level and disease activity, TNF-α, IL-6, ESR, and CRP level. Tumour M2-PK levels significantly correlated with disease activity and laboratory results of IL-6, ESR, and CRP, but did not show a correlation with TNF-α. Pearson's correlation analysis was performed to elucidate the relationship between tumour M2-PK levels and continuous variables. Tumour M2-PK, dimeric form of pyruvate kinase M2; TNF, tumour necrosis factor; IL, interleukin; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28: disease activity score in 28 joints.

low disease activity to 0.962. Fourth, tumour M2-PK levels were higher in patients with RA than in those with OA or controls, and tumour M2-PK differed between subjects with RA and OA, or controls. Lastly, in contrast to the level of tumour M2-PK in the plasma, the IHC for tumour M2-PK and PCR results for PKM2 in the synovial tissues of patients with RA and OA were comparable. Collectively, these findings suggested that tumour M2-PK in the circulation could be a feasible marker for disease severity and diagnosis in RA.

Alterations in metabolism are now increasingly considered to be closely related to aberrant immune responses, which has drawn special interest in the field of immunometabolism. This is because the upregulation of aerobic glycolysis, which is referred to as the Warburg effect, initially thought to be exclusively found in patients with cancers, has also been demonstrated in patients with autoimmune diseases (33). In particular, accumulating evidence emphasises that the Warburg effect is present in the affected joints in RA and may be a potential treatment target in

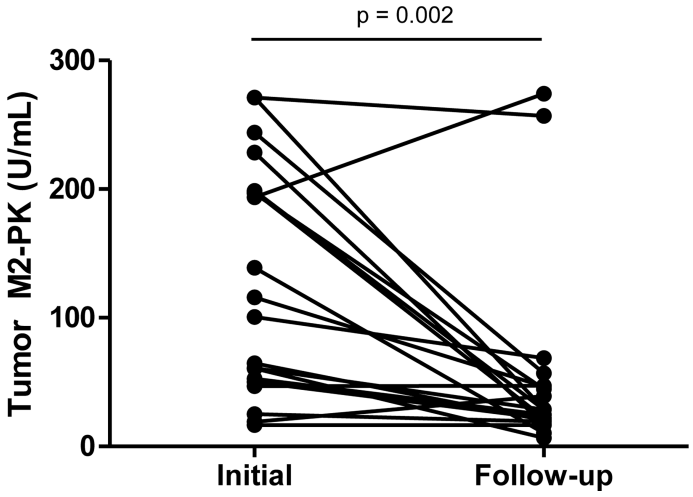


FIGURE 2
Serial testing of tumour M2-PK level after reduction in disease activity. Tumour M2-PK levels decreased significantly following improvement in disease activity. Changes in plasma tumour M2-PK levels were estimated by the Wilcoxon signed rank test. Tumour M2-PK: dimeric form of pyruvate kinase M2.

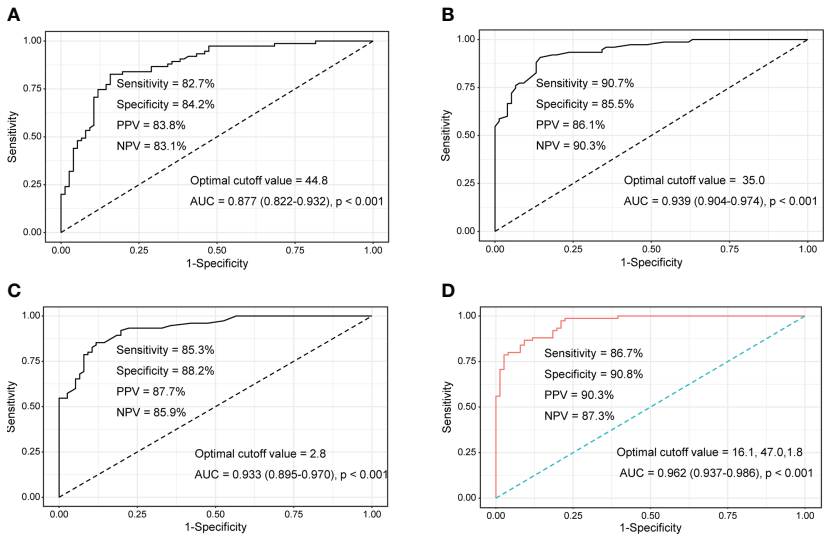


FIGURE 3
ROC curves of tumour M2-PK, ESR, and CRP to determine moderate/high disease activity and remission/low disease activity. Area under the ROC curve for **(A)** tumour M2-PK, **(B)** ESR, **(C)** CRP, and **(D)** integration of tumour M2-PK, ESR, and CRP for determining optimal cut-off values in discriminating moderate/high RA activity (DAS28-ESR ≥ 3.2) and remission/low disease activity (DAS28-ESR < 3.2). ROC: Receiver operating characteristic; Tumour M2-PK, dimeric form of pyruvate kinase M2; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RA, rheumatoid arthritis.

modulating articular inflammation (20, 34, 35). In this context, it is possible that plasma tumour M2-PK is associated with disease activity in RA through either glycolysis- or non-glycolysis-dependent mechanisms (36). First, tumour M2-PK may be a marker of increased glycolysis in RA. In general, highly proliferating cells, which include FLS and immune cells in RA, are thought to have an increased energy requirement (18). Therefore, facilitation of energy production *via* increased glycolysis is required in the RA microenvironment to meet the increased energy demand. Second, PKM2 is also involved in the

TABLE 2 Univariate and multivariate logistic regression of the association between laboratory results and moderate/high disease activity.

| Laboratory results | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | p value | OR | 95% CI | p value |
| WBC count | 1.000 | 1.000-1.001 | <0.001 | | | |
| Platelet count | 1.000 | 0.999-1.001 | 0.924 | | | |
| Neutrophil count | 1.001 | 1.001-1.001 | <0.001 | 1.001 | 1.000-1.001 | 0.006 |
| Lymphocyte count | 1.000 | 0.999-1.000 | 0.044 | | | |
| ESR | 1.108 | 1.073-1.144 | <0.001 | 1.108 | 1.060-1.158 | <0.001 |
| CRP | 1.533 | 1.307-1.796 | <0.001 | | | |
| Alkaline phosphatase | 1.033 | 1.014-1.053 | 0.001 | | | |
| AST | 0.958 | 0.913-1.005 | 0.079 | | | |
| ALT | 0.992 | 0.969-1.015 | 0.487 | | | |
| TNF-α | 1.000 | 0.996-1.003 | 0.894 | | | |
| IL-6 | 1.018 | 1.003-1.032 | 0.016 | | | |
| Tumour M2-PK > 43.9 U/mL | 25.436 | 10.776-60.040 | <0.001 | 3.672 | 1.047-12.878 | 0.042 |

WBC, white blood cell; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TNF, tumour necrosis factor; IL, interleukin; tumour M2-PK, dimeric form of pyruvate kinase M2; OR, odds ratio; CI, confidence interval.

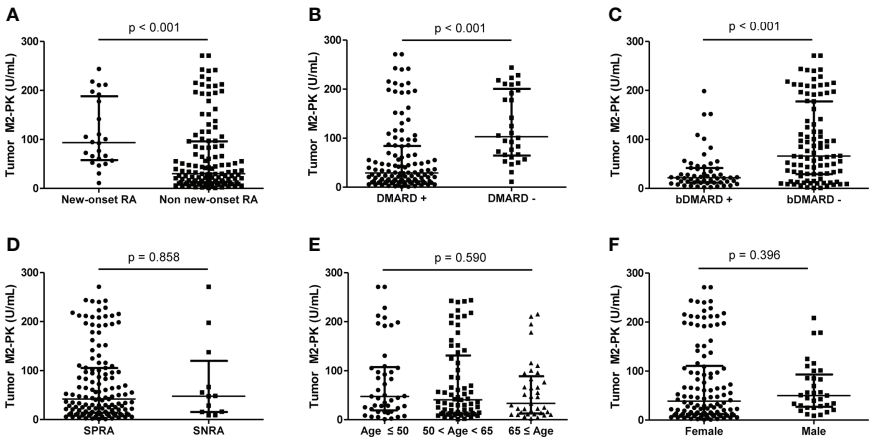


FIGURE 4
Comparison of tumour M2-PK level based on disease duration, DMARD usage, seropositivity, age, and sex. The differences in tumour M2-PK levels were assessed in RA patients with regard to **(A)** disease duration, **(B)** DMARD usage, **(C)** bDMARD usage, **(D)** serotype, **(E)** age, and **(F)** sex. Mann–Whitney U test and Kruskal–Wallis test was used for the comparison of two groups, and three groups, respectively. The error bars indicate median values and interquartile range. Tumour M2-PK, dimeric form of pyruvate kinase M2; DMARD, disease-modifying anti-rheumatic drug; bDMARD, biologic disease-modifying anti-rheumatic drug; SPRA, seropositive rheumatoid arthritis; SNRA, seronegative rheumatoid arthritis.

activation of key transcription factors in RA, such as signal transducer and activator of transcription (STAT) 1 and 3, hypoxia-inducible factor-1α, and nuclear factor-κB, which are closely linked to the inflammatory signalling pathway in RA (36). Notably, nuclear PKM2 exists in a dimeric form and acts as a protein kinase that influences the transcription of inflammation-related genes, indicating that it may represent heightened inflammation in RA (37). Third, tumor M2-PK was shown to promote angiogenesis in an *in vitro* analysis, which is also thought to be a characteristic feature observed within the inflamed RA joints (38, 39).

Based on the reasons provided above, we postulated that the tumour M2-PK level could parallel the activity of RA and would be lower in patients with OA and controls than in patients with

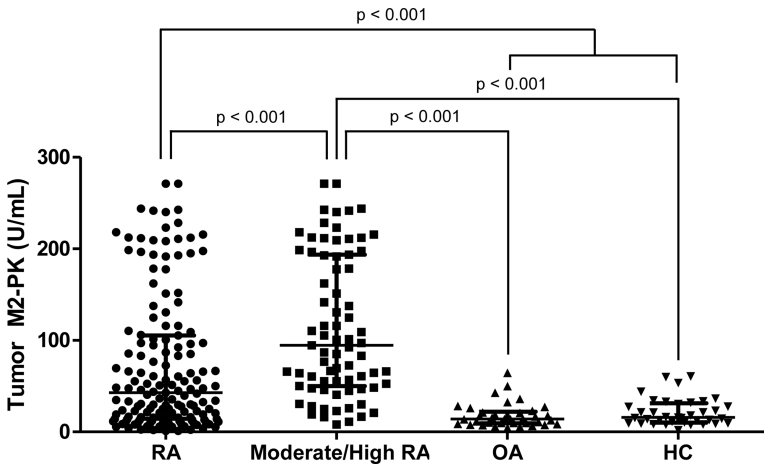


FIGURE 5
Comparison of tumour M2-PK levels in patients with RA, OA, and controls. Tumour M2-PK levels were compared between patients with RA with moderate/high disease activity, OA, and controls. Differences between the two groups were compared using the Mann–Whitney U test. The error bars indicate median values and interquartile range. Tumour M2-PK, dimeric form of pyruvate kinase M2; RA, rheumatoid arthritis; OA, osteoarthritis; HC, healthy controls.

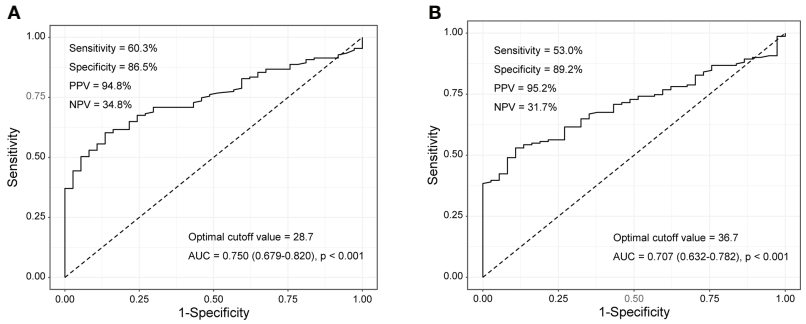


FIGURE 6
Tumour M2-PK cut-off levels for differentiating RA from OA and controls. Tumour M2-PK levels of 28.7 and 36.7 could be used to differentiate between **(A)** RA and OA patients, and **(B)** RA patients and controls, respectively. The optimal cut-off values were derived using the receiver operating characteristic curve. Tumour M2-PK: dimeric form of pyruvate kinase M2; RA: rheumatoid arthritis; OA: osteoarthritis.

RA. Supporting our hypothesis, plasma tumour M2-PK levels exhibited a significant correlation with disease activity and were predictive of disease activity status in RA, which was also found to be higher in RA patients than in those with OA and controls. Importantly, a longitudinal assessment of tumour M2-PK demonstrated that its decrease reflected a reduction in disease activity. ESR and CRP were the most commonly performed laboratory tests to estimate disease improvement or aggravation, and combining tumour M2-PK with these tests in the ROC curve analysis for the discrimination of disease severity showed an excellent performance, with an AUC which was higher than that of ESR and CRP alone; this implies that it could have an additional clinical value in measuring RA activity. However, it is well known that tumour M2-PK increase is relevant for the extent of disease and prognosis of cancers, especially in the gastrointestinal tract (40, 41). Thus, patients with cancer were excluded from our study. However, in patients with RA who show increased tumour M2-PK, it is apparent that the possibility of malignancy should be examined thoroughly before clinical application because malignancies could affect tumour M2-PK levels.

Similar to the results of this study, a recent publication by Han et al. demonstrated that extracellular PKM2 is increased in patients with RA and is related to disease activity. It was also demonstrated that extracellular PKM2 level was associated with radiographic progression in a subset of patients with early RA and recombinant PKM2 was involved in osteoclastogenesis in an *in vitro* analysis (42). Nonetheless, our study is unique that the clinical utility of tumour M2-PK in assessing the disease activity of RA has been analyzed detailedly in a separate cohort using ROC and logistic analyses. In addition, comparison of tumour M2-PK level in RA, OA, and controls and serial assessment of its level confirms that measuring plasma tumor M2-PK may be a practical test in discriminating RA and OA, as well as in the management of patients with RA. Of note, in our *in vitro* analysis using FLS, we did not observe a significant reduction of inflammatory cytokines when PKM2 activator TEPP-46 was used. This finding seems to be partly explained by a previous

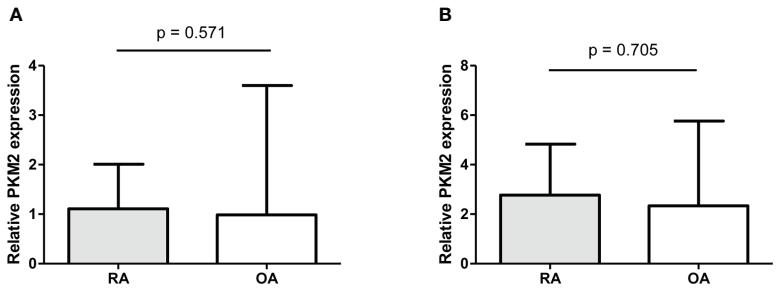


FIGURE 7
Real-time quantitative polymerase chain reaction of PKM2 in synovial tissues from RA and OA patients. The relative expression of PKM2 in knee synovial tissues was comparable between RA and OA patients (n=10); this was analysed using two different primer sequences: (i) **(A)** and (ii) **(B)**. Relative PKM2 expression was compared by the Mann-Whitney U test. The error bars indicate median values and interquartile range. PKM2: pyruvate kinase isoenzyme M2; RA: rheumatoid arthritis; OA: osteoarthritis.

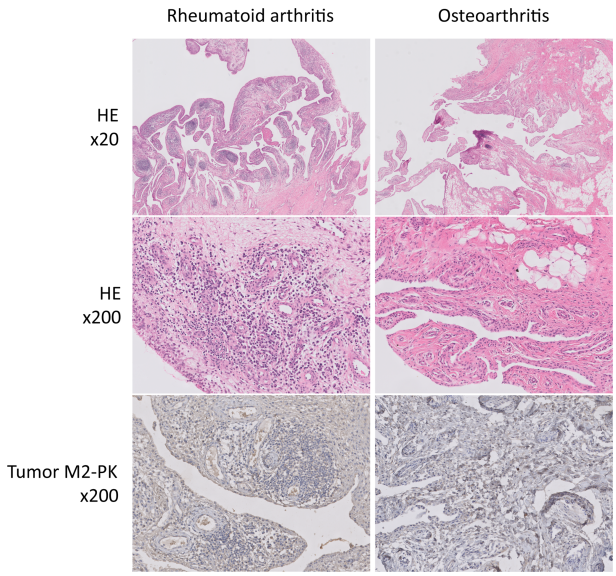


FIGURE 8
Immunohistochemical analysis of tumour M2-PK in knee synovial tissues of patients with RA and OA. In haematoxylin and eosin-stained synovial tissue from patients with RA, dense infiltration of plasma cells, lymphocytes, and macrophages; germinal centre formation; and villous synovial hyperplasia and hypertrophy were observed. In contrast, the OA synovium showed fatty ingrowth and synovial hyperplasia with myxoid degeneration. Tumour M2-PK immunohistochemical staining in synovial tissues from patients with RA and OA showed no definite expression of local tumour M2-PK. Tumour M2-PK: dimeric form of pyruvate kinase M2; RA, rheumatoid arthritis; OA, osteoarthritis.

observation that secretion of tumour M2-PK are more likely to be affected by monocytes than FLS (42), implying immune cells such as T-cells and monocyte/macrophages might be a greater source of elevated tumour M2-PK in patients with RA (43). On the other hand, a complex association of inflammation and metabolic deregulation could be also considered as a reason for this insignificant results. Interestingly, a previous publication revealed that the decrease of inflammation could intrinsically modify immunometabolism in RA (44). Overall, it is apparent that the dynamics of dimeric and tetrameric form of PKM2 and the role of tumour M2-PK in potentiating inflammation in RA deserves further investigation.

An earlier study showed that immunohistochemistry of PKM2 is enhanced in cells residing in the lining and sub lining of synovial tissues of patients with RA compared to those of patients with OA (45). However, in this study, qPCR analysis of PKM2 in synovial tissues showed that there was no significant difference in PKM2 expression between RA and OA. These discrepant results could be attributed to the fact that PKM2 IHC staining appears to be limited to a specific region within the synovium and may not sufficiently reflect the degree of local inflammation in the synovium. Alternatively, the increase in inflammatory mediators which was also observed in the OA microenvironment and changes in RA synovial tissue pathologic findings even after active treatment, could also contribute to the comparable PKM2 qPCR results (46, 47). IHC staining of tumour M2-PK in RA and OA synovium revealed that its expression was not noticeable. The relatively low proportion of dimeric PKM2 relative to its tetrameric isoform, even when there is a high energy demand, as shown in cancer cells, might explain the negligible tumour M2-PK staining in RA and OA synovium (48). Finally, it is possible that PKM2 and tumour M2-PK expression were affected by formalin fixation. Accordingly, in-depth research is necessary to further elucidate the role of tumour M2-PK and PKM2 in tissues from patients with RA.

Our study has several limitations. First, both incident and prevalent cases of RA were included, and the proportion of patients with new-onset RA was relatively low. Second, a detailed assessment of the impact of the treatment regimen on plasma tumour M2-PK levels could not be performed because of the present study design. Third, only the level of tumour M2-PK was analysed, and its expression relative to that of PKM2 was not evaluated. Fourth, although patients with RA with moderate// high disease activity had significantly higher levels of tumour M2-PK level than those with OA and controls, the number of patients were small and could be considered exploratory. Finally, the underlying pathophysiology of increased plasma tumour M2-PK in RA and its direct pathogenic role could not be determined. Future studies are required to better understand

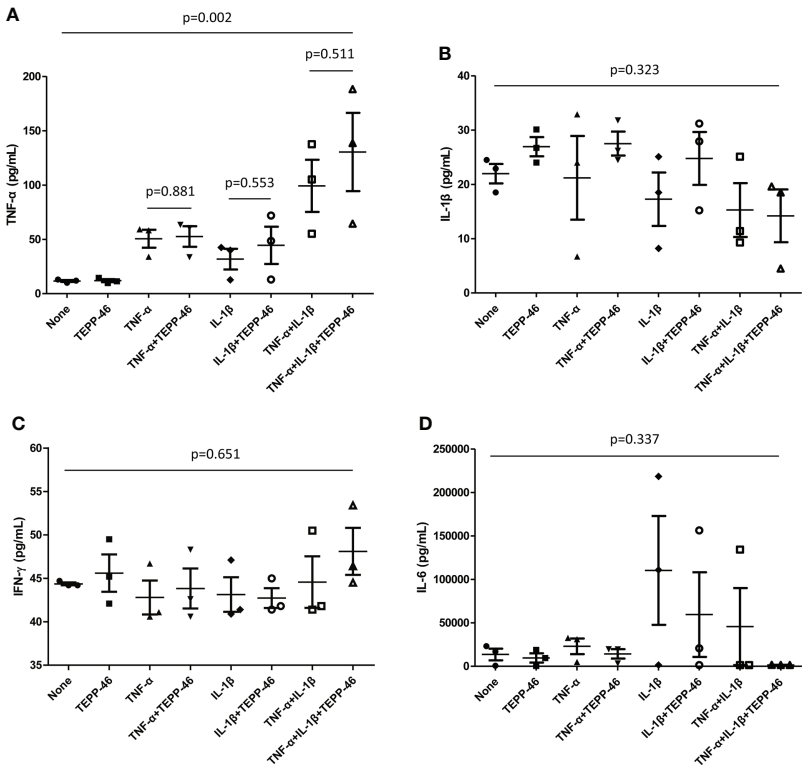


FIGURE 9
Cytokine assays of supernatant from FLS. FLS ($1.5\times10^5$ cells) obtained from patients with RA was stimulated by TNF-α or IL-β (100unit) in the presence and absence of TEPP-46 (10μM), a PKM2 activator. After 72 hours, the cytokines of **(A)** TNF-α, **(B)** IL-β, **(C)** IFN-γ, and **(D)** IL-6 in the supernatant was measured by duplicate experiments of enzyme-linked immunosorbent assay. Differences of two groups were evaluated by student's t-test, whereas Analysis of Variance was used to compared the differences of more than three groups. The error bars indicate mean and SEM. FLS: fibroblast-like synoviocytes; RA, rheumatoid arthritis; TNF, tumour necrosis factor; IL, interleukin; PKM2, pyruvate kinase isoenzyme M2; IFN, interferon.

whether tests evaluating changes in glucose metabolism can be used as surrogate markers of RA severity.

## Conclusions

In conclusion, we found that plasma tumour M2-PK levels significantly correlated with disease activity and independently predicted disease severity in RA, which decreased with reduction in disease activity. Furthermore, plasma tumour M2-PK was higher in patients with RA than in patients with OA and controls. Our results suggest that the assessment of plasma tumour M2-PK levels might be a clinically useful indicator for evaluating disease activity and RA diagnosis.

# 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 Severance Hospital's Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

# Author contributions

Conceptualization, SSA and HK. Methodology, SSA, HK and YP. Software, SSA and HK. Validation, SSA. Formal analysis, SSA. Investigation, SSA, HK and YP. Resources, SSA, HK and YP. Data curation, SSA. Writing—original draft preparation, SSA, HK and YP. Writing—review and editing, SSA, HK and YP. Visualization, SSA and HK. Supervision, YP. Project administration, SSA, HK and YP. Funding Acquisition, SSA. All authors have read and agreed to the final version 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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.901555/full#supplementary-material

## References

1. Israelsen WJ, Vander Heiden MG. Pyruvate kinase: Function, regulation and role in cancer. *Semin Cell Dev Biol* (2015) 43:43–51. doi: 10.1016/j.semcdb.2015.08.004

2. Wu S, Le H. Dual roles of PKM2 in cancer metabolism. *Acta Biochim Biophys Sin (Shanghai)* (2013) 45:27–35. doi: 10.1093/abbs/gms106

3. Zhang Z, Deng X, Liu Y, Liu Y, Sun L, Chen F. PKM2, function and expression and regulation. *Cell Biosci* (2019) 9:52. doi: 10.1186/s13578-019-0317-8

4. Hay N. Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nat Rev Cancer* (2016) 16:635–49. doi: 10.1038/nrc.2016.77

5. Prakasam G, Iqbal MA, Bamezai RNK, Mazurek S. Posttranslational modifications of pyruvate kinase M2: Tweaks that benefit cancer. *Front Oncol* (2018) 8:22. doi: 10.3389/fonc.2018.00022

6. Hugo F, Fischer G, Eigenbrodt E. Quantitative detection of tumor M2-PK in serum and plasma. *Anticancer Res* (1999) 19:2753–7.

7. Muñoz-Colmenero A, Fernández-Suárez A, Fatela-Cantillo D, Ocaña-Pérez E, Domínguez-Jiménez JL, Díaz-Iglesias JM. Plasma tumor M2-pyruvate kinase levels in different cancer types. *Anticancer Res* (2015) 35:4271–6.

8. Chhipa AS, Patel S. Targeting pyruvate kinase muscle isoform 2 (PKM2) in cancer: What do we know so far? *Life Sci* (2021) 280:119694. doi: 10.1016/j.lfs.2021.119694

9. Schulze G. The tumor marker tumor M2-PK: an application in the diagnosis of gastrointestinal cancer. *Anticancer Res* (2000) 20:4961–4.

10. Hardt PD, Ewald N. Tumor M2 pyruvate kinase: a tumor marker and its clinical application in gastrointestinal malignancy. *Expert Rev Mol Diagn* (2008) 8:579–85. doi: 10.1586/14737159.8.5.579

11. Bandara IA, Baltatzis M, Sanyal S, Siriwardena AK. Evaluation of tumor M2-pyruvate kinase (Tumor M2-PK) as a biomarker for pancreatic cancer. *World J Surg Oncol* (2018) 16:56. doi: 10.1186/s12957-018-1360-3

12. Schneider J, Neu K, Velcovsky HG, Morr H, Eigenbrodt E. Tumor M2-pyruvate kinase in the follow-up of inoperable lung cancer patients: a pilot study. *Cancer Lett* (2003) 193:91–8. doi: 10.1016/S0304-3835(02)00720-6

13. Hoopmann M, Warm M, Mallmann P, Thomas A, Göhring UJ, Schöndorf T. Tumor M2 pyruvate kinase–determination in breast cancer patients receiving trastuzumab therapy. *Cancer Lett* (2002) 187:223–8. doi: 10.1016/S0304-3835(02)00404-4

14. Zahra K, Dey T, Ashish, Mishra SP, Pandey U. Pyruvate kinase M2 and cancer: The role of PKM2 in promoting tumorigenesis. *Front Oncol* (2020) 10:159. doi: 10.3389/fonc.2020.00159

15. Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al. Rheumatoid arthritis. *Nat Rev Dis Primers* (2018) 4:18001. doi: 10.1038/nrdp.2018.1

16. Yoshitomi H. Regulation of immune responses and chronic inflammation by fibroblast-like synoviocytes. *Front Immunol* (2019) 10:1395. doi: 10.3389/fimmu.2019.01395

17. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol Rev* (2010) 233:233–55. doi: 10.1111/j.0105-2896.2009.00859.x

18. de Oliveira PG, Farinon M, Sanchez-Lopez E, Miyamoto S, Guma M. Fibroblast-like synoviocytes glucose metabolism as a therapeutic target in rheumatoid arthritis. *Front Immunol* (2019) 10:1743. doi: 10.3389/fimmu.2019.01743

19. Masoumi M, Mehrabzadeh M, Mahmoudzehi S, Mousavi MJ, Jamalzehi S, Sahebkar A, et al. Role of glucose metabolism in aggressive phenotype of fibroblast-like synoviocytes: Latest evidence and therapeutic approaches in rheumatoid arthritis. *Int Immunopharmacol* (2020) 89:107064. doi: 10.1016/j.intimp.2020.107064

20. Garcia-Carbonell R, Divakaruni AS, Lodi A, Vicente-Suarez I, Saha A, Cheroutre H, et al. Critical role of glucose metabolism in rheumatoid arthritis fibroblast-like synoviocytes. *Arthritis Rheumatol* (2016) 68:1614–26. doi: 10.1002/art.39608

21. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO3rd, et al. Rheumatoid arthritis classification criteria: an American college of Rheumatology/European league against rheumatism collaborative initiative. *Arthritis Rheum* (2010) 62:2569–81. doi: 10.1002/art.27584

22. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* (1995) 38:44–8. doi: 10.1002/art.1780380107

23. Wells G, Becker JC, Teng J, Dougados M, Schiff M, Smolen J, et al. Validation of the 28-joint disease activity score (DAS28) and European league against rheumatism response criteria based on c-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate. *Ann Rheum Dis* (2009) 68:954–60. doi: 10.1136/ard.2007.084459

24. Lau CS, Chia F, Dans L, Harrison A, Hsieh TY, Jain R, et al. 2018 Update of the APLAR recommendations for treatment of rheumatoid arthritis. *Int J Rheum Dis* (2019) 22:357–75. doi: 10.1111/1756-185X.13513

25. Singh JA, Saag KG, Bridges SLJr., Akl EA, Bannuru RR, Sullivan MC, et al. 2015 American College of rheumatology guideline for the treatment of rheumatoid arthritis. *Arthritis Care Res (Hoboken)* (2016) 68:1–25. doi: 10.1002/acr.22783

26. Anderson J, Caplan L, Yazdany J, Robbins ML, Neogi T, Michaud K, et al. Rheumatoid arthritis disease activity measures: American college of rheumatology recommendations for use in clinical practice. *Arthritis Care Res (Hoboken)* (2012) 64:640–7. doi: 10.1002/acr.21649

27. Zafari P, Rafiei A, Faramarzi F, Ghaffari S, Amiri AH, Taghadosi M. Human fibroblast-like synoviocyte isolation matter: a comparison between cell isolation from synovial tissue and synovial fluid from patients with rheumatoid arthritis. *Rev Assoc Med Bras (1992)* (2021) 67:1654–8. doi: 10.1590/1806-9282.20210706

28. Wu Z, Ma D, Yang H, Gao J, Zhang G, Xu K, et al. Fibroblast-like synoviocytes in rheumatoid arthritis: Surface markers and phenotypes. *Int Immunopharmacol* (2021) 93:107392. doi: 10.1016/j.intimp.2021.107392

29. Choi HS, Ryu CJ, Choi HM, Park JS, Lee JH, Kim KI, et al. Effects of the pro-inflammatory milieu on the dedifferentiation of cultured fibroblast-like synoviocytes. *Mol Med Rep* (2012) 5:1023–6. doi: 10.3892/mmr.2012.767

30. Nair A, Kanda V, Bush-Joseph C, Verma N, Chubinskaya S, Mikecz K, et al. Synovial fluid from patients with early osteoarthritis modulates fibroblast-like synoviocyte responses to toll-like receptor 4 and toll-like receptor 2 ligands *via* soluble CD14. *Arthritis Rheum* (2012) 64:2268–77. doi: 10.1002/art.34495

31. Angiari S, Runtsch MC, Sutton CE, Palsson-McDermott EM, Kelly B, Rana N, et al. Pharmacological activation of pyruvate kinase M2 inhibits CD4(+) T cell pathogenicity and suppresses autoimmunity. *Cell Metab* (2020) 31:391–405.e8. doi: 10.1016/j.cmet.2019.10.015

32. Liu H, Takagaki Y, Kumagai A, Kanasaki K, Koya D. The PKM2 activator TEPP-46 suppresses kidney fibrosis *via* inhibition of the EMT program and aberrant glycolysis associated with suppression of HIF-1α accumulation. *J Diabetes Investig* (2021) 12:697–709. doi: 10.1111/jdi.13478

33. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: the metabolic requirements of cell proliferation. *Science* (2009) 324:1029–33. doi: 10.1126/science.1160809

34. Xu J, Jiang C, Wang X, Geng M, Peng Y, Guo Y, et al. Upregulated PKM2 in macrophages exacerbates experimental arthritis *via* STAT1 signaling. *J Immunol* (2020) 205:181–92. doi: 10.4049/jimmunol.1901021

35. Kvacskay P, Yao N, Schnotz JH, Scarpone R, Carvalho RA, Klika KD, et al. Increase of aerobic glycolysis mediated by activated T helper cells drives synovial fibroblasts towards an inflammatory phenotype: new targets for therapy? *Arthritis Res Ther* (2021) 23:56. doi: 10.1186/s13075-021-02437-7

36. Xu D, Liang J, Lin J, Yu C. PKM2: A potential regulator of rheumatoid arthritis *via* glycolytic and non-glycolytic pathways. *Front Immunol* (2019) 10:2919. doi: 10.3389/fimmu.2019.02919

37. Gao X, Wang H, Yang JJ, Liu X, Liu ZR. Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol Cell* (2012) 45:598–609. doi: 10.1016/j.molcel.2012.01.001

38. Dhar DK, Olde Damink SW, Brindley JH, Godfrey A, Chapman MH, Sandanayake NS, et al. Pyruvate kinase M2 is a novel diagnostic marker and predicts tumor progression in human biliary tract cancer. *Cancer* (2013) 119:575–85. doi: 10.1002/cncr.27611

39. Elshabrawy HA, Chen Z, Volin MV, Ravella S, Virupannavar S, Shahrara S. The pathogenic role of angiogenesis in rheumatoid arthritis. *Angiogenesis* (2015) 18:433–48. doi: 10.1007/s10456-015-9477-2

40. Hathurusinghe HR, Goonetilleke KS, Siriwardena AK. Current status of tumor M2 pyruvate kinase (tumor M2-PK) as a biomarker of gastrointestinal malignancy. *Ann Surg Oncol* (2007) 14:2714–20. doi: 10.1245/s10434-007-9481-x

41. Wu J, Hu L, Chen M, Cao W, Chen H, He T. Pyruvate kinase M2 overexpression and poor prognosis in solid tumors of digestive system: evidence from 16 cohort studies. *Onco Targets Ther* (2016) 9:4277–88. doi: 10.2147/OTT.S106508

42. Han DW, Choi YS, Kim HW, Shin S, Ha YJ, Kang EH, et al. Extracellular pyruvate kinase M2 promotes osteoclastogenesis and is associated with radiographic progression in early rheumatoid arthritis. *Sci Rep* (2022) 12:4024. doi: 10.1038/s41598-022-07667-6

43. Qiu J, Wu B, Goodman SB, Berry GJ, Goronzy JJ, Weyand CM. Metabolic control of autoimmunity and tissue inflammation in rheumatoid arthritis. *Front Immunol* (2021) 12:652771. doi: 10.3389/fimmu.2021.652771

44. Koedderitzsch K, Zezina E, Li L, Herrmann M, Biesemann N. TNF induces glycolytic shift in fibroblast like synoviocytes *via* GLUT1 and HIF1A. *Sci Rep* (2021) 11:19385. doi: 10.1038/s41598-021-98651-z

45. McGarry T, Biniecka M, Gao W, Cluxton D, Canavan M, Wade S, et al. Resolution of TLR2-induced inflammation through manipulation of metabolic pathways in rheumatoid arthritis. *Sci Rep* (2017) 7:43165. doi: 10.1038/srep43165

46. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. *Ann Rheum Dis* (2005) 64:1263–7. doi: 10.1136/ard.2004.025270

47. Bresnihan B. Are synovial biopsies of diagnostic value? *Arthritis Res Ther* (2003) 5:271–8. doi: 10.1186/ar1003

48. Xie J, Dai C, Hu X. Evidence that does not support pyruvate kinase M2 (PKM2)-catalyzed reaction as a rate-limiting step in cancer cell glycolysis. *J Biol Chem* (2016) 291:8987–99. doi: 10.1074/jbc.M115.704825

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