

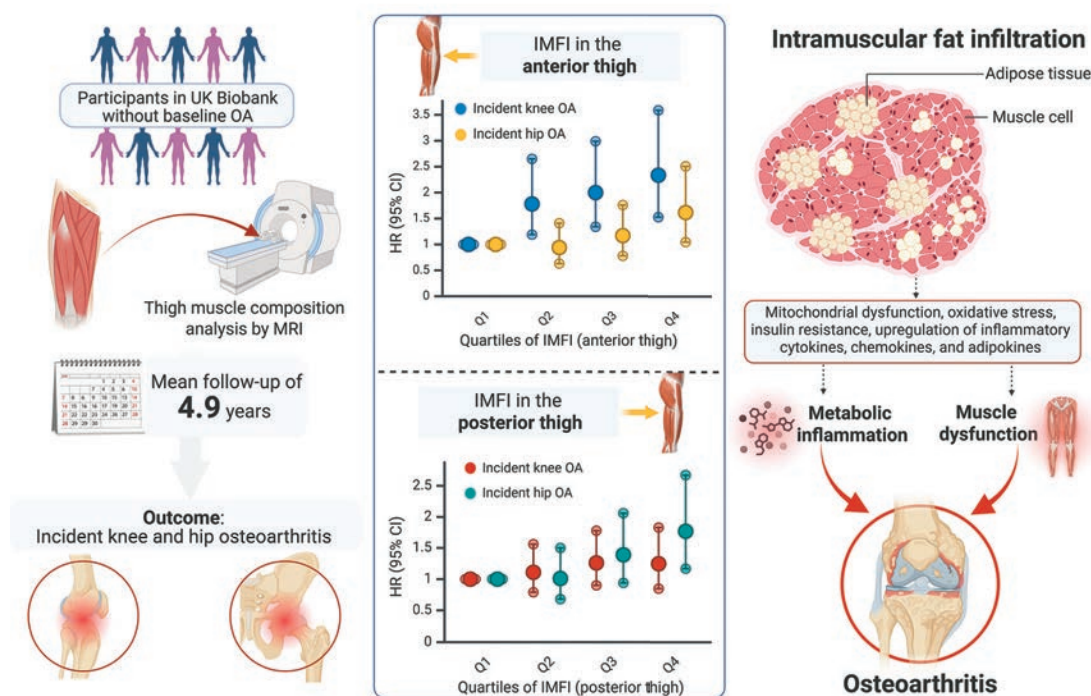
# Clinical Connections

## Association of Thigh Intramuscular Fat Infiltration With Incident Knee and Hip Osteoarthritis

Weng et al, *Arthritis Rheumatol.* 2025;77:1205-1215.

### CORRESPONDENCE

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### KEY POINTS

- Thigh IMFI was positively associated with an increased risk of incident knee and hip OA, with a strong dose-response relationship.
- Interaction and subgroup analyses suggest the effect of IMFI is independent of body mass index and sex.
- IMFI may serve as a novel risk factor and potential therapeutic target for clinical intervention of OA.

### SUMMARY

Targeting modifiable risk factors is key to preventing and managing osteoarthritis (OA), a major contributor to chronic pain and long-term disability. Intramuscular fat infiltration (IMFI), a hallmark of myopathic changes, increases by 6–8% annually in older adults and contributes to metabolic inflammation and muscle dysfunction. However, the temporal relationship between baseline IMFI and incident OA remains unexplored.

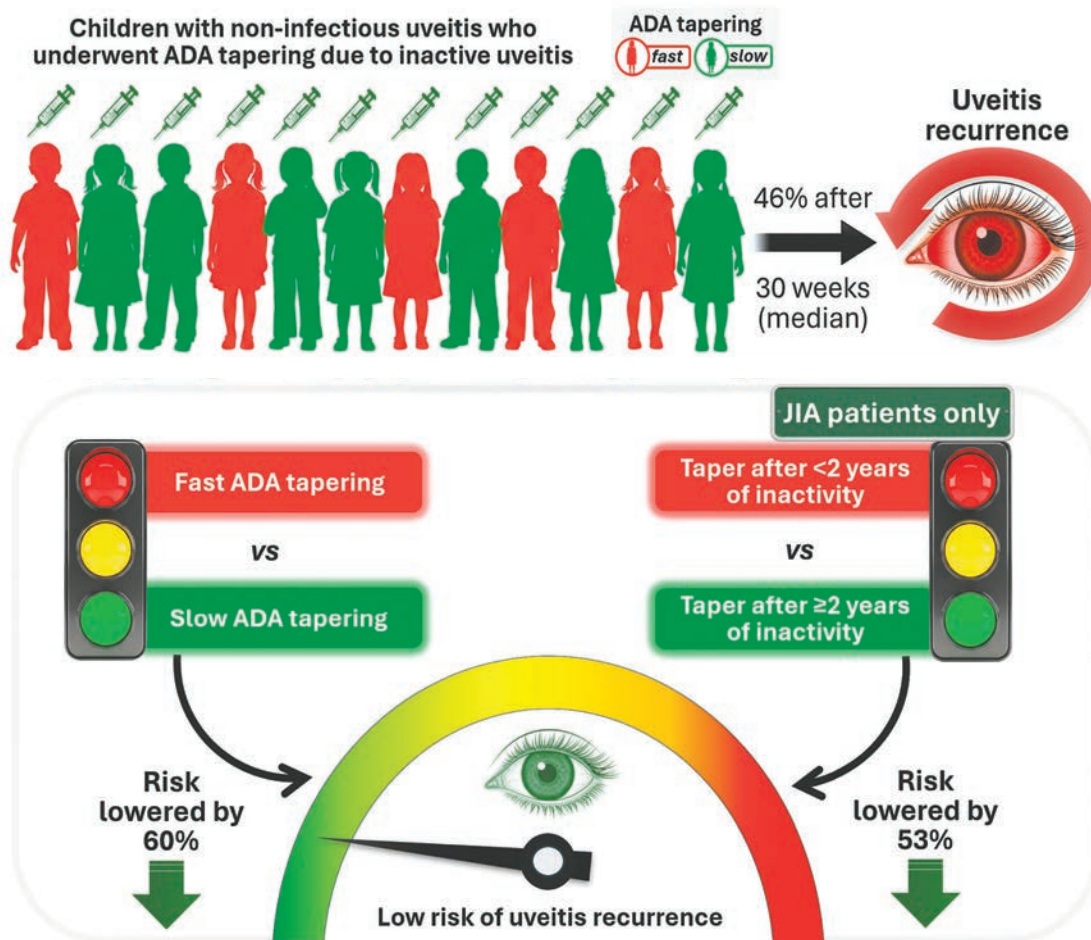
Weng et al performed a cohort study using UK Biobank data, including participants with baseline IMFI in thigh muscle assessed by magnetic resonance imaging and without history of knee or hip OA. The results indicated that elevated IMFI in the anterior thigh was associated with a higher incidence of knee OA, and elevated IMFI in both the anterior and posterior thigh were related to an increased risk of hip OA. Interaction and subgroup analyses suggest that the effect of IMFI is independent of sex and body mass index. Since IMFI is a modifiable risk factor, these findings may have important clinical and public health implications for the prevention and management of large joint OA.

# Recurrence Risk in Pediatric Non-Infectious Uveitis During Adalimumab Tapering

Marino et al, *Arthritis Rheumatol.* 2025;77:1254-1262.

## CORRESPONDENCE

**Achille Marino, MD, PhD:** achille.marino@asst-pini-cto.it



## KEY POINTS

- Nearly half of patients with NIU experienced relapse during ADA tapering, underscoring the need for careful monitoring.
- Gradual tapering of ADA reduces the risk of uveitis relapse in children with NIU.
- Starting ADA tapering after at least two years of inactive disease lowers recurrence rates in JIA-associated uveitis.

## SUMMARY

Non-infectious uveitis (NIU) is a serious inflammatory eye disease that can cause vision loss in children, particularly those with juvenile idiopathic arthritis (JIA). Many children are treated successfully with adalimumab (ADA), but questions remain about when and how to safely reduce or discontinue the drug after remission.

Marino et al assessed the risk for NIU relapse in a multicenter cohort of pediatric patients undergoing ADA tapering and evaluated potential predictors of this risk. The cohort included 114 children with NIU who began tapering ADA. Almost half (46%) experienced a relapse, typically within 30 weeks of starting the taper. Importantly, a slower tapering schedule significantly reduced the risk of relapse and extended the time without disease flare-ups. In addition, Marino et al found a significant decrease in recurrences among patients with JIA who began ADA tapering at least two years after achieving disease inactivity. These findings suggest that clinicians should taper ADA gradually and monitor patients closely to maintain remission.

# In this Issue

Highlights from this issue of *A&R* | By Lara C. Pullen, PhD

## Patients With OA Have an Expansion of CD25<sup>hi</sup> Switched Memory B Cells

Clinical evaluation of patients for osteoarthritis (OA) typically includes assessment of pain and imaging of affected joints, which often shows joint space narrowing and osteophytes. Unfortunately, many patients are not seen until the disease is relatively advanced, when irreversible damage has occurred. Thus, researchers have sought robust biomarkers for early disease that might facilitate earlier therapeutic or preventative interventions. Recently they have focused their attention on the role of memory B cells in inflammation, pathogenesis, and disease activity of OA.

**p.1228** In this issue, Sahu et al (p. 1228) describe a CD25<sup>hi</sup> switched memory B cell population in the blood of patients with early OA, which may be a potential biomarker of OA in its earlier stages. Their study highlights the potential of using patient-specific immune cell features, particularly in combination with advanced

magnetic resonance imaging, to improve current clinical methods for OA prognosis.

In their study, the investigators compared peripheral blood mononuclear cells (PBMCs) from healthy donors, patients with OA, and patients with degenerative meniscal tears (DMTs). Compared with healthy donors, there were distinct perturbations in the PBMCs of patients with OA, specifically, the CD25<sup>hi</sup>CXCR5<sup>hi</sup>CD27<sup>+</sup>IgD<sup>+</sup> switched memory B cell subpopulation. In contrast, there were fewer naive B cell populations in the same samples. These findings were confirmed in a smaller validation cohort.

Additionally, they found that the immune landscape of patients with DMT, a population at enhanced risk for OA, had a similar expansion of CD25<sup>hi</sup> switched memory B cells.

Single-cell RNA sequencing revealed dysfunction of interleukin-2/Stat5 and tumor necrosis factor signaling in the CD25<sup>hi</sup> switched

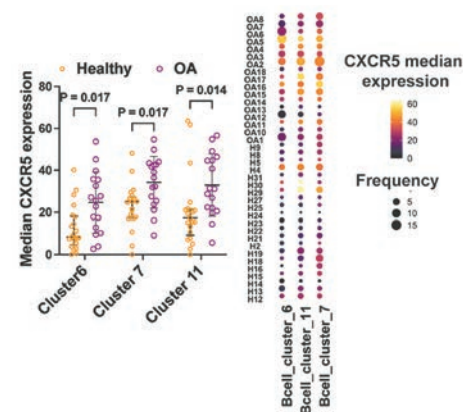


**Figure 1.** FlowSOM identifies perturbations in the naive and memory B cell repertoire in OA. Comparative analysis of median expression of CXCR5 in differentially abundant switched B cell memory clusters. Statistical significance was calculated by pairwise Wilcoxon rank sum test.

memory B cells. The authors conclude that further study of the role of the ILR2A/CD25 gene and the newly identified population of memory B cells in patients with OA may lead to a biomarker of early OA.

## Anti-PAD4 Autoantibodies Activate Monocytes and Exacerbate Inflammatory Arthritis

Previous studies have demonstrated the presence of interleukin-17A (IL-17A)-producing Th17 cells in the synovial tissue of patients with rheumatoid arthritis (RA). In this issue,

**p.1150** Won et al (p. 1150) describe a model in which the binding of autoantibodies targeting peptidylarginine deiminase 4 (PAD4) to monocytes triggers an inflammatory cascade that promotes immune cell recruitment to the joint and T cell activation. Their data support the hypothesis that antibody binding leads to the activation of synovial fibroblasts and the progression of more severe arthritis.

The researchers investigated the cellular targets and pathogenic role of anti-PAD4

autoantibodies derived from patients with RA in vitro, and in a collagen-induced arthritis (CIA) mouse model. They hypothesized that the increased production of proinflammatory chemokines by monocytes treated with anti-PAD4 antibodies was responsible for driving the notable infiltration of myeloid cells into the joints of arthritic mice treated with anti-PAD4 antibodies.

When they treated mice with anti-PAD4 autoantibodies, the mice exhibited enhanced T cell activity, as well as elevated IL-17A levels in the serum. They also found an increase in TNF $\alpha$ -producing lymph node CD4<sup>+</sup> or CD8<sup>+</sup> cells, including subsets that produced IL-17A and/or interferon- $\gamma$ ,

respectively. The treated mice also exhibited increased synovial fibroblast hyperplasia, which accompanied the severe inflammation. There were no differences in the deposition of citrullinated proteins in CIA mice between the anti-PAD4 antibody and isotype control treatment groups.

The authors acknowledge that while their study demonstrated that the binding of anti-PAD4 antibody clones to monocytes exacerbated CIA and triggered the release of inflammatory cytokines, it did not reveal the precise mechanism by which these antibodies activate monocytes. Their data are, however, consistent with the notion that anti-PAD4 antibodies can act as amplifiers of RA severity.

# Comprehensive Autoantibody Profiling of Patients With RA

Smoking and other lung exposures contribute to risk for rheumatoid arthritis (RA), suggesting that mucosal immunity may play a central role in its pathogenesis. In this issue,

p.1179

Mathsson Alm et al (p. 1179) report the results of a comprehensive serologic analysis of 6,907 patients with RA from five Scandinavian cohorts. The investigators detected rheumatoid factor and anticyclic citrullinated peptide isotypes along with antibodies to various modified peptides to characterize different anticitrullinated protein

antibody (ACPA) profiles in the patients. A panel of 33 patient-derived monoclonal antibodies were used to validate assays.

They found that citrulline-glycine motifs are prevalent targets of ACPA. Of note, ACPAs from patients with HLA-DRB1 alleles containing the RA shared epitope (SE) were more dominated by reactivity to non-glycine citrulline motifs. In contrast, patients with a history of smoking had higher reactivity to citrulline-glycine motifs. The authors conclude that examining ACPA specificity based on the recognition motif rather than

recognition of individual peptides, reveals distinct HLA-DR associations.

These observations provide additional insights into the B cell and T cell recognition of citrulline residues in RA. Specifically, the weak association between HLA SE alleles and glycine-containing peptides could implicate differences in T cell dependence in epitope spreading between different ACPA responses. They acknowledge, however, that while the findings provide insight into ACPA T cell dependence, the significance of ACPA targeting different protein structures remains unknown.

## Journal Club

*A monthly feature designed to facilitate discussion on research methods in rheumatology.*

## Clinical Factors Associated With PJP Despite Its Primary Prophylaxis: When to Stop Prophylaxis?

Kim et al, *Arthritis Rheumatol.* 2025;77:1263-1272.

*Pneumocystis jirovecii* pneumonia (PJP) is a potentially fatal complication in patients with systemic rheumatic diseases undergoing immunosuppressive therapy. Although its primary prophylaxis using trimethoprim-sulfamethoxazole (TMP-SMX) is known to be effective, clinical guidance on when it is safe to discontinue prophylaxis remains unclear. To address this gap, this study aimed to identify clinical factors associated with the development of PJP in patients receiving prophylaxis to inform tailored strategies regarding the timing of prophylaxis discontinuation.

This study retrospectively reviewed prophylactic TMP-SMX administration as primary PJP prophylaxis during immunosuppressive therapy for rheumatic diseases in a tertiary referral center in South Korea between 2010 and 2022. Because PJP incidence in patients receiving primary PJP prophylaxis is expected to be very low, a case-control design was selected. The observation period extended from the date of TMP-SMX initiation until occurrence of PJP, loss to follow-up, or 52 weeks after initiation. A Cox proportional hazards model with least absolute shrinkage and selection operator (LASSO) was used to identify the risk factors associated with PJP.

Among 1,294 prophylactic episodes, 10 cases of PJP occurred. Notably, 9 of these cases occurred after discontinuation of TMP-SMX, and the glucocorticoid (GC) dose at that time point was significantly higher in the PJP group compared to the control group. This observation prompted further

evaluation of GC dose thresholds. Discontinuation of prophylaxis while patients were receiving >12.5 mg/day of prednisone equivalent was significantly associated with increased PJP risk (crude HR 18.55 [95% CI] 2.35-146.42). This finding remained consistent after adjusting for relevant risk factors (age, impaired renal function, lymphopenia, and rituximab use) selected by LASSO (adjusted HR 13.84 [95% CI] 1.71-111.80). These results suggest that the optimal safest dose of prednisone to consider withdrawing PJP prophylaxis is 12.5 mg/day, but additional studies are needed.

### Questions

1. What are the strengths and limitations of using a case-control design in this context? What alternative study designs could be considered to strengthen causal inference in the absence of a randomized controlled trial?
2. What might be the impact of a follow-up period longer or shorter than 52 weeks?
3. What are the advantages of using LASSO for variable selection, especially in studies with rare outcome occurrences? What other methods could be considered?
4. How does defining each prophylactic episode as the unit of analysis (rather than the patient) influence the interpretation of results?

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2024 American College of Rheumatology (ACR) Guideline for the Screening, Treatment, and Management of Lupus Nephritis

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**Cover image:** The image on the cover (from Liu et al; pages 1216–1227) shows Safranin O/Fast Green staining of mouse knee joint articular cartilage, highlighting extensive proteoglycan loss and structural degradation in a Sirt5-deficient mouse subjected to high-fat diet. The loss of red staining reflects diminished cartilage matrix integrity, accompanied by surface fibrillation and hypocellularity. This image illustrates the exacerbated osteoarthritic changes linked to impaired Sirt5-mediated regulation of lysine malonylation and disrupted chondrocyte metabolism during obesity.

# 2024 American College of Rheumatology (ACR) Guideline for the Screening, Treatment, and Management of Lupus Nephritis

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**Objective.** The objective is to provide evidence-based and expert guidance for the screening, treatment, and management of lupus nephritis.

**Methods.** The Core Team developed clinical questions for screening, treatment, and management of lupus nephritis using the PICO format (population, intervention, comparator, and outcome). Systematic literature reviews were completed for each PICO question, and the Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology was used to assess the quality of evidence and to formulate recommendations. The Voting Panel achieved a consensus  $\geq 70\%$  on the direction (for or against) and strength (strong or conditional) of each recommendation.

**Results.** We present 28 graded recommendations (7 strong, 21 conditional) and 13 ungraded, consensus-based good practice statements for the screening and management of lupus nephritis. Our recommendations focus on the unifying principle that lupus nephritis therapy is continuous and ongoing, rather than consisting of discrete induction/initial and maintenance/subsequent therapies. Therapy should include pulse glucocorticoids followed by oral glucocorticoid taper and two additional immunosuppressive agents for 3–5 years for those achieving complete renal response.

**Conclusion.** This guideline provides direction for clinicians regarding screening and treatment decisions for management of lupus nephritis. These recommendations should not be used to limit or deny access to therapies, as treatment decisions may vary due to the unique clinical situation and personal preferences of each individual patient.

#### SIGNIFICANCE/HIGHLIGHTS:

- Lupus nephritis (LN) therapy should be initiated as soon as possible after diagnosis.
- Conditionally recommended treatment for Class III/IV (with or without Class V) LN includes triple therapy with intravenous glucocorticoids followed by oral glucocorticoid ( $\leq 0.5$  mg/kg/day prednisone, maximum dose 40 mg/day) taper and:
  - a. Mycophenolic acid analog (MPAA) plus belimumab -or-
  - b. MPAA plus a calcineurin inhibitor (CNI) -or-
  - c. Euro-Lupus Nephritis Trial (ELNT) low-dose cyclophosphamide (CYC) plus belimumab (with substitution of MPAA after completion of CYC).
- Conditionally recommended therapy for pure Class V LN ( $\geq 1$  g proteinuria) includes combination therapy with intravenous glucocorticoids followed by oral glucocorticoid ( $\leq 0.5$  mg/kg/day prednisone, maximum dose 40 mg/day) taper and MPAA plus a CNI.
- A glucocorticoid taper goal of  $\leq 5$  mg prednisone daily by 6 months is conditionally recommended.
- The conditionally recommended duration of immunosuppressive therapy (beyond hydroxychloroquine) for people with LN who achieve a complete renal response (CRR) is 3-5 years.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease with a prevalence of 72/100,000 persons in the United States.<sup>1</sup> Lupus nephritis (LN) occurs in close to half of SLE patients and carries a mortality rate of up to 30% at 10 years; 10–22% of people with LN will develop end stage kidney disease (ESKD).<sup>2,3</sup> Among those with SLE, male sex, younger age, and

African, Hispanic, American Indian/Alaska Native, and Asian ancestry increase the likelihood of LN and ESKD.<sup>4–8</sup> Socially disadvantaged individuals in medically underserved areas have worse kidney outcomes.<sup>9–11</sup>

The American College of Rheumatology (ACR) last published LN clinical practice guidelines in 2012.<sup>12</sup> Recommendations called for induction therapy with high-dose glucocorticoids plus mycophenolate mofetil (MMF) or cyclophosphamide (CYC) and endorsed mycophenolate for maintenance therapy. Since then, belimumab and voclosporin<sup>13,14</sup> have been approved by the US Food and Drug Administration (FDA) for LN treatment, prompting a conceptual shift from induction/initial and maintenance/subsequent therapy to one of combination, ongoing therapy targeting different arms of the immune system.<sup>15–17</sup> Evidence on the relative effectiveness and toxicity of systemic glucocorticoids has also evolved.<sup>18</sup>

Recommendations in this guideline follow certain guiding principles (Table 1) and assume the exclusion of alternative diagnoses. Most are conditional; they are based on systematic literature reviews, values, and preferences elicited from an LN Patient Panel, and the expert opinion of adult and pediatric rheumatologists and nephrologists and a rheumatology physician assistant. The recommendations are intended to promote optimal outcomes for the most encountered LN scenarios; they include therapies available in the United States as of 2024 and apply to LN in adults and children.<sup>19–21</sup> Additional pediatric-specific or older adult concerns are addressed in Good Practice Statements (GPS). We acknowledge that therapeutic decisions vary depending on clinical presentation and patient preferences, and are limited by access to specialists, procedures, and medications. When recommended medications are not available, this guideline should not preclude the use of available traditional therapies. Recommendations are not based on patient-reported race or ethnicity, as evidence

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**Table 1.** Guiding Principles\*

|                                                                                                                                                                                       |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>The goals of LN treatment</b> are to preserve kidney function, reduce morbidity and mortality associated with chronic kidney disease, and minimize medication-related toxicities.  |
| <b>Collaborative care from rheumatology and nephrology</b> should be offered to people with LN whenever possible.                                                                     |
| <b>Shared decision-making</b> between clinicians and patients is essential as it respects patient values and preferences, leading to better adherence and outcomes.                   |
| <b>Healthcare disparities</b> may impact outcomes in people with LN; equitable implementation of treatment recommendations aims to improve outcomes and alleviate health disparities. |
| <b>Pediatric and geriatric</b> good practice statements are included when applicable.                                                                                                 |

\* LN, lupus nephritis.

for race- or ethnicity-specific treatment efficacy is limited and confounded by socioeconomic factors. We present 28 Grading of Recommendations Assessment, Development and Evaluation (GRADE)-generated recommendations (7 strong, 21 conditional) and 13 ungraded, consensus-based GPS.

# METHODS

This guideline follows the ACR guideline development process and policy directing management of conflicts of interest and disclosures (<https://rheumatology.org/clinical-practice-guidelines>), which includes GRADE methodology.<sup>22,23</sup> (Supplementary Materials 1). The Core Leadership Team (LRS, RAM, AAskanase, BLB, MD, AD, LTH, BHR, MBFS) drafted clinical population, intervention, comparator, and outcomes (PICO) questions (Supplementary Materials 2). The Literature Review Team performed systematic literature reviews for the PICO questions, graded the quality of evidence (high, moderate, low, very low), and produced an evidence report (Supplementary Materials 3). The evidence was reviewed, recommendations were formulated by the Core Team and voted on by an expert Voting Panel. Additionally, a Patient Panel comprised of 15 people with LN (two of whom also served on the Voting Panel) informed the Voting Panel on patients' perspectives and preferences.

Consensus required ≥70% agreement on direction (for or against) and strength (strong or conditional) of each recommendation. A recommendation is categorized as *strong* if the panel is confident that the benefits of an intervention clearly outweigh the harms (or vice versa); a *conditional* recommendation denotes uncertainty

regarding the balance of benefits and harms, low quality of evidence, or that the recommendation is particularly sensitive to individual patient preferences and patient-provider discussion.

The strength of a recommendation determines its clinical implications and should be considered when interpreting and using it for patient care. For patients, a strong recommendation suggests that most people in their situation would want the recommended course of action and only a small proportion would not; for clinicians, it means most patients should receive the recommended course of action. With a conditional recommendation, the implication for patients is that most people in their situation would want the recommended course of action, but many would not; for clinicians, it means they should recognize that different choices will be appropriate for different patients and they must engage in shared decision-making with each patient to arrive at a management decision.

GPS are made when panel members are confident that there is unequivocal benefit or harm despite indirect or inadequate evidence. Some of the original 249 PICO-generated recommendations were combined into broader recommendations, some generated good practice statements, and some were relegated to a future research agenda.

Rosters of the Core Leadership Team, Literature Review Team, Voting Panel, and Patient Panel are included in Supplementary Materials 4. Search strategies and study selection details are provided in Supplementary Materials 5 and 6. Approval from Human Studies Committees was not required.

# Scope

This guideline addresses screening and treatment for all people with LN regardless of age, race, ethnicity, and other individual patient variables. It is the first part of a broader ACR SLE guideline project; the second part will include a general approach to SLE therapy as well as organ-specific treatment recommendations.

# RESULTS/RECOMMENDATIONS

Terminology, definitions, and abbreviations are summarized in Table 2; recommendations and good practice statements are listed in Table 3.

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## Screening

**In people with SLE without known kidney disease, we strongly recommend screening for proteinuria at least every 6–12 months, OR when experiencing extra-renal flares.**

The Voting Panel stated that for recent onset, or recently active SLE, LN surveillance every 6 months is most appropriate, consistent with the 2023 ACR SLE quality measures.<sup>24</sup> Conversely, for those with longstanding and mild and inactive SLE, annual testing is adequate. This recommendation is strong,

despite a lack of high-certainty evidence, because the risk of missing new onset LN requiring urgent treatment far outweighs the minimal risk of obtaining a urine sample.

## Kidney biopsy

**GPS: Prompt percutaneous kidney biopsy should be performed in people with SLE when LN is suspected (unless contraindicated or not feasible), as histopathologic biopsy**

**Table 2.** Guideline terminology, definitions, and abbreviations\*

| Terminology                               | ACR LN Guideline Definitions <sup>a</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|-------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Kidney biopsy                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| Diagnostic                                | Biopsy performed to establish diagnosis and guide treatment                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| For cause                                 | Biopsy performed in response to clinical indications or change in patient status                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Per protocol                              | Biopsy performed according to a predetermined schedule or study protocol, regardless of clinical response                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| Therapy                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| Initial / induction therapy               | Prior terminology: Therapy prescribed immediately after diagnosis of new LN or flare of LN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| Subsequent / maintenance therapy          | Prior terminology: Therapy prescribed to patients on initial therapy for 6–12 months who have achieved at least a PRR                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Lupus nephritis therapy                   | Preferred terminology:<br>Ongoing therapy (ie, initial plus subsequent therapy) based on current recommendations for combination therapy that starts at diagnosis and continues throughout the treatment course<br>TRIPLE therapy:<br>GC (pulse intravenous: 250–1000 mg methylprednisolone daily × 1–3 days, followed by oral 0.5 mg/kg/day (maximum dose 40 mg/day) taper<br>Plus:<br>two immunosuppressive therapies, usually<br>a) MPAA plus belimumab OR<br>b) MPAA plus CNI OR<br>c) ELNT low-dose CYC plus belimumab (MPAA substituted for CYC after CYC course is completed).<br>DUAL therapy:<br>GC plus one immunosuppressive therapy, usually MPAA or ELNT low-dose CYC |
| Renal response                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| Complete renal response (CRR)             | Within 6–12 months of starting therapy (may take >12 months):<br>• Reduction in proteinuria <0.5 g/g (50 mg/mmol) (24-hour collection or urine protein/creatinine ratio); AND<br>• Stabilization or improvement in kidney function (+ 20% baseline i.e. at least 80% of baseline) <sup>b</sup>                                                                                                                                                                                                                                                                                                                                                                                     |
| Partial renal response (PRR)              | Within 6–12 months of starting therapy:<br>• Reduction in proteinuria by at least 50% and to <3 g/g (300 mg/mmol) (24-hour collection or urine protein/creatinine ratio); AND<br>• Stabilization of kidney function (+ 20% baseline i.e., at least 80% baseline) <sup>b</sup>                                                                                                                                                                                                                                                                                                                                                                                                      |
| Inadequate renal response/<br>Nonresponse | Lack of achieving at least a PRR despite adherence to appropriate treatment for active LN of any class by 6–12 months                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Refractory disease                        | Persistently active disease and absence of at least a PRR to at least two different appropriate 6-month courses of therapy for active LN of any class                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Proteinuria                               | Protein as measured by 24-hour collection (g/24hr) or random urine protein-creatinine ratio (g/g)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| Glomerular hematuria                      | Urine sediment positive for acanthocytes, ≥5%, RBC casts                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Decreased kidney function                 | Abnormal eGFR below expected level for age and clinical history, or decreasing eGFR with no attribution other than SLE <sup>c</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |

\* ACR, American College of Rheumatology; CNI, calcineurin inhibitor therapy; CYC, cyclophosphamide; eGFR, estimated glomerular filtration rate; ELNT, Euro-Lupus Nephritis Trial; GC, glucocorticoid; LN, lupus nephritis; MPAA, mycophenolic acid analogs; RBC, red blood cell; SLE, systemic lupus erythematosus.

<sup>a</sup> Terminology and definitions vary across specialties, guidelines, and clinical trials. Those listed here reflect the consensus of the Voting Panel as being both reasonable and relevant; however, no systematic analyses were performed, and others may prefer alternative definitions.

<sup>b</sup> Some experts and clinical trials have included a requirement for low dose of prednisone (eg, ≤5 mg/d equivalent) in addition to proteinuria and renal function requirements for CRR and PRR definitions; however, many do not. This was extensively discussed when definitions were created. Although GC dose is a part of validated SLE remission criteria, and while we recommend a goal of ≤5 mg/d prednisone equivalent by 6 months of therapy in this guideline, we did not consider this to be an appropriate mandatory criterion for the renal response definitions.

<sup>c</sup> Variably defined across studies – both irreversible damage and active disease impact kidney function and proteinuria and may require kidney biopsy to distinguish.

**Table 3.** Recommendations and good practice statements\*

| Recommendations and Good Practice Statements                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | Strength                                                | Level of Evidence                                                   | PICOs addressed                                                                       |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| <b>SCREENING:</b><br><b>In people with SLE without known kidney disease, we strongly recommend screening for proteinuria at least every 6–12 months, OR when experiencing extra-renal flares.</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Strong                                                  | Indirect evidence;<br>Very low                                      | P16(a)<br>(revision)                                                                  |
| <b>KIDNEY BIOPSY:</b><br><i>GPS: Prompt kidney biopsy should be performed in people with SLE when LN is suspected (unless contraindicated or not feasible) as histopathologic biopsy features will confirm the diagnosis, rule out mimicking diseases, and impact therapy decisions.</i><br><b>In people with SLE who have proteinuria &gt;0.5 g/g and/or impaired kidney function not otherwise explained, we conditionally recommend performing a percutaneous kidney biopsy.</b><br><b>For people with treated LN in remission who present with suspected LN flare (increased proteinuria, hematuria, and/or worsening kidney function), OR for people with ≥6 months of appropriate treatment and ongoing or worsening proteinuria, hematuria, and/or decreased kidney function, we conditionally recommend repeat percutaneous kidney biopsy.</b>                                                                                                                                                                                     | Conditional<br><br>Conditional                          | Low-<br>Very low<br><br>Low-<br>Very low                            | P1(a-e)<br>P3(e-h)<br><br>P2(a-e)<br>P4(a-c)                                          |
| <b>TREATMENT OF ACTIVE LN (CLASS III/IV OR CLASS V)</b><br><i>GPS: Prompt glucocorticoid treatment should be administered for suspected LN to suppress acute inflammation while awaiting a kidney biopsy and the histopathology results.</i><br><i>GPS: Dosage of LN medications should be adjusted in people with decreased GFR at initiation of therapy and periodically.</i><br><i>GPS: Adjunctive treatment with systemic anticoagulation for people with LN and significant risk factors for thrombosis (eg, low serum albumin in context of severe proteinuria) should be discussed with nephrology.</i>                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                         |                                                                     |                                                                                       |
| <b>IN PEOPLE WITH ACTIVE, NEW ONSET OR FLARE OF CLASS III/IV OR CLASS V LN:</b><br><b>...If not already on HCQ treatment, we strongly recommend initiation and continuation of HCQ to manage and prevent lupus clinical manifestations, unless contraindicated.</b><br><b>...With any elevation in level of proteinuria, including &lt;0.5g/g, we conditionally recommend the addition of RAAS-I therapy.</b><br><b>...We conditionally recommend pulse intravenous glucocorticoids followed by oral prednisone (≤0.5 mg/kg/d, max of 40 mg/d) with taper to a target dose of ≤5mg/day by 6 months.</b><br><b>... Who have achieved and sustained a complete response after treatment with any (triple or dual) immunosuppressive therapy, we conditionally recommend a total duration of therapy of at least 3–5 years.</b>                                                                                                                                                                                                               | Strong<br><br>Conditional<br>Conditional<br>Conditional | Low-<br>Very low<br><br>Low-<br>Very low<br>Moderate-low<br><br>Low | P15(a)<br><br>P7(d)<br>P9(a)<br>P7(a-c)<br>P8(a,b)<br>P9 (a-c)<br>P8(o,p)<br>P10(l,m) |
| <b>IN PEOPLE WITH ACTIVE, NEW ONSET, OR FLARE OF CLASS III/IV (WITH OR WITHOUT CONCOMITANT CLASS V LN):</b><br><b>... We conditionally recommend therapy with a triple immunosuppressive regimen consisting of pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper, plus:</b><br><b>a) MPAA plus belimumab -or-</b><br><b>b) MPAA plus CNI -or-</b><br><b>c) Euro-Lupus Nephritis Trial (ELNT) low-dose CYC plus belimumab (MPAA substituted for CYC after CYC course is complete).</b><br><b>... We conditionally recommend an MPAA-based regimen over a CYC-based regimen.</b><br><b>... With proteinuria ≥3g/g, we conditionally recommend a triple immunosuppressive regimen containing pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper, plus MPAA plus CNI over a regimen containing belimumab.</b> | Conditional<br><br>Conditional<br>Conditional           | Moderate-<br>Low<br><br>Low-<br>Very low<br>Low                     | P7 (j,k,n-q)<br>P8 (f-h,k-m)<br><br>P7(g,h)<br>P7 (l,p)                               |

(Continued)

**Table 3.** (Cont'd)

| Recommendations and Good Practice Statements                                                                                                                                                                                                                                                                                                                                | Strength              | Level of Evidence    | PICOs addressed                 |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|----------------------|---------------------------------|
| ... With extra-renal manifestations, we conditionally recommend a triple immunosuppressive therapy that contains belimumab over a regimen containing a CNI.                                                                                                                                                                                                                 | Conditional           | Low                  | P7(p2) (revision)               |
| ... We conditionally recommend a target MMF dose of 2–3g/d (or equivalent).                                                                                                                                                                                                                                                                                                 | Conditional           | Very low             | P7(l)                           |
| ... Receiving a CYC-based regimen, we conditionally recommend the ELNT low-dose CYC regimen over a high-dose monthly pulse IV regimen;                                                                                                                                                                                                                                      | Conditional<br>Strong | Very low<br>Very low | P7(e)<br>P7(f)                  |
| <b>We also strongly recommend the ELNT-low dose CYC regimen over a daily oral CYC regimen.</b>                                                                                                                                                                                                                                                                              |                       |                      |                                 |
| ... Who have undergone triple immunosuppressive therapy and achieved a complete renal response, we conditionally recommend continuing the same immunosuppressive regime.                                                                                                                                                                                                    | Conditional           | Moderate-Low         | P8.3 (revision)<br>P8 (f-h,k-m) |
| ...Who have undergone triple immunosuppressive therapy and achieved a partial renal response, we conditionally recommend individualizing therapy depending on clinical factors that include the trajectory of response.                                                                                                                                                     | Conditional           | None                 | P8.4 (revision)                 |
| ... Who have undergone dual immunosuppressive therapy (glucocorticoids plus either CYC or MPAA) and achieved a complete renal response, we conditionally recommend continuing therapy with MPAA over AZA.                                                                                                                                                                   | Conditional           | Low                  | P8.1 (revision)<br>P8(d,e,j)    |
| ... who have undergone dual immunosuppressive therapy (glucocorticoids plus either CYC or MPAA) and achieved a partial renal response, we conditionally recommend escalating therapy to a triple immunosuppressive regimen.                                                                                                                                                 | Conditional           | None                 | P8.2 (revision) P8 (v-x, aa-cc) |
| <b>IN PEOPLE WITH ACTIVE, NEW ONSET, OR FLARE OF (PURE) CLASS V LN:</b>                                                                                                                                                                                                                                                                                                     |                       |                      |                                 |
| ...With proteinuria $\geq 1$ g/g we conditionally recommend treatment with a triple immunosuppressive regimen consisting of pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily x 1–3 days) followed by oral glucocorticoid ( $\leq 0.5$ mg/kg/day, maximum dose 40 mg/day) taper, and MPAA plus CNI (over MPAA plus belimumab, or CYC plus belimumab). | Conditional           | Indirect: Very low   | P9(p)                           |
| ...with proteinuria $< 1$ g/g, we conditionally recommend treatment with glucocorticoids and/or immunosuppressant therapy (MPAA, AZA, or CNI) over no glucocorticoid or other immunosuppression.                                                                                                                                                                            | Conditional           | None                 | P9(b)                           |
| <b>NON-RESPONSIVE OR REFRACTORY LN:</b>                                                                                                                                                                                                                                                                                                                                     |                       |                      |                                 |
| <i>GPS: Medication dose and patient adherence should be assessed as an important first step in evaluating inadequate response or refractory LN, as insufficient treatment is an important cause of non-response.</i>                                                                                                                                                        |                       |                      |                                 |
| In people with any LN class with nonresponse (i.e., have not achieved at least a partial renal response by 6–12 months) we conditionally recommend escalation of treatment:                                                                                                                                                                                                 | Conditional           | Very low-None        | P11.1, P11.2 (revision)         |
| • For initial dual therapy, escalate to triple therapy (pulse intravenous glucocorticoids, 250–1000 mg methylprednisolone daily for 1–3 days, followed by oral glucocorticoid $\leq 0.5$ mg/kg/day, maximum dose 40 mg/day taper, plus either MPAA plus belimumab, MPAA plus CNI, or ELNT CYC plus belimumab).                                                              |                       |                      |                                 |
| • For initial triple therapy, change to an alternative triple therapy or consider addition of an anti-CD20 agent as a second immunosuppressive.                                                                                                                                                                                                                             |                       |                      |                                 |
| In people with any LN class with refractory disease (i.e., failed two standard therapy courses), we conditionally recommend treatment escalation to a more intensive regimen, including addition of anti-CD20 agents, combination therapy with three non-glucocorticoid immunosuppressives (i.e., MPAA, belimumab and CNI), or referral for investigational therapy.        | Conditional           | Very low- None       | P12.1, P12.2 (revision)         |
| <b>OTHER LUPUS KIDNEY DISEASE:</b>                                                                                                                                                                                                                                                                                                                                          |                       |                      |                                 |
| <i>GPS: Alternative etiologies of kidney dysfunction in people with SLE should be carefully excluded, including non-inflammatory etiologies such as hypertensive, diabetic, and medication-induced nephropathy.</i>                                                                                                                                                         |                       |                      |                                 |

(Continued)

**Table 3.** (Cont'd)

| Recommendations and Good Practice Statements                                                                                                                                                                                                                                                            | Strength    | Level of Evidence              | PICOs addressed     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|--------------------------------|---------------------|
| <b>ADJUNCTIVE / NON-IMMUNOLOGIC TREATMENT:</b>                                                                                                                                                                                                                                                          |             |                                |                     |
| <i>GPS: Adjunctive and non-immunologic therapies and practices should be initiated in addition to appropriate immunosuppressive therapy to improve overall kidney health (Table 4).</i>                                                                                                                 |             |                                |                     |
| <i>GPS: In children with childhood-onset SLE (cSLE) and LN, glucocorticoid regimens should be reduced to pediatric-appropriate doses for children, as reduction of cumulative glucocorticoid dosing is critically important given the early age of onset of cSLE onset and attendant comorbidities.</i> |             |                                |                     |
| <i>GPS: In children with cSLE and LN, clinicians should monitor for delayed pubertal onset and decreased growth velocity that can result from disease activity and glucocorticoid treatment and consider referral to pediatric endocrinology if indicated.</i>                                          |             |                                |                     |
| <i>GPS: For children with cSLE, a structured, intentional transition from pediatric to adult rheumatology care is indicated to avoid poor outcomes during this vulnerable period.</i>                                                                                                                   |             |                                |                     |
| <i>GPS: For older people with LN, medication number, type, and dosage should be regularly assessed, given the risks of polypharmacy and age-related decline in GFR in this population.</i>                                                                                                              |             |                                |                     |
| <b>MONITORING LN ACTIVITY:</b>                                                                                                                                                                                                                                                                          |             |                                |                     |
| <b>In people with SLE and LN who have not achieved CRR, we strongly recommend quantifying proteinuria at least every 3 months.</b>                                                                                                                                                                      | Strong      | Indirect evidence;<br>Very low | P16(b,c) (revision) |
| <b>In people with SLE with known nephritis in sustained clinical renal remission, we strongly recommend quantifying proteinuria every 3–6 months.</b>                                                                                                                                                   | Strong      | Indirect evidence;<br>Very low | P16(d) (revision)   |
| <i>GPS: In people with LN, serum complement and anti-dsDNA antibody should be measured at every clinic visit (but not more frequently than monthly).</i>                                                                                                                                                |             |                                |                     |
| <b>RENAL REPLACEMENT THERAPIES:</b>                                                                                                                                                                                                                                                                     |             |                                |                     |
| <i>GPS: Decisions for initiation and type of dialysis and timing for kidney transplant require close collaboration with nephrology.</i>                                                                                                                                                                 |             |                                |                     |
| <b>In people with LN and ESKD, we strongly recommend kidney transplantation over dialysis.</b>                                                                                                                                                                                                          | Strong      | High                           | P18(a)              |
| <b>In people with LN who have progressive loss of kidney function and are nearing ESKD (defined as an eGFR of 15 ml/min/1.73m<sup>2</sup>), we conditionally recommend preemptive kidney transplant over dialysis/no preemptive kidney transplant.</b>                                                  | Conditional | Very low                       | P22(a)              |
| <b>In people with LN and ESKD, we conditionally recommend proceeding with kidney transplantation without requiring complete clinical or serologic remission, provided there is no other major organ involvement.</b>                                                                                    | Conditional | Very low                       | P23(a,b)            |
| <b>In people with LN on current dialysis or after kidney transplantation, we strongly recommend regular follow up with rheumatology.</b>                                                                                                                                                                | Strong      | Very low                       | P20(a,b)            |

\* Anti-CD20 therapy: rituximab or obinutuzumab. AZA, azathioprine; CNI, calcineurin inhibitor therapies (cyclosporine, tacrolimus, voclosporin); CRR, complete renal response; CYC, cyclophosphamide; dsDNA, double-stranded DNA; eGFR, estimated glomerular filtration rate (various definitions are used in clinical studies; calculations of eGFR from creatinine in recent research do not include coefficients for race; however, earlier literature does); ESKD, end stage kidney disease; GFR, glomerular filtration rate; GPS, Good Practice Statements; HCQ, hydroxychloroquine; LN, lupus nephritis; MMF, mycophenolate mofetil; MPAA, mycophenolic acid analogs (including mycophenolate mofetil, or MMF, and mycophenolic acid, or MPA); PICO, population, intervention, comparator, outcome; RAAS-I, renin-angiotensin-aldosterone system inhibitors (including angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, mineralocorticoid receptor antagonists); SLE, systemic lupus erythematosus.

**features will confirm the diagnosis, rule out mimicking diseases, and impact therapy decisions.**

Biopsy should be read by a nephropathologist using the International Society of Nephrology (ISN) and Renal Pathology Society (RPS) classification<sup>25</sup> and include LN class and activity/chronicity indices. Risk of major bleeding with kidney biopsy, ie, requiring a blood transfusion or embolization procedure, is very low (~1–2%).<sup>26–31</sup> For people with SLE, risk may be higher (up to 3%) in specific subgroups including those with thrombocytopenia, decreased kidney function, and antiphospholipid

syndrome.<sup>26,32–35</sup> Patient representatives shared concerns about the invasive nature of biopsy and emphasized the importance of physicians discussing the procedure's benefits and risks.

While we recommend prompt kidney biopsy with treatment based on histology, biopsy may not always be possible. In the absence of a kidney biopsy, those with nephritic features (eg, hematuria, hypertension, impaired kidney function) are usually best treated according to Class III/IV recommendations, and those with nephrotic features (eg, proteinuria, ≥3.5 g/g, hypoalbuminemia) according to Class V recommendations.

**In people with SLE who have proteinuria >0.5 g/g and/or impaired kidney function not otherwise explained, we conditionally recommend performing a percutaneous kidney biopsy.**

Kidney biopsy has value in people with SLE with isolated impaired kidney function that is not otherwise explained because histologic disease activity can occur without proteinuria.<sup>36–39</sup>

**For people with treated LN previously in remission who later present with suspected LN flare (increased proteinuria, hematuria, and/or worsening kidney function), OR for people with ≥6 months of appropriate treatment and ongoing or worsening proteinuria, hematuria, and/or decreased kidney function, we conditionally recommend repeat percutaneous kidney biopsy.**

Clinical judgment and patient preference are essential in deciding when to repeat kidney biopsy. With appropriate medication dosing and adherence, worsening kidney function or proteinuria should prompt consideration of repeat biopsy. Change in kidney histology is found in 40–50% of repeat biopsies.<sup>40–42</sup> While repeat biopsy for isolated significant/increasing hematuria can be considered when other etiologies are excluded, the value of biopsy in the setting of chronic low-level hematuria is uncertain. The Voting Panel did not issue a recommendation on per protocol (ie, scheduled) repeat kidney biopsies but considered this an important research item.

## Treatment of LN:

**GPS: Prompt glucocorticoid treatment should be administered for suspected LN to suppress acute inflammation while awaiting a kidney biopsy and the histopathology results.**

**GPS: Dosage of LN medications should be adjusted in people with decreased glomerular filtration rate (GFR) at the initiation of therapy and periodically as indicated during the disease course (Supplementary Materials 7).**

**GPS: Adjunctive treatment with systemic anticoagulation for people with LN and significant risk factors for thrombosis (e.g., low serum albumin in the context of severe proteinuria) should be discussed with nephrology.**

Nephrology guidelines recommend treating patients with a serum albumin concentration below 2.0–2.5 g/dl in the setting of nephrotic range proteinuria with full-dose anticoagulation to prevent clotting unless the risk of bleeding is high.<sup>43</sup>

## Class III/IV or Class V LN:

**In people with LN who are not already on hydroxychloroquine (HCQ), we strongly recommend initiation and continuation of HCQ to manage and prevent extra-renal manifestations, unless contraindicated.**

This is a strong recommendation based on low certainty evidence due to the well-established role for HCQ in overall SLE management. HCQ reduces risk of mortality in people with SLE, including those with lupus nephritis.<sup>44–46</sup> Dose adjustment for low GFR should be considered because kidney disease is a risk factor for retinal toxicity<sup>46</sup> (Supplement Materials 7).

**In people with active, new onset or flare of LN with any elevation in proteinuria, including <0.5 g/g, we conditionally recommend the addition of renin-angiotensin-aldosterone system inhibitor (RAAS-I) therapy.**

This recommendation applies to any level of persistent proteinuria above the normal range and is based on studies showing the kidney protective effects of RAAS-I in proteinuric LN and advanced chronic kidney disease (CKD).<sup>47</sup> Additionally, a pediatric study demonstrated that addition of RAAS-I led to earlier glucocorticoid discontinuation.<sup>48</sup> Use may be limited by blood pressure or estimated glomerular filtration rate (eGFR).

**In people with active, new onset or flare of LN, we conditionally recommend pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) with taper to a target dose of ≤5 mg/day by 6 months.**

A recent systematic review and meta-analysis determined that pulse glucocorticoids followed by oral glucocorticoids (up to 40 mg/day) maximized complete renal response while minimizing toxicities.<sup>18,49–51</sup> A range of pulse therapy dosing is presented to accommodate individualized treatment approaches.<sup>52</sup> Lower doses have been utilized in some recent treatment trials,<sup>14</sup> and patients emphasized their preference for minimizing glucocorticoid dose. The tapering regimen in clinical practice should be individualized and based on monitoring of both renal and extra-renal disease activity. Data informing the optimal dosing of glucocorticoids for pure Class V LN are limited.

**In people with new onset or flare of LN who have achieved and sustained a complete renal response after treatment with any (triple or dual) immunosuppressive therapy, we conditionally recommend a total duration of immunosuppressive therapy of at least 3–5 years.**

The advent of triple therapies blurred the distinction between induction therapy and maintenance therapy. Traditionally, patients were initially treated with one drug plus glucocorticoid followed by a “less toxic” drug for maintenance. Induction implied remission was achieved; however, in the short exposure to induction therapy (usually 3–6 months), most patients did not achieve remission. Maintenance implied maintenance of remission; but for most patients, maintenance served the initial purpose of consolidation.<sup>53</sup>

Current regimens aim to provide initial glucocorticoid and immunosuppressive therapies to rapidly reduce disease activity, with continuation of immunosuppressive therapies until disease is inactive, which often takes at least 12 months. Typically, some immunosuppressive therapy should be continued for at least

3–5 years of total treatment before considering withdrawal.<sup>54,55</sup> Support for a relatively long exposure to immunosuppression comes from repeat biopsy studies showing persistence of immunologic activity and immune complexes for several years after starting therapy; risk of LN flare is increased with withdrawal of immunosuppression while histologic activity remains.<sup>56</sup>

Over time, immunosuppressive therapy dosage may be tapered in stable patients as determined by renal and extra-renal disease activity and medication tolerability. No evidence provides robust guidance regarding optimal tapering practice; these decisions are currently made based on clinical expertise and patient preference. Risk of nephrotoxicity may impact decisions regarding the total duration of therapy with CNIs. HCQ should be continued indefinitely if there are no contraindications.

### **Class III/IV LN (with or without Class V LN):**

Class III/IV LN lesions, characterized by endocapillary hypercellularity, are highly inflammatory and destructive. When occurring concomitantly with Class V, the presence of Class III/IV lesions drives therapy choice. Until complete renal response (CRR) is achieved, patients should be closely monitored and have therapies adjusted accordingly based on individual risk factors including blood pressure, proteinuria, and kidney function (Figure 1).

**In people with active, new onset or flare of Class III/IV (±V) LN, we conditionally recommend therapy with a triple immunosuppressive regimen consisting of pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper plus:**

- a. MPAA plus belimumab -or-
- b. MPAA plus CNI -or-
- c. Euro-Lupus Nephritis Trial (ELNT) low-dose CYC<sup>53</sup> plus belimumab (MPAA substituted for CYC after CYC course complete).

Recent randomized controlled trials (RCTs) suggesting overall improved outcomes with triple versus dual therapies guided discussion and voting for this recommendation.<sup>13,14</sup> While the trials were randomized and controlled, the certainty of evidence was assessed as low-moderate. The recommendation for triple therapy is conditional, ie, sensitive to individual patient preferences and patient-clinician discussion. A sensitivity analysis excluding Voting Panel members who had relevant conflicts of interest for this recommendation (5 of 21 members) resulted in no change in direction or strength.

Numerous factors will impact a decision regarding type of triple LN therapy. With eGFR ≤45, blood pressure >165/105, or significant chronicity on kidney biopsy, a belimumab regimen

is preferred over a CNI regimen because of potential CNI-associated nephrotoxicity and hypertension.

Randomized controlled trials demonstrated similar rates of response in people treated with MPAA and CYC-based regimens, however, the Voting Panel favored MPAA because of the better toxicity profile including lower risk of malignancy and lack of impact on fertility.<sup>57</sup> A CYC-based regimen might be favored in certain circumstances, however, including patient preference, medication non-adherence or intolerance, or the presence of rapidly progressive glomerulonephritis with numerous crescents and/or fibrinoid necrosis on biopsy and declining kidney function.

Data in support of ELNT low-dose CYC plus belimumab is more limited because only 26% of Belimumab International Study in Lupus Nephritis (BLISS-LN) trial participants were treated with background ELNT CYC.<sup>58</sup> Subgroup analysis of participants on background ELNT CYC showed a numerically higher but not statistically significant rate of renal response with addition of belimumab versus placebo. In a post-hoc analysis, addition of belimumab to ELNT CYC resulted in fewer LN flares and a reduced rate of eGFR decline compared to placebo<sup>59</sup>; for this reason, this combination was included as a recommended triple therapy.

The combination of ELNT CYC plus CNI has not been studied in RCTs; for this reason, it is not recommended here as triple therapy. However, this combination may be considered despite the lack of supporting data, especially if other therapy options are unavailable, ineffective, or not tolerated. Patient Panel members repeatedly emphasized the challenges of high pill burden, and preference for the route of medication administration (eg, parenteral or oral) may influence the choice of therapy.

**In people with active, new onset or flare of Class III/IV (±V) LN with proteinuria ≥3 g/g, we conditionally recommend a triple immunosuppressive regimen containing pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper, plus MPAA plus CNI over a regimen containing belimumab.**

This recommendation was based on observed rapid reduction of proteinuria with CNIs<sup>14</sup> and the limited efficacy with belimumab in people with baseline proteinuria ≥3 g/g.<sup>59</sup>

**In people with active, new onset or flare of Class III/IV (±V) LN with moderate to severe extra-renal manifestations, we conditionally recommend a triple immunosuppressive therapy that contains belimumab over a regimen containing a CNI.**

Belimumab is associated with reduction in disease activity and severe flares in nonrenal SLE<sup>54</sup>; in post-hoc analysis it appears especially beneficial for mucocutaneous and musculoskeletal manifestations.<sup>60</sup>

**In people with active, new onset or flare of Class III/IV (±V) LN on treatment with MPAA, we conditionally recommend a target MMF dose of 2–3g/d (or equivalent).**

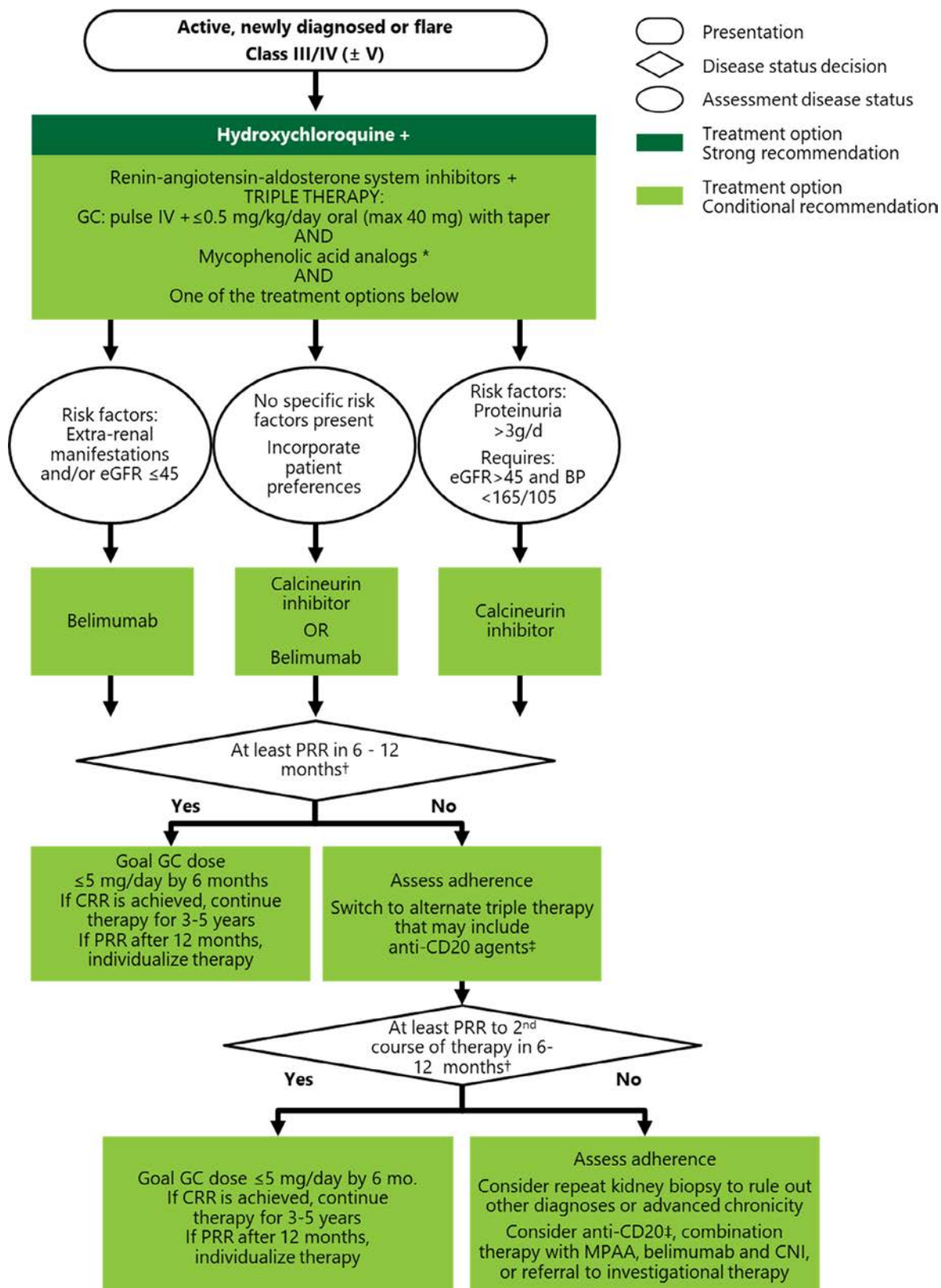


**Figure 1.** Recommendations for the treatment of class III, IV with or without class V lupus nephritis. \* = Alternative triple therapy: glucocorticoids and Euro-Lupus Nephritis Trial low-dose cyclophosphamide and belimumab with mycophenolic acid analogs substituted for cyclophosphamide after the cyclophosphamide course is completed. Mycophenolic acid analogs regimens are preferred over cyclophosphamide regimens. † = Treatment should be escalated or changed earlier, even at ≤3 months, in patients with rapidly declining GFR or increasing proteinuria due to risk for potentially irreversible damage. ‡ = Rituximab, obinutuzumab, or others.

The dose of MPAA should be tailored to the individual patient, balancing the considerations of tolerability, safety, and efficacy. For pediatric patients, the usual starting dose is 1.2–1.4 g/m<sup>2</sup>/d; mycophenolic acid levels may aid in tailoring dosage.

**In people with active, new onset or flare of Class III/IV (±V) LN receiving a CYC-based regimen, we conditionally recommend the ELNT low-dose CYC regimen over a high-dose monthly pulse IV CYC regimen; we strongly recommend the ELNT low-dose CYC regimen over a daily oral CYC regimen.**

An RCT and post-hoc analysis demonstrated that the ELNT regimen of CYC was as effective as intravenous monthly, high-dose CYC in achieving renal response.<sup>61,62</sup> The ELNT regimen is favored because of its better tolerability and toxicity profile, including a lower risk for infertility. Although pediatric data are limited to non-randomized, observational studies, the use of the ELNT regimen is preferred given the potential for multiple CYC courses over time.<sup>63</sup> The Voting Panel unanimously preferred an intravenous regimen over a daily oral CYC regimen because of the cumulative toxicities associated with oral CYC. It is important to provide fertility protective therapies to women and men of reproductive age when using a CYC-based regimen, particularly with high-dose pulse monthly IV CYC or >1 course of the ELNT CYC regimen.

**In people with new onset or flare of Class III/IV (±V) LN who have undergone triple immunosuppressive therapy (pulse intravenous glucocorticoids 250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper, plus either MPAA plus belimumab, MPAA plus CNI, or CYC plus belimumab) and achieved a complete renal response, we conditionally recommend continuing the same immunosuppressive regimen.**

**In people with active, new onset or flare of Class III/IV (±V) LN who have undergone triple immunosuppressive therapy with pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper, plus either MPAA plus belimumab, MPAA plus CNI, or CYC plus belimumab) and achieved a partial renal response (PRR), we conditionally recommend individualizing therapy depending on clinical factors that include the trajectory of response.**

If the patient with PRR is improving with reduction in proteinuria and increasing/stabilization of eGFR, the Voting Panel concurred that continuation of the initial triple immunosuppressive regimen with continued glucocorticoid taper is reasonable. However, if the patient shows indications of worsening disease activity (increasing proteinuria, worsening eGFR), we suggest altering therapy. A repeat kidney biopsy may be helpful to clarify proteinuria etiology (ongoing activity versus fixed damage). A specific duration of therapy is not recommended due to variability in clinical presentations.

**In people with new onset or flare of Class III/IV (±V) LN who have undergone dual immunosuppressive therapy (glucocorticoids plus either CYC or MPAA) and achieved a complete renal response, we conditionally recommend continuing therapy with MPAA over switching to azathioprine (AZA).**

People planning pregnancy or intolerant of MPAA should be treated with AZA.

**In people with new onset or flare of Class III/IV (±V) LN who have undergone dual immunosuppressive therapy (glucocorticoids plus either CYC or MPAA) and achieved a partial renal response, we conditionally recommend escalating therapy to a triple immunosuppressive regimen.**

### Class V LN:

Class V (membranous) LN accounts for 20% of cases and is characterized by the presence of global or segmental subepithelial immune complex deposits. Class V LN can occur in isolation or in combination with Class III/IV.<sup>64–68</sup> There is limited evidence for management of pure Class V.

**In people with active, newly diagnosed or flare of pure Class V lupus nephritis with proteinuria ≥1 g/g, we conditionally recommend treatment with a triple immunosuppressive regimen consisting of glucocorticoids (pulse intravenous glucocorticoids (250–1000 mg methylprednisolone daily × 1–3 days) followed by oral glucocorticoid (≤0.5 mg/kg/day, maximum dose 40 mg/day) taper, and MPAA plus CNI (over MPAA plus belimumab or CYC plus belimumab).**

Post-hoc analyses from clinical trials support that voclosporin, but not belimumab, added to MPAA and low-dose glucocorticoids achieve earlier reductions in proteinuria in pure class V LN.<sup>69</sup> Alternative regimens include initial therapy with glucocorticoids and MPAA, CNI, CYC, azathioprine, or anti-CD20 therapy<sup>65–68</sup> (Figure 2).

The importance and optimal dosing of glucocorticoid for Class V LN is not certain, as suggested by the conditional nature of this recommendation. The Voting Panel opted to include pulse/oral glucocorticoid therapy with taper plus two immunosuppressive agents here based on improved outcomes in recent pivotal clinical trials of triple therapy<sup>13,14</sup> that included individuals with pure Class V. The certainty of the level of evidence was very low (due to indirectness). Glucocorticoid therapy, sometimes at very high dose, has been used consistently across prior trials that included participants with pure Class V in addition to Class III/IV LN. An RCT of pure Class V (single versus dual) therapies did not support benefit of glucocorticoid monotherapy<sup>64</sup> but showed the combination of prednisone plus CNI or CYC to be more effective than prednisone alone. While we may be able to use lower doses of glucocorticoids for pure Class V than for Class III/IV, we do not have high-level data to inform different dosing levels for Class V vs. III/IV. Clinician-patient discussion should guide

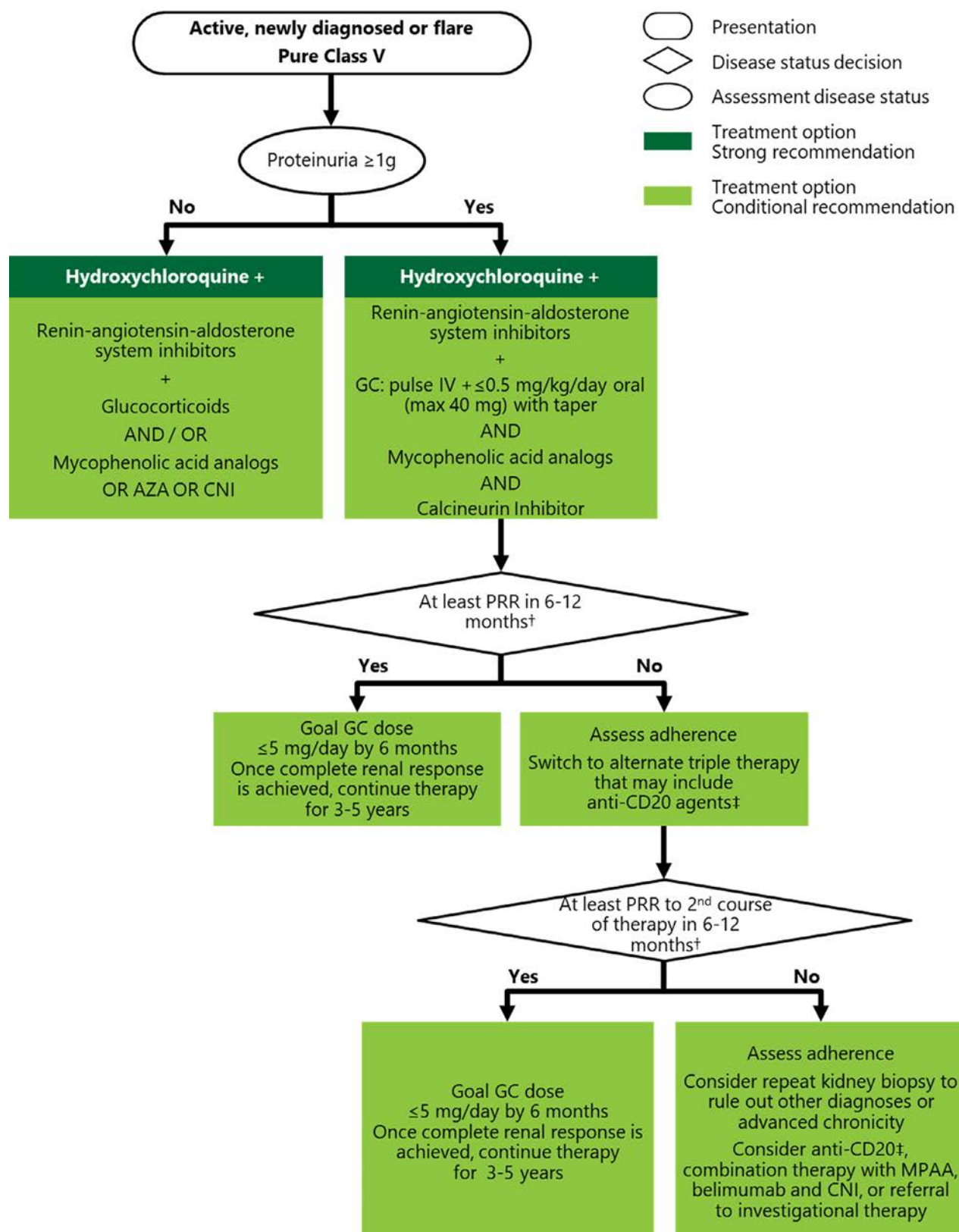


**Figure 2.** Recommendations for the treatment of pure class V lupus nephritis. <sup>†</sup> = Treatment should be escalated or changed earlier, even at  $\leq 3$  months, in patients with rapidly declining GFR or increasing proteinuria due to risk for potentially irreversible damage. <sup>‡</sup> = Rituximab, obinutuzumab or others. AZA, azathioprine; CNI, calcineurin inhibitors; GC, glucocorticoid; GFR, glomerular filtration rate; kg, kilogram; mg, milligram; MPAA, mycophenolic acid analogs; PRR, partial renal response.

therapy decisions, including the lack of high-quality data, the clinical presentation, and the patient's values and preferences.

**In people with active, newly diagnosed or flare of pure Class V lupus nephritis with proteinuria <1 g/g, we conditionally recommend treatment with glucocorticoid and/or immunosuppressant therapy (MPAA, AZA, or CNI) over no glucocorticoid or immunosuppressive therapy.**

The Voting Panel acknowledged the paucity of high-quality evidence for the treatment of Class V with low-level proteinuria because such patients were not included in clinical trials but expressed concern that low-grade proteinuria might progress to proteinuria >1g/g that could be less responsive to treatment.

### Inadequate renal response/refractory LN

**GPS: Medication dose and patient adherence should be assessed regularly throughout the course of treatment as an important first step in evaluating inadequate response or refractory LN, as insufficient treatment is a key cause of non-response.**

Discussion regarding barriers to adherence (e.g., cost, side effects) is an important first step; strategies to monitor adherence (e.g., medication levels) may also be helpful.<sup>70</sup>

**In people with any LN class with inadequate renal response (i.e., have not achieved at least a partial renal response by 6–12 months), we conditionally recommend escalation of treatment:**

- For initial dual therapy: escalate to triple therapy (glucocorticoids plus either MPAA plus belimumab, MPAA plus CNI, or ELNT CYC plus belimumab).
- For initial triple therapy: change to an alternative (listed) triple therapy or consider addition of an anti-CD20 agent to MPAA or ELNT CYC.

There are limited uncontrolled data<sup>71–75</sup> to guide therapy – including optimal timing – for inadequate renal response. Choice of therapy in the setting of inadequate response varies depending on several factors including the medication used initially, patient and clinician preference, and tolerability. Close monitoring is essential: treatment should be escalated or changed earlier, even at ≤3 months, in patients with rapidly declining GFR or increasing proteinuria due to risk of potentially irreversible damage.

**In people with any LN class with refractory disease (ie, failed two standard therapy courses), we conditionally recommend treatment escalation to a more intensive regimen, including the addition of anti-CD20 agents, combination therapy with three non-glucocorticoid immunosuppressive agents (ie, MPAA, belimumab and CNI), or referral for investigational therapy.**

When refractory LN is diagnosed, one may consider a kidney biopsy to assess the extent of chronic damage and determine

whether escalating therapy is warranted. In cases of true refractory LN, meta-analyses suggest that 50–80% of patients convert to partial or complete responders with rituximab.<sup>76,77</sup> Other B cell targeted approaches,<sup>78–80</sup> as well as combination B cell therapies,<sup>81–83</sup> show utility in refractory LN and may offer future therapy options. (See Figure 3 for a Treatment Overview.)

### Other lupus kidney disease

**GPS: Alternative etiologies of kidney dysfunction in people with SLE should be carefully excluded, including non-inflammatory etiologies such as hypertensive, diabetic, and medication-induced nephropathy.**

Less common manifestations of lupus kidney disease include thrombotic microangiopathy (TMA), Class II LN, and lupus podocytopathy. These were discussed by the Voting Panel but not formally voted upon given their lower incidence relative to Classes III/IV and V LN. The 2024 KDIGO clinical practice guideline for the treatment of lupus nephritis<sup>84</sup> provides details regarding clinical presentation and suggested management for these less common lupus kidney issues.

### TMA

TMA is a histopathologic finding indicative of endothelial injury. Underlying causes include acute antiphospholipid antibody (aPL) nephropathy,<sup>85</sup> thrombotic thrombocytopenic purpura, complement-mediated TMA, and others. Because these conditions require different treatments, accurate diagnosis is important and often requires hematology consultation. While there was insufficient consensus to form a recommendation regarding aPL nephropathy or other TMAs in the context of LN, there have been reports of treatment with anticoagulation, plasma exchange, or C5 inhibitor therapy in this situation.<sup>86,87</sup>

### Class II LN

Class II (mesangial) LN is characterized by expanded matrix with immune complexes confined primarily to the mesangium. Extensive podocyte effacement suggests lupus podocytopathy.<sup>88</sup>

The Voting Panel did not reach a consensus to formulate a recommendation for treatment although RAAS-I therapy is usual; however, repeat biopsy to assess for class switch or lupus podocytopathy may be considered in the setting of increasing proteinuria noted on follow-up.

### Lupus podocytopathy

Podocytopathy usually presents with nephrotic range proteinuria; electron microscopy shows diffuse podocyte foot process effacement without subepithelial or subendothelial deposition.<sup>89</sup> Glucocorticoid and other immunosuppressive

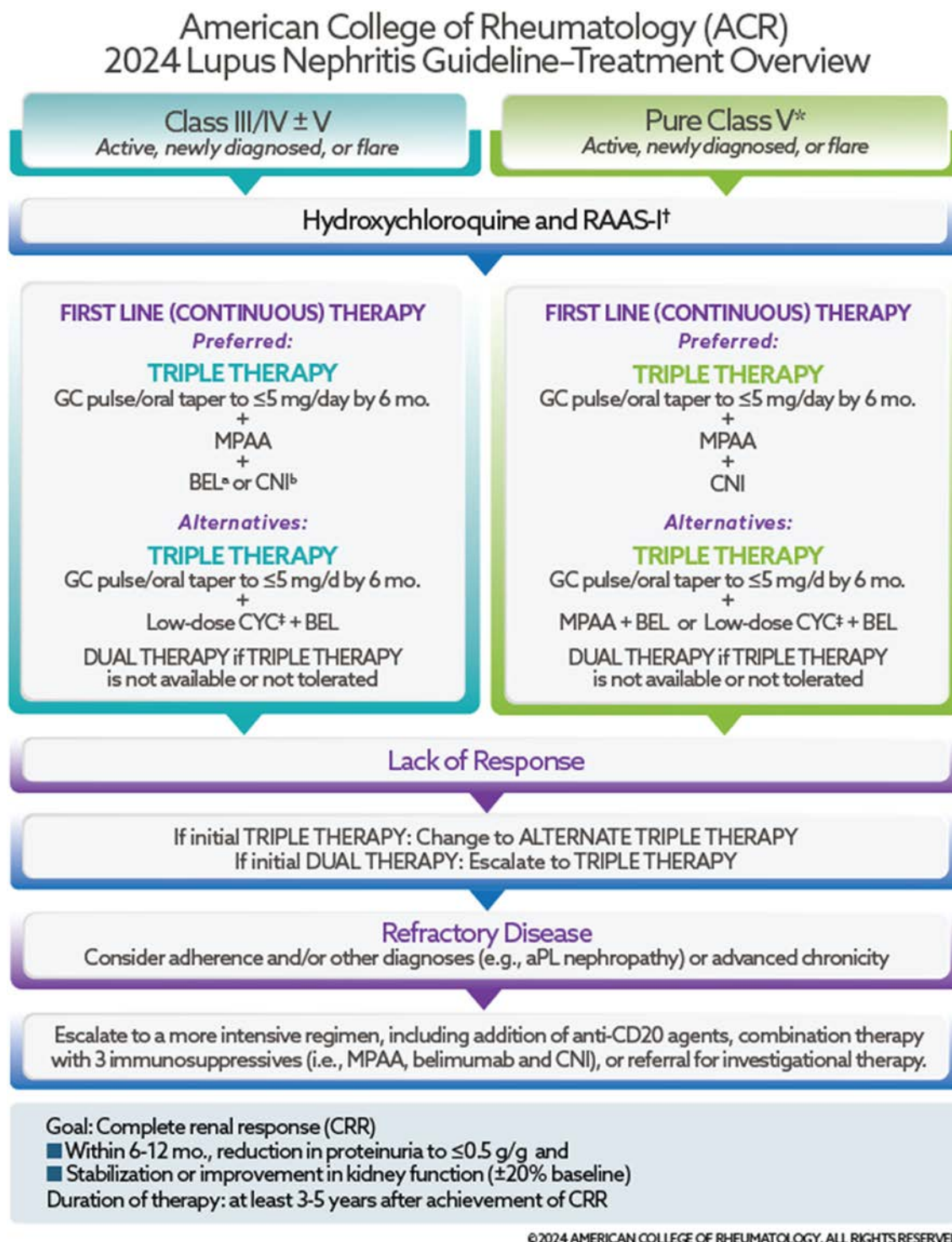


**Figure 3.** American College of Rheumatology 2024 Lupus Nephritis Guideline treatment overview. \* For  $\geq 1$  gm protein; for  $< 1$  gm, treat with GC and/or immunosuppression. † Discuss adjunctive treatment with systemic anticoagulation with nephrology for patients with LN and significant factors for thrombosis (eg, low serum albumin in context of severe proteinuria). ‡ Substitute MPAA once low-dose CYC cycle is completed. a: Recommended preferentially when significant extrarenal manifestations are present. b: Recommended preferentially when proteinuria is  $\geq 3.0$  gm. GC pulse/oral taper: pulse intravenous GCs (250–1,000 mg methylprednisolone daily for 1–3 days) followed by oral GC  $\leq 0.5$  mg/kg/day (maximum dose 40 mg/day) and taper. Low-dose CYC: as per Euro-Lupus Nephritis Trial protocol,<sup>61</sup> 500 mg IV CYC every 2 weeks for 6 doses. Dual therapy: GC plus/oral taper plus one immunosuppressive agent, usually MPAA or low-dose CYC. RAAS-I, renin-angiotensin-aldosterone system inhibitors; GC, glucocorticoid; MPAA, mycophenolic acid analogs (including mycophenolate mofetil [MMF]; BEL, belimumab; CNI, calcineurin inhibitor; CYC, cyclophosphamide.

treatments are common<sup>90</sup>; therapy is best managed in collaboration with nephrology colleagues.

### Adjunctive / non-immunologic treatments and good practice guidance

**GPS:** *Adjunctive and non-immunologic therapies and practices should be added to appropriate immunosuppressive therapy to improve overall kidney health.*

In addition to non-immunosuppressive kidney therapies such as RAAS-I, management of cardiovascular health, bone health, infection risk, and reproductive concerns should be addressed as summarized in Table 4.

**GPS:** *In children with childhood-onset SLE (cSLE) nephritis, glucocorticoid regimens should use pediatric-appropriate doses for children, as reduction of cumulative glucocorticoid dosing is critically important given the early age of cSLE onset and attendant comorbidities.*

**GPS:** *In children with cSLE nephritis, clinicians should monitor for delayed pubertal onset and decreased growth velocity that can result from disease activity and glucocorticoid treatment and consider referral to pediatric endocrinology if indicated.*

**GPS:** *For children with cSLE nephritis, a structured, intentional transition<sup>91–94</sup> from pediatric to adult rheumatology*

*care is indicated to avoid poor outcomes during this vulnerable period.*

**GPS:** *For older people with LN, medication number, type, and dosage should be regularly assessed, given the risks of polypharmacy and age-related decline in GFR in this population.*

### Monitoring LN activity

Treatment trials in SLE measure proteinuria rather than albuminuria. The gold standard for assessing proteinuria, the 24-hour urine collection, is challenging to implement in clinical practice; random urine protein-to-creatinine ratios are usually adequate. The first void of the day sample<sup>95,96</sup> is the most accurate for the spot urine collection but may not be feasible. Unexpected results on random testing should be followed by a 24-hour collection, especially before any change in therapy.

**In people with LN who have not achieved CRR, we strongly recommend quantifying proteinuria at least every 3 months.**

**In people with LN in sustained clinical renal remission, we strongly recommend quantifying proteinuria every 3–6 months.**

These recommendations are strong despite a lack of high-certainty evidence because in people undergoing treatment for LN who have not achieved complete renal response, quantifying

**Table 4.** Good practice guidance: adjunctive therapies for patients with lupus nephritis\*

| General considerations |                         | Guidance                                                                                                                                                                                                   |
|------------------------|-------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Kidney health:         | Diet                    | Limit sodium intake (suggest ≤2g sodium/day)                                                                                                                                                               |
|                        | Non-pharmacologic       | Avoid high protein intake if eGFR <60 (suggest <1g/kg/day)                                                                                                                                                 |
| Kidney health:         | RAAS-I                  | Recommended for all LN patients, if tolerated                                                                                                                                                              |
|                        | Pharmacologic           | Consider for stable LN patients with DM, CKD, moderate-high proteinuria, or heart failure (use with caution in patients on high-dose immunosuppression due to increased risk of urinary tract infection)   |
| Cardiovascular health  | Lifestyle               | Avoid smoking, exercise, optimize BMI                                                                                                                                                                      |
|                        | Blood pressure          | Systolic BP <120 if tolerated                                                                                                                                                                              |
|                        | Lipid management        | Dyslipidemia management per CVD risk reduction guidelines                                                                                                                                                  |
|                        | CVD risk assessment     | Estimate 10-year cardiovascular risk using a validated risk tool                                                                                                                                           |
| Bone health            | Screening and treatment | See ACR Glucocorticoid-induced Osteoporosis Guideline <sup>125</sup>                                                                                                                                       |
| Infection              | Screening               | Screening for hepatitis B, hepatitis C, and tuberculosis                                                                                                                                                   |
|                        | Vaccination             | See ACR Vaccine Guideline <sup>126</sup>                                                                                                                                                                   |
|                        | Prophylactic therapies  | Consider prophylaxis for PJP and hepatitis B when indicated                                                                                                                                                |
| Reproductive health    | Contraception           | See ACR Reproductive Health Guideline <sup>127</sup><br>Use highly effective method (eg, IUD)<br>If on MPAA, use IUD or two other forms                                                                    |
|                        | Pregnancy               | See ACR Reproductive Health Guideline <sup>127</sup> Contraindicated with active LN<br>Azathioprine and tacrolimus are pregnancy-compatible: use when LN is in remission but ongoing treatment is required |
|                        | Fertility               | See ACR Reproductive Health Guideline <sup>127</sup><br>Gonadotropin releasing hormone agonist co-therapy recommended in females treated with CYC.                                                         |
|                        |                         | Consider IVF for oocyte/embryo cryopreservation if stable disease still requiring ongoing teratogenic therapies and concern for age-related infertility                                                    |
|                        |                         |                                                                                                                                                                                                            |

\* ACR, American College of Rheumatology; BMI, body mass index; BP, blood pressure; CKD, chronic kidney disease; CVD, cardiovascular disease; CYC, cyclophosphamide; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; IVF, in vitro fertilization; IUD, intrauterine device; LN, lupus nephritis; MPAA, mycophenolic acid analogs; PJP, pneumocystis jirovecii pneumonia; RAAS-I, renin-angiotensin-aldosterone system inhibitors; SGLT2-I, sodium-glucose cotransporter-2 inhibitors.

proteinuria every three months may prompt adjustment of treatment regimen. In people with LN who have sustained complete renal response, quantifying proteinuria every 3–6 months will minimize the risk of missing an LN flare that would require more aggressive treatment. While we are certain about the beneficial effects of monitoring and screening for proteinuria in both diagnosis and prognosis, there is a lack of evidence regarding the optimal screening interval, and the overall certainty of the evidence is very low. The benefit of potentially preserving long-term kidney function far outweighs the minimal risk of obtaining a urine sample.

**GPS: For people with LN, serum complement and anti-double-stranded DNA (dsDNA) antibody should be measured at every clinic visit (but not more frequently than monthly).**

While hypocomplementemia and elevated anti-dsDNA antibodies have only modest sensitivity and specificity for LN activity, several studies<sup>97–99</sup> suggest they may herald new onset LN or LN flare. Changes in these levels should prompt careful clinical and laboratory assessment but should not necessarily trigger preemptive treatment in the absence of clinical manifestations, unless previous individual clinical experience suggests otherwise. Anti-C1q antibodies<sup>100</sup> correlate better with LN flares<sup>101,102</sup>; however, this antibody testing may not be universally available. Emerging biomarkers' utility will be reviewed for future guideline updates as these become validated.

## Renal replacement therapies (dialysis and transplant)

**GPS: Decisions for initiation and type of dialysis and timing of kidney transplant require close collaboration with nephrology.**

Ten to twenty-two percent of people with LN will develop ESKD.<sup>2,3</sup> Treatment options include hemodialysis or peritoneal dialysis, or a kidney transplant. Individual patient characteristics and preferences should impact dialysis modality choice. People undergoing peritoneal dialysis have a higher risk of infections, especially peritonitis. Hemodialysis has inherent complications – bloodstream infections and thrombosis – related to vascular access. People with antiphospholipid antibodies are at higher risk of vascular access complications and allograft thrombosis.<sup>103,104</sup>

**In people with LN and ESKD, we strongly recommend kidney transplantation over dialysis without kidney transplantation.**

Transplantation significantly reduces mortality, cardiovascular disease events, infections, and risk of flares compared to dialysis.<sup>105</sup> The Patient Panel highlighted both the poor quality of life associated with dialysis and the challenges of accessing transplantation. People with ESKD due to LN are less likely to receive a kidney transplant compared to people with other glomerulonephritides.<sup>106</sup>

**In people with LN who have progressive loss of kidney function and are nearing ESKD (defined as an eGFR of 15 ml/min/1.73m<sup>2</sup>), we conditionally recommend preemptive kidney transplant over dialysis/no preemptive kidney transplant.<sup>107</sup>**

Preemptive kidney transplantation improves survival compared to non-preemptive approaches in people with CKD,<sup>108,109</sup> and an observational study suggested that preemptive kidney transplantation improves survival in people with LN compared to non-preemptive approaches.<sup>110</sup> The Voting Panel emphasized the benefits of avoiding dialysis morbidity but recognized transplant access limitations.

**In people with LN and ESKD, we conditionally recommend proceeding with kidney transplantation without requiring complete clinical or serologic remission of SLE, provided there is no other major organ involvement.**

Limited data indicate that lupus activity does not significantly affect allograft function.<sup>111</sup> The Voting Panel emphasized that transplant eligibility should not be based on serologic activity as it does not appear to have an impact on transplant outcome.<sup>112</sup> The recurrence of LN in the allograft is rare (10%) and often mild, with predominantly mesangial lesions.<sup>113</sup>

**In people with LN on current dialysis or after kidney transplantation, we strongly recommend regular follow up with rheumatology.**

Despite a low recurrence rate of LN in transplanted kidneys, regular rheumatology follow up is recommended even for people with SLE who have ESKD or are post kidney transplant. The recommendation is strong despite low certainty of evidence supporting the benefit of regular rheumatology follow up, due to the essential role of rheumatologists in managing the broader health issues associated with lupus. At least 50% of people with ESKD due to LN in the US remain on immunosuppression<sup>114</sup>; those who are co-managed with a rheumatologist (≥2 rheumatology visits per year) have higher survival rates.<sup>107</sup>

## DISCUSSION

Lupus nephritis is among the most common severe manifestation of SLE. In this guideline, we propose treatment with triple therapy (glucocorticoids plus two immunosuppressive medications) as the most desirable therapy for LN, preferring MPAA regimens over CYC regimens. We also propose a lower dose glucocorticoid regimen (after initial intravenous pulse) to minimize toxicity, with a prednisone goal of ≤5 mg/day by 6 months of therapy. These recommendations are conditional and require discussion between clinicians and patients because multiple factors impact therapy choice.

We do not specify a particular CNI because comparative effectiveness and safety studies are not available, and accessibility may dictate the choice of CNI. Ongoing monitoring is essential as long-term nephrotoxicity is an important concern with any CNI.

These recommendations apply to adults and children with LN. The Guideline Team analyzed pediatric-specific LN data when available, as LN affects up to half of individuals with cSLE.<sup>115,116</sup> Since cSLE LN treatment includes higher cumulative doses of glucocorticoids and CYC,<sup>117</sup> these recommendations propose corticosteroid regimens that differ from other pediatric-specific options.<sup>118</sup> While efficacy evidence for this change is indirect, it acknowledges pediatric-specific concerns regarding glucocorticoid effects on growth and pubertal development. We also emphasize the necessity of structured transition to adult rheumatology and nephrology care.<sup>118–121</sup> Despite recent improvements in LN outcomes, youth of historically marginalized groups remain at higher risk for ESKD and dialysis.<sup>122,123</sup>

Two major themes emerged from the Patient Panel discussion. First, shared decision-making is a dynamic, ongoing process influenced by the patient's values, individual disease course, stage of life, medication tolerance, efficacy, and side effects; as such, an individual patient's decisions regarding management evolve over time. Second, patients emphasized the importance for clinicians to recognize pill burden, discuss all medication options, and provide close monitoring with the shared goals of preservation of kidney function, overall health, and optimal quality of life.

Current gaps in the LN literature identified through our systemic literature review and evidence analysis helped to identify important areas of study for a future research agenda (Supplementary Materials 8), including new agents and strategies to improve outcomes for people with LN. During this guideline's manuscript preparation, a positive phase 3 trial reported that addition of the humanized anti-CD20 therapy obinutuzumab to standard therapy (glucocorticoid and mycophenolate mofetil) led to a significantly greater likelihood of complete renal response at 76 weeks than did standard therapy alone, although infectious risk (particularly COVID-19) was higher.<sup>124</sup> Further studies on this and other agents may lead to more targeted and effective LN strategies and will be reflected in updated revisions of this guideline.

With the development of this guideline, the ACR recognizes the key role of clinical rheumatologists in managing LN. Important goals of this guideline are to provide substance and direction for therapy decisions after clinician-patient discussions, and to encourage close working relationships between rheumatologists and nephrologists to enhance collaborative care.

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## REFERENCES

1. Izmirly PM, Parton H, Wang L, et al. Prevalence of systemic lupus erythematosus in the United States: Estimates from a meta-analysis of the Centers for Disease Control and Prevention National Lupus Registries. *Arthritis Rheumatol* 2021;73:991–996.
2. Hocaoglu M, Valenzuela-Almada MO, Dabit JY, et al. Incidence, prevalence, and mortality of lupus nephritis: a population-based study over four decades using the Lupus Midwest Network. *Arthritis Rheumatol* 2023;75:567–573.
3. Tektonidou MG, Dasgupta A, Ward MM. Risk of end-stage renal disease in patients with lupus nephritis, 1971–2015: a systematic review and Bayesian meta-analysis. *Arthritis Rheumatol* 2016;68:1432–1441.
4. Dall'Era M, Cisternas MG, Snipes K, et al. The incidence and prevalence of systemic lupus erythematosus in San Francisco County, California: The California Lupus Surveillance Project. *Arthritis Rheumatol* 2017;69:1996–2005.
5. Drenkard C, Lim SS. Update on lupus epidemiology: advancing health disparities research through the study of minority populations. *Curr Opin Rheumatol* 2019;31:689–696.
6. Izmirly PM, Wan I, Sahl S, et al. The incidence and prevalence of systemic lupus erythematosus in New York County (Manhattan), New York: The Manhattan Lupus Surveillance Program. *Arthritis Rheumatol* 2017;69:2006–2017.
7. Lim SS, Bayakly AR, Helmick CG, et al. The incidence and prevalence of systemic lupus erythematosus, 2002–2004: The Georgia Lupus Registry. *Arthritis Rheumatol* 2014;66:357–368.
8. Somers EC, Marder W, Cagnoli P, et al. Population-based incidence and prevalence of systemic lupus erythematosus: the Michigan Lupus Epidemiology and Surveillance program. *Arthritis Rheumatol* 2014;66:369–378.
9. Feldman CH, Hiraki LT, Liu J, et al. Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000–2004. *Arthritis Rheum* 2013;65:753–763.
10. Mahmood SB, Aziz M, Malepati D, et al. Evaluating sex differences in the characteristics and outcomes of lupus nephritis: a systematic review and meta-analysis. *Glomerular Dis* 2024;4:19–32.
11. Yelin E, Trupin L, Yazdany J. A prospective study of the impact of current poverty, history of poverty, and exiting poverty on accumulation of disease damage in systemic lupus erythematosus. *Arthritis Rheumatol* 2017;69:1612–1622.
12. Hahn BH, McMahon MA, Wilkinson A, et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care Res (Hoboken)* 2012;64:797–808.
13. Furie R, Rovin BH, Houssiau F, et al. Two-year, randomized, controlled trial of belimumab in lupus nephritis. *N Engl J Med* 2020;383:1117–1128.
14. Rovin BH, Teng YKO, Ginzler EM, et al. Efficacy and safety of voclosporin versus placebo for lupus nephritis (AURORA 1): a double-blind,

- randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet* 2021;397:2070–2080.
15. Anders HJ, Lei Y, Rovin BH. Induction and maintenance therapy of lupus nephritis: an obituary. *Kidney Int* 2021;99:288–291.
  16. Alamillo-Sanchez ME, Alcala-Salgado MA, Alonso-Bello CD, et al. Mechanism of action and efficacy of immunosuppressors in lupus nephritis. *Int J Nephrol Renovasc Dis* 2021;14:441–458.
  17. Crow MK. Pathogenesis of systemic lupus erythematosus: risks, mechanisms and therapeutic targets. *Ann Rheum Dis* 2023;82:999–1014.
  18. Figueroa-Parra G, Cuéllar-Gutiérrez MC, González-Treviño M, et al. Impact of glucocorticoid dose on complete response, serious infections, and mortality during the initial therapy of lupus nephritis: a systematic review and meta-analysis of the control arms of randomized controlled trials. *Arthritis Rheumatol* 2024;76:1408–1418.
  19. Balevic SJ, Niu J, Chen J, et al. Extrapolation of adult efficacy data to pediatric systemic lupus erythematosus: evaluating similarities in exposure-response. *J Clin Pharmacol* 2023;63:105–118.
  20. Brunner HI, Martini A, Lovell DJ, et al. Clinical trials in children and adolescents with systemic lupus erythematosus: methodological aspects, regulatory landscape and future opportunities. *Ann Rheum Dis* 2019;78:162–170.
  21. Pottackal G, Travis J, Neuner R, et al. Application of Bayesian Statistics to Support Approval of Intravenous Belimumab in Children with Systemic Lupus Erythematosus in the United States. 2019;71(suppl 10). <https://acrabstracts.org/abstract/application-of-bayesian-statistics-to-support-approval-of-intravenous-belimumab-in-children-with-systemic-lupus-erythematosus-in-the-united-states/>.
  22. Andrews JC, Schünemann HJ, Oxman AD, et al. GRADE guidelines: 15. Going from evidence to recommendation—determinants of a recommendation's direction and strength. *J Clin Epidemiol* 2013;66:726–735.
  23. Guyatt GH, Oxman AD, Vist GE, et al. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *BMJ* 2008;336:924–926.
  24. Bartels CM, Jorge A, Feldman CH, et al. Development of American College of Rheumatology quality measures for systemic lupus erythematosus: a modified Delphi process with Rheumatology Informatics System for Effectiveness (RISE) registry data review. *Arthritis Care Res (Hoboken)* 2023;75:2295–2305.
  25. Bajema IM, Wilhelmus S, Alpers CE, et al. Revision of the International Society of Nephrology/Renal Pathology Society classification for lupus nephritis: clarification of definitions, and modified National Institutes of Health activity and chronicity indices. *Kidney Int* 2018;93:789–796.
  26. Corapi KM, Chen JL, Balk EM, et al. Bleeding complications of native kidney biopsy: a systematic review and meta-analysis. *Am J Kidney Dis* 2012;60:62–73.
  27. Deonaraine KK, Carlucci PM, Fava A, et al. Safety of procuring research tissue during a clinically indicated kidney biopsy from patients with lupus: data from the Accelerating Medicines Partnership RA/SLE Network. *Lupus Sci Med* 2021;8:e000522.
  28. Halimi JM, Gatault P, Longuet H, et al. Major bleeding and risk of death after percutaneous native kidney biopsies: a French nationwide cohort study. *Clin J Am Soc Nephrol* 2020;15:1587–1594.
  29. Palsson R, Short SAP, Kibbelaar ZA, et al. Bleeding complications after percutaneous native kidney biopsy: results from the Boston Kidney Biopsy Cohort. *Kidney Int Rep* 2020;5:511–518.
  30. Poggio ED, McClelland RL, Blank KN, et al. Systematic review and meta-analysis of native kidney biopsy complications. *Clin J Am Soc Nephrol* 2020;15:1595–1602.
  31. Schorr M, Roshanov PS, Weir MA, et al. Frequency, timing, and prediction of major bleeding complications from percutaneous renal biopsy. *Can J Kidney Health Dis* 2020;7:2054358120923527.
  32. Chen TK, Estrella MM, Fine DM. Predictors of kidney biopsy complication among patients with systemic lupus erythematosus. *Lupus* 2012;21:848–854.
  33. Jordan N, Chaib A, Sangle S, et al. Association of thrombotic microangiopathy and intimal hyperplasia with bleeding post-renal biopsy in antiphospholipid antibody-positive patients. *Arthritis Care Res (Hoboken)* 2014;66:725–731.
  34. Kang ES, Ahn SM, Oh JS, et al. Risk of bleeding-related complications after kidney biopsy in patients with systemic lupus erythematosus. *Clin Rheumatol* 2023;42:751–759.
  35. Sun YS, Sun IT, Wang HK, et al. Risk of complications of ultrasound-guided renal biopsy for adult and pediatric patients with systemic lupus erythematosus. *Lupus* 2018;27:828–836.
  36. Carlucci PM, Li J, Fava A, et al. High incidence of proliferative and membranous nephritis in SLE patients with low proteinuria in the Accelerating Medicines Partnership. *Rheumatology (Oxford)* 2022;61:4335–4343.
  37. De Rosa M, Rocha AS, De Rosa G, et al. Low-grade proteinuria does not exclude significant kidney injury in lupus nephritis. *Kidney Int Rep* 2020;5:1066–1068.
  38. Stamenkovic I, Favre H, Donath A, et al. Renal biopsy in SLE irrespective of clinical findings: long-term follow-up. *Clin Nephrol* 1986;26:109–115.
  39. Wakasugi D, Gono T, Kawaguchi Y, et al. Frequency of class III and IV nephritis in systemic lupus erythematosus without clinical renal involvement: an analysis of predictive measures. *J Rheumatol* 2012;39:79–85.
  40. Fava A, Fenaroli P, Rosenberg A, et al. History of proliferative glomerulonephritis predicts end stage kidney disease in pure membranous lupus nephritis. *Rheumatology (Oxford)* 2022;61:2483–2493.
  41. Gao JJ, Cai GY, Liu SW, et al. Characteristics and influence factors of pathologic transformation in the subclasses of class IV lupus nephritis. *Rheumatol Int* 2012;32:1751–1759.
  42. Moroni G, Porata G, Raffiotta F, et al. Predictors of increase in chronicity index and of kidney function impairment at repeat biopsy in lupus nephritis. *Lupus Sci Med* 2022;9:e000721.
  43. KDIGO 2021 Clinical practice guideline for the management of glomerular diseases. *Kidney Int* 2021;100:S1–S276.
  44. Pons-Estel GJ, Alarcón GS, McGwin G, Jr., et al. Protective effect of hydroxychloroquine on renal damage in patients with lupus nephritis: LXV, data from a multiethnic US cohort. *Arthritis Rheum* 2009;61:830–839.
  45. Cai T, Zhao J, Yang Y, et al. Hydroxychloroquine use reduces mortality risk in systemic lupus erythematosus: a systematic review and meta-analysis of cohort studies. *Lupus* 2022;31:1714–1725.
  46. Rosenbaum JT, Costenbader KH, Desmarais J, et al. American College of Rheumatology, American Academy of Dermatology, Rheumatologic Dermatology Society, and American Academy of Ophthalmology 2020 Joint Statement on hydroxychloroquine use with respect to retinal toxicity. *Arthritis Rheumatol* 2021;73:908–911.
  47. Ku E, Inker LA, Tighiouart H, et al. Angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers for advanced chronic kidney disease: a systematic review and retrospective individual participant-level meta-analysis of clinical trials. *Ann Intern Med* 2024;177:953–963.
  48. Chang JC, Weiss PF, Xiao R, et al. Use of renin angiotensin aldosterone system inhibitors in children with lupus and time to glucocorticoid discontinuation. *Kidney Int* 2022;102:395–404.

49. Bandhan IH, Islam MN, Ahmad HI, et al. Outcome of low-dose prednisolone use for the induction of remission in lupus nephritis patients. *Int J Rheum Dis* 2022;25:121–130.
50. Bharati J, Rath M, Ramachandran R, et al. Comparison of two steroid regimens in induction therapy of proliferative lupus nephritis: a randomized controlled trial. *Indian J Nephrol* 2019;29:373–375.
51. Zeher M, Doria A, Lan J, et al. Efficacy and safety of enteric-coated mycophenolate sodium in combination with two glucocorticoid regimens for the treatment of active lupus nephritis. *Lupus* 2011;20:1484–1493.
52. Webel ML, Ritts RE, Jr., Taswell HF, et al. Cellular immunity after intravenous administration of methylprednisolone. *J Lab Clin Med* 1974;83:383–392.
53. Malvar A, Alberton V, Lococo B, et al. Remission of lupus nephritis: the trajectory of histological response in successfully treated patients. *Lupus Sci Med* 2023;10:e000932.
54. Chakravarty EF, Utset T, Kamen DL, et al. Mycophenolate mofetil withdrawal in patients with systemic lupus erythematosus: a multicentre, open-label, randomised controlled trial. *Lancet Rheumatol* 2024;6:e168–e177.
55. Jourde-Chiche N, Costedoat-Chalumeau N, Baumstarck K, et al. Weaning of maintenance immunosuppressive therapy in lupus nephritis (WIN-Lupus): results of a multicentre randomised controlled trial. *Ann Rheum Dis* 2022;81:1420–1427.
56. Malvar A, Alberton V, Lococo B, et al. Kidney biopsy-based management of maintenance immunosuppression is safe and may ameliorate flare rate in lupus nephritis. *Kidney Int* 2020;97:156–162.
57. Appel GB, Contreras G, Dooley MA, et al. Mycophenolate mofetil versus cyclophosphamide for induction treatment of lupus nephritis. *J Am Soc Nephrol* 2009;20:1103–1112.
58. Furie R, Petri M, Zamani O, et al. A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum* 2011;63:3918–3930.
59. Rovin BH, Furie R, Teng YKO, et al. A secondary analysis of the Belimumab International Study in Lupus Nephritis trial examined effects of belimumab on kidney outcomes and preservation of kidney function in patients with lupus nephritis. *Kidney Int* 2022;101:403–413.
60. Manzi S, Sánchez-Guerrero J, Merrill JT, et al. Effects of belimumab, a B lymphocyte stimulator-specific inhibitor, on disease activity across multiple organ domains in patients with systemic lupus erythematosus: combined results from two phase III trials. *Ann Rheum Dis* 2012;71:1833–1838.
61. Houssiau FA, Vasconcelos C, D'Cruz D, et al. Immunosuppressive therapy in lupus nephritis: the Euro-Lupus Nephritis Trial, a randomized trial of low-dose versus high-dose intravenous cyclophosphamide. *Arthritis Rheum* 2002;46:2121–2131.
62. Wofsy D, Diamond B, Houssiau FA. Crossing the Atlantic: the Euro-Lupus nephritis regimen in North America. *Arthritis Rheumatol* 2015;67:1144–1146.
63. Wang CS, Sadun RE, Zhou W, et al. Renal response outcomes of the EuroLupus and National Institutes of Health cyclophosphamide dosing regimens in childhood-onset proliferative lupus nephritis. *Arthritis Rheumatol* 2024;76:469–478.
64. Austin HA, 3rd, Illei GG, Braun MJ, et al. Randomized, controlled trial of prednisone, cyclophosphamide, and cyclosporine in lupus membranous nephropathy. *J Am Soc Nephrol* 2009;20:901–911.
65. Chavarot N, Verhelst D, Pardon A, et al. Rituximab alone as induction therapy for membranous lupus nephritis: a multicenter retrospective study. *Medicine (Baltimore)* 2017;96:e7429.
66. Liu Z, Zhang H, Liu Z, et al. Multitarget therapy for induction treatment of lupus nephritis: a randomized trial. *Ann Intern Med* 2015;162:18–26.
67. Mok CC, Ying KY, Yim CW, et al. Very long-term outcome of pure lupus membranous nephropathy treated with glucocorticoid and azathioprine. *Lupus* 2009;18:1091–1095.
68. Radhakrishnan J, Moutzouris DA, Ginzler EM, et al. Mycophenolate mofetil and intravenous cyclophosphamide are similar as induction therapy for class V lupus nephritis. *Kidney Int* 2010;77:152–160.
69. Arriens C, Teng YKO, Ginzler EM, et al. Update on the efficacy and safety profile of voclosporin: an integrated analysis of clinical trials in lupus nephritis. *Arthritis Care Res (Hoboken)* 2023;75:1399–1408.
70. Garg S, Plantinga LC. A bitter pill to swallow: the challenge of medication nonadherence in systemic lupus erythematosus. *J Rheumatol* 2024;51:841–844.
71. Cortés-Hernández J, Torres-Salido MT, Medrano AS, et al. Long-term outcomes--mycophenolate mofetil treatment for lupus nephritis with addition of tacrolimus for resistant cases. *Nephrol Dial Transplant* 2010;25:3939–3948.
72. Dall'Era M, Stone D, Levesque V, et al. Identification of biomarkers that predict response to treatment of lupus nephritis with mycophenolate mofetil or pulse cyclophosphamide. *Arthritis Care Res (Hoboken)* 2011;63:351–357.
73. Houssiau FA, Vasconcelos C, D'Cruz D, et al. Early response to immunosuppressive therapy predicts good renal outcome in lupus nephritis: lessons from long-term followup of patients in the Euro-Lupus Nephritis Trial. *Arthritis Rheum* 2004;50:3934–3940.
74. Kapitsinou PP, Boletis JN, Skopouli FN, et al. Lupus nephritis: treatment with mycophenolate mofetil. *Rheumatology (Oxford)* 2004;43:377–380.
75. Mackay M, Dall'Era M, Fishbein J, et al. Establishing surrogate kidney end points for lupus nephritis clinical trials: development and validation of a novel approach to predict future kidney outcomes. *Arthritis Rheumatol* 2019;71:411–419.
76. Alshaiki F, Obaid E, Almuallim A, et al. Outcomes of rituximab therapy in refractory lupus: a meta-analysis. *Eur J Rheumatol* 2018;5:118–126.
77. Arora S, Rovin BH. Expert perspective: an approach to refractory lupus nephritis. *Arthritis Rheumatol* 2022;74:915–926.
78. Roccatello D, Fenoglio R, Caniggia I, et al. Daratumumab monotherapy for refractory lupus nephritis. *Nat Med* 2023;29:2041–2047.
79. Segarra A, Arredondo KV, Jaramillo J, et al. Efficacy and safety of bortezomib in refractory lupus nephritis: a single-center experience. *Lupus* 2020;29:118–125.
80. Zhang H, Liu Z, Huang L, et al. The short-term efficacy of bortezomib combined with glucocorticoids for the treatment of refractory lupus nephritis. *Lupus* 2017;26:952–958.
81. Aranow C, Allaart CF, Amoura Z, et al. Efficacy and safety of sequential therapy with subcutaneous belimumab and one cycle of rituximab in patients with systemic lupus erythematosus: the phase 3, randomised, placebo-controlled BLISS-BELIEVE study. *Ann Rheum Dis* 2024;83:1502–1512.
82. Atisha-Fregoso Y, Malkiel S, Harris KM, et al. Phase II randomized trial of rituximab plus cyclophosphamide followed by belimumab for the treatment of lupus nephritis. *Arthritis Rheumatol* 2021;73:121–131.
83. Shipa M, Embleton-Thirsk A, Parvaz M, et al. Effectiveness of belimumab after rituximab in systemic lupus erythematosus: a randomized controlled trial. *Ann Intern Med* 2021;174:1647–1657.
84. KDIGO 2024 Clinical practice guideline for the management of lupus nephritis. *Kidney Int* 2024;105:S1–S69.
85. Barbhaiya M, Zuily S, Naden R, et al. The 2023 ACR/EULAR Antiphospholipid Syndrome Classification Criteria. *Arthritis Rheumatol* 2023;75:1687–1702.

86. Sciascia S, Yazdany J, Dall'Era M, et al. Anticoagulation in patients with concomitant lupus nephritis and thrombotic microangiopathy: a multicentre cohort study. *Ann Rheum Dis* 2019;78:1004–1006.
87. Wright RD, Bannerman F, Beresford MW, et al. A systematic review of the role of eculizumab in systemic lupus erythematosus-associated thrombotic microangiopathy. *BMC Nephrol* 2020; 21:245.
88. Weening JJ, D'Agati VD, Schwartz MM, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 2004;65:521–530.
89. Chen D, Hu W. Lupus podocytopathy: a distinct entity of lupus nephritis. *J Nephrol* 2018;31:629–634.
90. Xia W, Deng J, Zhuang L, et al. Risk factors for acute kidney injury and kidney relapse in patients with lupus podocytopathy. *Clin Kidney J* 2024;17:sfae148.
91. Pediatric to Adult Rheumatology Care Transition. Accessed April 14, 2025. <https://rheumatology.org/pediatric-to-adult-rheumatology-care-transition>
92. Cintron D, Chang JC, Sadun RE. Transition to adult rheumatology care: a disease-specific guide. *J Clin Rheumatol* 2024;30:159–167.
93. Ciosek AL, Makris UE, Kramer J, et al. Health literacy and patient activation in the pediatric to adult transition in systemic lupus erythematosus: patient and health care team perspectives. *ACR Open Rheumatol* 2022;4:782–793.
94. Dushnick MJ, Fine C, Cellucci T, et al. Mind the gap: a longitudinal analysis of the experiences of adolescents and parents in a rheumatology transition clinic. *J Clin Rheumatol* 2023;29:235–239.
95. Kamińska J, Dymicka-Piekarska V, Tomaszewska J, et al. Diagnostic utility of protein to creatinine ratio (P/C ratio) in spot urine sample within routine clinical practice. *Crit Rev Clin Lab Sci* 2020;57: 345–364.
96. Rahman MM, Azad K, Ahmed N, et al. Spot morning urine protein creatinine ratio and 24 hour urinary total protein excretion rate. *Mymensingh Med J* 2006;15:146–149.
97. Barnado A, Carroll RJ, Casey C, et al. Phenome-wide association study identifies dsDNA as a driver of major organ involvement in systemic lupus erythematosus. *Lupus* 2019;28:66–76.
98. Birmingham DJ, Irshaid F, Nagaraja HN, et al. The complex nature of serum C3 and C4 as biomarkers of lupus renal flare. *Lupus* 2010;19: 1272–1280.
99. Fasano S, Pierro L, Borgia A, et al. Biomarker panels may be superior over single molecules in prediction of renal flares in systemic lupus erythematosus: an exploratory study. *Rheumatology (Oxford)* 2020;59:3193–3200.
100. Akhter E, Burlingame RW, Seaman AL, et al. Anti-C1q antibodies have higher correlation with flares of lupus nephritis than other serum markers. *Lupus* 2011;20:1267–1274.
101. Fava A, Wagner CA, Guthridge CJ, et al. Association of autoantibody concentrations and trajectories with lupus nephritis histologic features and treatment response. *Arthritis Rheumatol* 2024;76:1611–1622.
102. Izmirly PM, Kim MY, Carlucci PM, et al. Longitudinal patterns and predictors of response to standard-of-care therapy in lupus nephritis: data from the Accelerating Medicines Partnership Lupus Network. *Arthritis Res Ther* 2024;26:54.
103. Taghavi M, Jabrane A, Jacobs L, et al. Antiphospholipid antibodies associated with native arteriovenous fistula complications in hemodialysis patients: a comprehensive review of the literature. *Antibodies (Basel)* 2024;13:1.
104. Wagenknecht DR, Becker DG, LeFor WM, et al. Antiphospholipid antibodies are a risk factor for early renal allograft failure. *Transplantation* 1999;68:241–246.
105. Jorge A, Wallace ZS, Lu N, et al. Renal transplantation and survival among patients with lupus nephritis: a cohort study. *Ann Intern Med* 2019;170:240–247.
106. O'Shaughnessy MM, Montez-Rath ME, Lafayette RA, et al. Differences in initial treatment modality for end-stage renal disease among glomerulonephritis subtypes in the USA. *Nephrol Dial Transplant* 2016;31:290–298.
107. Broder A, Khattri S, Patel R, et al. Undertreatment of disease activity in systemic lupus erythematosus patients with endstage renal failure is associated with increased all-cause mortality. *J Rheumatol* 2011; 38:2382–2389.
108. Azegami T, Kounoue N, Sofue T, et al. Efficacy of pre-emptive kidney transplantation for adults with end-stage kidney disease: a systematic review and meta-analysis. *Ren Fail* 2023;45:2169618.
109. Rana Magar R, Knight SR, Maggiore U, et al. What are the benefits of preemptive versus non-preemptive kidney transplantation? A systematic review and meta-analysis. *Transplant Rev (Orlando)* 2023; 37:100798.
110. Naveed A, Nilubol C, Melancon JK, et al. Preemptive kidney transplantation in systemic lupus erythematosus. *Transplant Proc* 2011; 43:3713–3714.
111. Chung MC, Yu TM, Shu KH, et al. Influence of pretransplantation dialysis time and lupus activity on outcome of kidney transplantation in systemic lupus erythematosus. *Transplant Proc* 2014;46: 336–338.
112. Yap KS, Urowitz MB, Mahood Q, et al. The utility of lupus serology in predicting outcomes of renal transplantation in lupus patients: systematic literature review and analysis of the Toronto lupus cohort. *Semin Arthritis Rheum* 2017;46:791–797.
113. Burgos PI, Alarcón GS, McGwin G, Jr., et al. Disease activity and damage are not associated with increased levels of fatigue in systemic lupus erythematosus patients from a multiethnic cohort: LXVII. *Arthritis Rheum* 2009;61:1179–1186.
114. Broder A, Mowrey WB, Valle A, et al. Prescribing patterns of hydroxychloroquine and glucocorticoids among lupus patients after new-onset end-stage renal disease. *Arthritis Care Res (Hoboken)* 2022; 74:2024–2032.
115. Hiraki LT, Feldman CH, Liu J, et al. Prevalence, incidence, and demographics of systemic lupus erythematosus and lupus nephritis from 2000 to 2004 among children in the US Medicaid beneficiary population. *Arthritis Rheum* 2012;64:2669–2676.
116. Vazzana KM, Daga A, Goilav B, et al. Principles of pediatric lupus nephritis in a prospective contemporary multi-center cohort. *Lupus* 2021;30:1660–1670.
117. Hiraki LT, Hamilton J, Silverman ED. Measuring permanent damage in pediatric systemic lupus erythematosus. *Lupus* 2007;16: 657–662.
118. Chalhoub NE, Wenderfer SE, Levy DM, et al. International consensus for the dosing of corticosteroids in childhood-onset systemic lupus erythematosus with proliferative lupus nephritis. *Arthritis Rheumatol* 2022;74:263–273.
119. de Gruijter NM, Naja M, Peckham H, et al. A systematic review exploring the bidirectional relationship between puberty and autoimmune rheumatic diseases. *Pediatr Rheumatol Online J* 2021;19:47.
120. Rygg M, Pistorio A, Ravelli A, et al. A longitudinal PRINTO study on growth and puberty in juvenile systemic lupus erythematosus. *Ann Rheum Dis* 2012;71:511–517.
121. Sontichai W, Liao F, Dominguez D, et al. Timing of childhood-onset systemic lupus erythematosus diagnosis relative to menarche and the impact on final adult height. *Arthritis Care Res (Hoboken)* 2022; 74:199–207.

122. Chang JC, Sears C, Torres V, et al. Racial disparities in renal outcomes over time among hospitalized children with systemic lupus erythematosus. *Arthritis Rheumatol* 2022;74:1430–1439.
123. Hiraki LT, Lu B, Alexander SR, et al. End-stage renal disease due to lupus nephritis among children in the US, 1995–2006. *Arthritis Rheum* 2011;63:1988–1997.
124. Furie RA, Rovin BH, Garg JP, et al. Efficacy and safety of obinutuzumab in active lupus nephritis. *N Engl J Med* Preprint posted online February 7, 2025. <https://doi.org/10.1056/NEJMoa2410965>.
125. Humphrey MB, Russell L, Danila MI, et al. 2022 American College of Rheumatology guideline for the prevention and treatment of glucocorticoid-induced osteoporosis. *Arthritis Rheumatol* 2023;75(12):2088–102.
126. Bass AR, Chakravarty E, Akl EA, et al. 2022 American College of Rheumatology guideline for vaccinations in patients with rheumatic and musculoskeletal diseases. *Arthritis Care Res* 2023;75(3):449–64.
127. Sammaritano LR, Bermas BL, Chakravarty EE, et al. 2020 American College of Rheumatology guideline for the management of reproductive health in rheumatic and musculoskeletal diseases. *Arthritis Rheumatol* 2020;72(4):529–56.

**EDITORIAL**

# Ever-Expanding Connections Between Peptidylarginine Deiminase 4 and Rheumatoid Arthritis

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When anticitrullinated protein antibodies (ACPAs) were discovered to be highly specific biomarkers for rheumatoid arthritis (RA),<sup>1</sup> rheumatologists and immunologists suddenly became very interested in a posttranslational modification that they had likely never previously even heard of: citrullination. Citrullination, also called deimination, is the conversion of arginines in a protein to citrullines. This modification, now understood to be one of more than one hundred posttranslational modifications that alter protein function and fate,<sup>2</sup> is found in a wide variety of proteins in the RA joint.<sup>3</sup> Over the years, citrullination has been detected in many normal biologic processes, including inflammation in tissue sites owing to a wide variety of causes. However, ACPAs have remained primarily unique to people with RA, and indeed their presence aids in defining the seropositive subset.

Citrullination is catalyzed by the peptidylarginine deiminases (PADs or PADIs). Although there are five PADs, PAD4 often dominates the spotlight in relation to RA and for good reason. Early on, PAD4 was detected in RA synovial tissue,<sup>4</sup> and functional haplotypes of *PADI4* were found to be associated with RA.<sup>5</sup> Later, PAD4 was shown to be required for the formation of citrullinated neutrophil and macrophage extracellular traps (ETs), both of which are bound by ACPAs.<sup>6–9</sup> These observations suggested that PAD4 may contribute to the generation of citrullinated antigens bound by ACPAs through a role in ET formation. Additionally, knocking out or specifically pharmacologically inhibiting PAD4 in different murine RA models reduced inflammatory arthritis.<sup>10,11</sup> However, the reduction in arthritis appeared to be due to altered immune cell function, not impaired citrullinated antigen generation.<sup>10,11</sup> The mechanisms by which PAD4 directly and indirectly impacts immune cells are still emerging, but citrullination of nuclear factor  $\kappa$ B (NF $\kappa$ B) p65 in neutrophils to enhance its nuclear import and induce interleukin (IL) 1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) transcription may be key.<sup>12</sup>

The discovery of anti-PAD4 antibodies suggested a new potentially pathogenic role for PAD4 in RA. As reviewed in Curran

et al,<sup>13</sup> anti-PAD4 antibodies were hypothesized to act as amplifiers of RA. In longitudinal studies, they were detected in a subset of individuals in the pre-RA period before the onset of clinical RA and, in most cases, after ACPAs appeared.<sup>14</sup> These results, along with their increased prevalence with the duration of clinical RA and their association with severe joint destruction, suggested that anti-PAD4 antibodies are part of the expansion of autoimmunity to additional targets and, in some individuals, could play a role in both the transition to arthritis and the development of more severe disease over time. Additional support for the amplifier hypothesis was provided by the finding that some autoantibodies could increase the catalytic activity of PAD4.<sup>13</sup> However, other studies have shown that human RA anti-PAD4 antibodies impair citrullination.<sup>15</sup> Thus, the mechanisms by which anti-PAD4 antibodies might be pathogenic in vivo have remained speculative.

This issue of *Arthritis & Rheumatology* brings new insights regarding the mechanisms by which anti-PAD4 antibodies might amplify disease in clinical RA. Won et al<sup>16</sup> demonstrate that treatment with certain RA patient-derived anti-PAD4 monoclonal antibodies (mAbs), created by transferring human antibody variable domain sequences into a murine IgG2a backbone, exacerbate disease in the murine collagen-induced arthritis (CIA) model of RA. Further, the anti-PAD4 mAb-treated mice exhibited more myeloid cells and synovial fibroblasts in the joint; increased TNF $\alpha$ -, IFN $\gamma$ -, and IL-17-producing T cells in the draining lymph nodes, and increased serum IL-17. The anti-PAD4 mAbs bound fairly exclusively to murine joint monocytes as well as to human monocytes, resulting in enhancement of IL-8, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ) secretion. Finally, TNF $\alpha$ , IFN $\gamma$ , and IL-17 together were shown to up-regulate synovial fibroblast activation, providing a potential pathogenic link between the enhanced T cell cytokines and the synovium. Based on these data, the authors propose a model in which anti-PAD4 antibodies exacerbate inflammatory arthritis by binding to monocytes, resulting in

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enhanced production of cytokines that attract and activate neutrophils, monocytes, and lymphocytes. Recruited and activated T cells then produce  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$ , and IL-17, which stimulate synovial fibroblast hyperplasia. This model fits well with the timing of anti-PAD4 antibody appearance, the procatalytic properties of certain anti-PAD4 antibodies, and the association of anti-PAD4 antibodies with severe joint disease in human RA.

Thus, this article provides several important insights. In the basic science realm, this study provides a very plausible mechanism by which anti-PAD4 antibodies could be pathogenic in human RA. Moreover, it adds to our growing understanding of how different RA-related autoantibodies can be pathogenic or protective. In addition, this article suggests a new and interesting mechanism by which PAD4 may contribute to RA, namely as a target of pathogenic autoantibodies (Figure 1). In the clinical realm, this study adds to the rationale for the use of anti-PAD4 antibodies as informative biomarkers in pre-RA, for identifying individuals with more severe clinical RA, or potentially for personalized treatment strategies or monitoring. To that end, this study supports the development of therapeutics that interfere with anti-PAD4 activity to ameliorate RA in anti-PAD4 antibody-positive patients.

Although this article describes important contributions to the field, it also leaves some questions unanswered. For example, to what do the anti-PAD4 antibodies bind on monocytes? Do they bind PAD4, or do they bind to a different protein in a cross-reactive manner? Some anti-PAD4 antibodies preferentially bind citrullinated PAD4,<sup>17</sup> and ACPAs can be multireactive against many posttranslationally modified proteins.<sup>18</sup> Thus, anti-PAD4 antibodies might bind proteins other than PAD4 on the monocyte surface. The anti-PAD4 antibodies used in this study were reported not to be polyreactive, but they were not tested against a broad range of RA autoantigens, only cyclic citrullinated peptide 3.<sup>19</sup> An experiment that determines if anti-PAD4 antibodies exacerbate CIA in PAD4 knockout mice would shed light on this question.

Additional mechanisms are also not yet explored. For example, do the mAbs have to engage multiple cell surface protein(s) to generate a biologic effect, most likely activating Fc receptors on either the endogenous cell or in a cross-cell manner? Also, are other known drivers of *in vivo* inflammation and damage in the CIA model, like complement activation, necessary for anti-PAD4 antibody effects? The authors did not detect an obvious increase in complement C3 deposition in the synovium, but the most

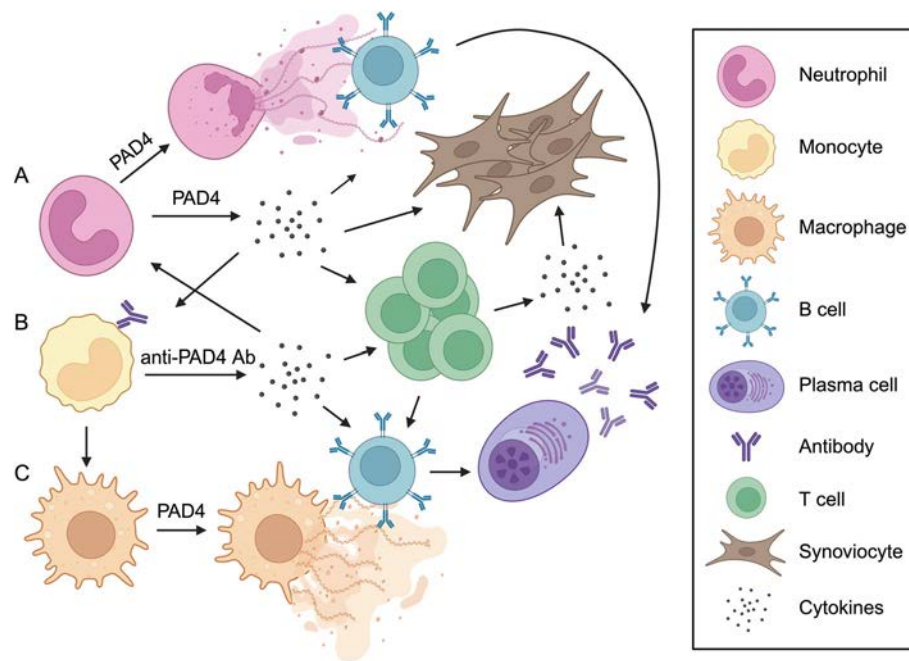


**Figure 1.** Multiple roles of PAD4 in rheumatoid arthritis pathogenesis. (A) PAD4 in neutrophils citrullinates histones as part of NETosis and also citrullinates nuclear factor  $\kappa\text{B}$  p65, leading to the production of  $\text{TNF}\alpha$  and IL-1 $\beta$ , which have inflammatory effects on many types of immune cells. Antigens in NETs are recognized by B cells that become ACPA-producing plasma cells. (B) As newly described in Won et al,<sup>16</sup> anti-PAD4 Abs bind to monocytes, inducing IL-8, monocyte chemoattractant protein 1 and macrophage inflammatory protein 1 $\beta$ , which recruit and activate neutrophils, monocytes, and lymphocytes. The T cells produce  $\text{TNF}\alpha$ , interferon  $\gamma$ , and IL-17, which stimulate synovial fibroblasts and provide help to B cells. (C) In macrophages, PAD4 citrullinates histones enabling METosis. Like NETs, macrophage extracellular traps are recognized by B cells that will differentiate into plasma cells that secrete ACPAs. Ab, antibody; ACPA, anti-citrullinated protein antibody; IL, interleukin; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4;  $\text{TNF}\alpha$ , tumor necrosis factor  $\alpha$ . Figure created in BioRender (<https://biorender.com/m63l781>).

informative means by which to test this hypothesis would be to use complement-deficient mice or anti-PAD4 mAbs in which the C1q engagement site is modified.

Assuming that anti-PAD4 antibodies are binding to PAD4 on monocytes, questions remain regarding how they are driving arthritis. The authors considered the generation of citrullinated antigens, but no increase in gross paw citrullination was detected, consistent with no loss of gross joint citrullination in arthritic mice in which PAD4 was knocked out or inhibited pharmacologically.<sup>11,20</sup> Citrullinated antigens could be increased by anti-PAD4 antibodies on the cell surface at a level too low to be detected in whole paw lysates. However, autoantibodies in CIA are primarily not citrulline-specific<sup>21</sup>; thus, an increase in citrullinated antigens is unlikely to be a major pathogenic driver.

Perhaps more likely is the binding of anti-PAD4 antibodies to PAD4 either on the monocyte surface or possibly in the cytoplasm, as autoantibodies appear to transit the cell membrane in some activated macrophages.<sup>9</sup> PAD4, activated by bound autoantibodies, might citrullinate NF $\kappa$ B p65 with resultant inflammatory cytokine production. As noted above, citrullinated p65 enhances TNF $\alpha$  and IL-1 $\beta$  expression in neutrophils.<sup>12</sup> Won et al found that these cytokines were not secreted by anti-PAD4 stimulated monocytes but IL-8, MCP-1, and MIP-1 $\beta$  were. Because IL-8, MCP-1, and MIP-1 $\beta$  are NF $\kappa$ B targets in general, they also may be targets in this model.<sup>16</sup> Additionally, hyperactivated PAD4 could citrullinate histones as part of ET formation. ETs were not evaluated in this study.

Finally, it is not clear how or why loss of self-tolerance occurs and anti-PAD4 antibodies develop, although several theories have been posited.<sup>13</sup> For at least some autoantibodies, PAD4 appears to be a specific target. These autoantibodies, which are not polyreactive, arise from PAD4-reactive precursors with their agonist activity enhanced by somatic mutation.<sup>19</sup> PAD4 presence on ETs, which are inflammatory, may contribute to loss of tolerance for PAD4. Alternatively, molecular mimicry with the PAD enzyme from *Porphyromonas gingivalis*, which has a long-standing association with RA, or epitope spreading from citrullinated antigens to bound PAD4 could play a role. Of note, PAD4-derived peptides can bind to shared epitope-containing HLA alleles, providing a link to genetic risk for RA.<sup>22</sup> Finally, PAD4 can autocitrullinate, and some autoantibodies preferentially bind citrullinated PAD4.<sup>17</sup> Interestingly, of the 19 citrullines identified by PAD4 autocitrullination, 5 are flanked by glycine, creating the citrulline-glycine motif for multireactive ACPA binding.<sup>18</sup> An additional three citrullines are next to serine, another motif for ACPA binding.<sup>23</sup> Thus, some autoantibodies may bind citrullinated PAD4 nonspecifically as part of their multireactivity.

So, with this article, the PAD4 story continues. This study provides a novel mechanism for a pathogenic autoantibody, a new role for PAD4 in RA pathogenesis, a potential target for immunomodulation in RA, and exciting new avenues for exploration.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Shelef confirms that all authors have provided the final approval of the version to be published and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

- Schellekens GA, Visser H, de Jong BA, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 2000;43(1):155–163.
- Kitamura N, Galligan JJ. A global view of the human post-translational modification landscape. *Biochem J* 2023;480(16):1241–1265.
- Tutturen AE, Fleckenstein B, de Souza GA. Assessing the citrullinome in rheumatoid arthritis synovial fluid with and without enrichment of citrullinated peptides. *J Proteome Res* 2014;13(6):2867–2873.
- Foulquier C, Sebbag M, Clavel C, 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):3541–3553.
- Suzuki A, Yamada R, Chang X, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003;34(4):395–402.
- Li P, Li M, Lindberg MR, et al. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 2010;207(9):1853–1862.
- Holmes CL, Shim D, Kernien J, et al. Insight into neutrophil extracellular traps through systematic evaluation of citrullination and peptidylarginine deiminases. *J Immunol Res* 2019;2019:2160192.
- Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013;5(178):178ra40.
- Bashar SJ, Holmes CL, Shelef MA. Macrophage extracellular traps require peptidylarginine deiminase 2 and 4 and are a source of citrullinated antigens bound by rheumatoid arthritis autoantibodies. *Front Immunol* 2024;15:1167362.
- Shelef MA, Sokolove J, Lahey LJ, et al. Peptidylarginine deiminase 4 contributes to tumor necrosis factor  $\alpha$ -induced inflammatory arthritis. *Arthritis Rheumatol* 2014;66(6):1482–1491.
- Willis VC, Banda NK, Cordova KN, et al. Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis. *Clin Exp Immunol* 2017;188(2):263–274.
- Sun B, Dwivedi N, Bechtel TJ, et al. Citrullination of NF- $\kappa$ B p65 promotes its nuclear localization and TLR-induced expression of IL-1 $\beta$  and TNF $\alpha$ . *Sci Immunol* 2017;2(12):eaal3062.
- Curran AM, Naik P, Giles JT, et al. PAD enzymes in rheumatoid arthritis: pathogenic effectors and autoimmune targets. *Nat Rev Rheumatol* 2020;16(6):301–315.
- Kolfenbach JR, Deane KD, Derber LA, et al. Autoimmunity to peptidyl arginine deiminase type 4 precedes clinical onset of rheumatoid arthritis. *Arthritis Rheum* 2010;62(9):2633–2639.
- Auger I, Martin M, Balandraud N, et al. Rheumatoid arthritis-specific autoantibodies to peptidyl arginine deiminase type 4 inhibit citrullination of fibrinogen. *Arthritis Rheum* 2010;62(1):126–131.
- Won T, Naik P, Wood MK, et al. Anti-peptidylarginine-4 autoantibodies derived from patients with rheumatoid arthritis exert

- pathogenic effects by activating monocytes and exacerbating inflammatory arthritis. *Arthritis Rheumatol* 2025;77(9):1150–1165. <https://doi.org/10.1002/art.43168>
17. Darrah E, Davis RL, Curran AM, et al. Citrulline not a major determinant in the recognition of peptidylarginine deiminase 2 and 4 by autoantibodies in rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(9):1476–1482.
  18. Steen J, Forsström B, Sahlström P, et al. Recognition of amino acid motifs, rather than specific proteins, by human plasma cell-derived monoclonal antibodies to posttranslationally modified proteins in rheumatoid arthritis. *Arthritis Rheumatol* 2019;71(2):196–209.
  19. Shi J, Darrah E, Sims GP, et al. Affinity maturation shapes the function of agonistic antibodies to peptidylarginine deiminase type 4 in rheumatoid arthritis. *Ann Rheum Dis* 2018;77(1):141–148.
  20. Bawadekar M, Shim D, Johnson CJ, et al. Peptidylarginine deiminase 2 is required for tumor necrosis factor alpha-induced citrullination and arthritis, but not neutrophil extracellular trap formation. *J Autoimmun* 2017;80:39–47.
  21. Willis VC, Gizinski AM, Banda NK, et al. N- $\alpha$ -benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. *J Immunol* 2011;186(7):4396–4404.
  22. Auger I, Balandraud N, Massy E, et al. Peptidylarginine deiminase autoimmunity and the development of anti-citrullinated protein antibody in rheumatoid arthritis: the Hapten-Carrier model. *Arthritis Rheumatol* 2020;72(6):903–911.
  23. Zheng Z, Mergaert AM, Fahmy LM, et al. Disordered antigens and epitope overlap between anti-citrullinated protein antibodies and rheumatoid factor in rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(2):262–272.

**EDITORIAL**

# New Insights Into the Multistage Development of Rheumatoid Arthritis

Lars Klareskog

In this issue of *Arthritis & Rheumatology*, Zaim et al have used long-term spared serum samples from the US military to investigate changes in biomarkers that occur before the onset of rheumatoid arthritis (RA).<sup>1</sup> The major novelty resides in the analysis of a large number of inflammatory biomarkers using the very sensitive Olink technology. They found that as many as 42 of 197 investigated serum proteins of relevance for inflammation and immunity were altered as early as >5 years before the onset of RA as compared with a well-matched control population. An additional 42 of these proteins were found to be altered during the 5 years that preceded the onset of RA, with altogether 104 proteins having a different expression at some time before or at diagnosis of RA as compared with the controls. In line with earlier findings, they also describe how levels of anticitrullinated peptide antibodies (ACPAs) measured with the cyclic citrullinated peptide (CCP3) assay as well as rheumatoid factor IgA and rheumatoid factor IgM increased gradually before the diagnosis of RA and showed the greatest differences between cases and controls in the 5-year period closest to the onset of RA. One important conclusion is thus that not only immunity as measured by antibodies but also many proteins related to inflammation are altered a long time before the onset of disease and that changes in anti-CCP3 titers correlated with distinct changes in protein patterns; this study adds substantially to earlier smaller studies<sup>2</sup> in this respect. The other conclusion is that different molecular pathways, as indicated from these protein measurements, are active in different stages of the pre-RA period and that there may be a particularly important timepoint at approximately 5 years before the onset of arthritis, after which new molecular pathways are activated.

The cohort that the authors used, with samples taken from individuals in the military service in the United States, provides a rich resource for this kind of studies. However, the authors also emphasize that results from this cohort may not be directly transferable to other more typical populations of individuals at risk

for RA; for example, almost half of the patients were men, and the average age at onset of RA was 34.9 years.

The study fits well into the very active field of research aiming to understand the origins and trajectories of RA development seen as a continuous process beginning far before the onset of clinical symptoms. As happens so often and also in this case, progress comes from the use of new technologies. The Olink technology is based on a congenial invention with which a completely novel way of determining extremely low levels of proteins is made possible.<sup>3</sup> As described in Figure 1, instead of using the usual immunoassay techniques, such as enzyme-linked immunosorbent assay, the inventor used DNA technology and a so-called “proximity extension assay” of complementary DNA sequences bound to two antibodies recognizing the same protein. As this enables multiple rounds of DNA replication of unique sequences that represent each protein, the technology allows parallel and very sensitive detection of many thousands of proteins using a few microliters of serum or other body fluids.

The now featured work builds on many previous studies from the current and other groups. Thus, it was shown >30 years, ago first from Aho and colleagues in Finland,<sup>4</sup> followed by several studies first from Sweden<sup>5</sup> and the Netherlands,<sup>6</sup> that the RA-specific immunity to proteins posttranslationally modified by citrullination and other means precedes the development of RA by many years. This is in line with studies in other autoimmune diseases, such as type 1 diabetes<sup>7</sup> and systemic lupus erythematosus,<sup>8</sup> and with a logic that autoimmunity is likely to emerge before the onset of the actual autoimmune disease. What is special for RA though, is that we begin to understand where and how this immunity is triggered. Thus, both early studies on gene-environment interactions in RA<sup>9</sup> and subsequent studies on immunity in lungs and gums<sup>10</sup> indicate that external agents, such as noxious agents and microbes, may trigger immunity at mucosal surfaces that may later contribute to symptoms such as arthralgia as well as subsequent arthritis development, which happens in some but not all

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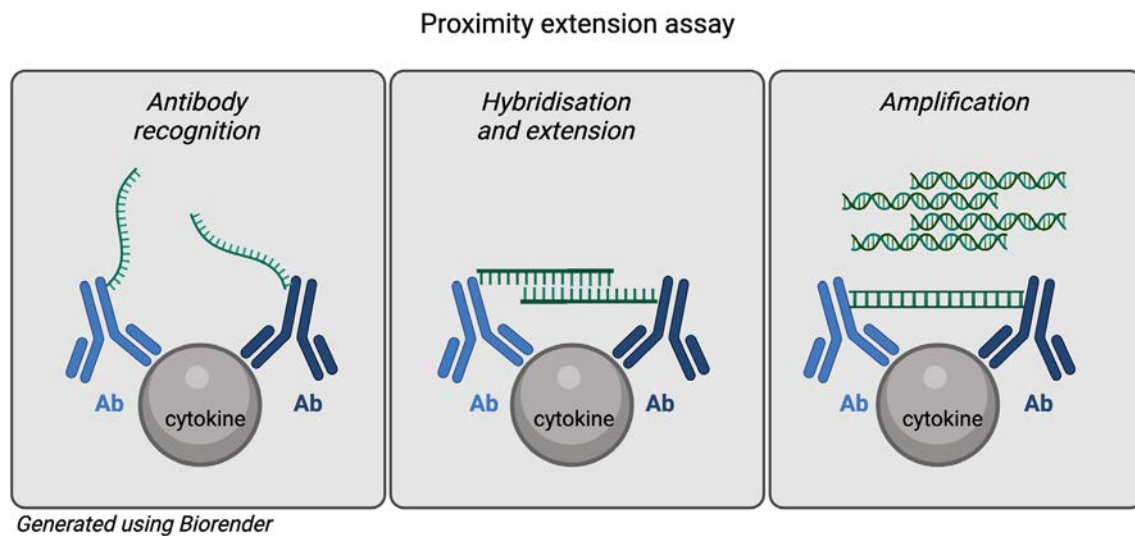


**Figure 1.** The proximity extension assay is based on the use of two Abs that recognize the same target and are both linked to nucleotide sequences (first step) that are complementary and can hybridize if the Abs bind closely to the same target (second step). The sequence formed from the hybridization can now be amplified (third step) by polymerase chain reaction and the now greatly amplified sequences, unique for each protein, can then be detected in a very sensitive way. In this way, large numbers of targets such as cytokines and chemokines can be quantified in parallel using only a few microliters of serum or other body fluids. Ab, antibody.

individuals with this immunity. The now-published identification of pathways related to the intestinal immune network for IgA production is in line with this concept of a mucosal origin of RA-associated immunity.

The now-published article sheds additional light on the process from autoimmunity to autoimmune arthritis in several ways. One highlight of the article is the sophisticated bioinformatics analysis that uses different Olink protein profiles to indicate which molecular pathways are likely to be active in different phases of the pre-RA period. More specifically, the authors' analysis indicates that 21 different pathways are active within 5 years preceding RA diagnosis, including pathways related to disease or infection and adaptive immunity (T cell and B cell receptor signaling). Interestingly, a pathway related to Epstein-Barr virus infection is also identified.

The authors propose that better understanding of these pathways may pave the way for the development of different therapies for prevention in different phases of the transformation from autoimmunity to autoimmune arthritis. However, as with all bioinformatics-based pathway analysis, these proposals need to be experimentally verified before becoming the basis for new therapeutic trials. In line also with earlier studies and based on the timing of changes in antibody reactivity patterns,<sup>5,6</sup> they propose that the initial break of tolerance may occur >10 years before the onset of RA but that major additional changes in autoreactivity may occur <5 years before onset. These latter changes, which coincide with major changes in the proteome patterns, may be decisive for the transformation from autoimmunity to autoimmune arthritis. Whereas the design of the present study did not enable analysis of which factors might be responsible for such changes

at this "inflection point," there are earlier studies that indicate that environmental factors may be very decisive in triggering the initial autoimmunity whereas genetics and, in particular, the influence of HLA-DR variants may be relatively more important in the final stage before the onset of RA.<sup>11</sup> This is the stage when a substantial broadening of the autoimmune repertoire occurs. Notably, this broadening is also accompanied by increased glycosylation of the Fab parts of IgG ACPA.<sup>12</sup> Taken together, the present and earlier findings indicate that we may be able to identify modifiable factors that determine whether the autoimmunity at this "inflection point" will proceed toward arthritis or not.

An important and novel observation in the present study is the finding of different protein patterns before the onset of RA in ACPA-positive versus ACPA negative individuals. It has been known that very different genetic and environmental risk factors are active in these two subsets of RA.<sup>9</sup> The new data from the proteomic analysis further demonstrate that different molecular pathways are used in the evolution of RA in these different disease subsets.

One of the most challenging questions in the field of autoimmunity preceding RA is that a large fraction of ACPA-positive individuals actually never develop RA. Whereas the samples in this study were selected only from individuals who had later developed RA, Zaim et al speculate that the finding of certain biomarkers, in particular increased levels of interleukin 10 and transforming growth factor  $\beta$  in some samples, may represent immune events involved in a potential protection of arthritis. This observation is of particular interest in light of recent studies that indicate that some ACPAs may indeed be involved in the active suppression of arthritis development so far seen in mouse

models.<sup>13</sup> These early observations suggest that it may be possible to manipulate the immune pathways toward protective ones. It will thus be very exciting to investigate the now-observed proteomic patterns in other cohorts in which some of the ACPA-positive individuals have developed RA and others have not.

This brings us to the major aim of all these studies on molecular events preceding RA, that is, how we can use such knowledge for the prevention of RA. Here, the clues from the present article that different molecular mechanisms may be active in different stages of the trajectory toward disease may be helpful in designing new prevention studies. As also discussed in the article, such further efforts toward prevention studies are highly needed as are better prediction models that can separate individuals who will develop arthritis from those who will not. The so far successful prevention studies using abatacept for targeting T cell immunity have demonstrated very pronounced preventive effects during the 6 and 12 months of treatment, respectively, in the two recently published studies.<sup>14,15</sup> However, many but not all of the abatacept-treated individuals developed RA during a 1-year follow-up period. Although these groundbreaking studies have provided proof of concept that pharmacologic prevention of RA will be possible for ACPA-positive risk for those with RA, there is a high need for complementary approaches that may provide long-lasting prevention. Studies like this article by Zaim et al will provide important pieces of the puzzle needed to accomplish the aim of preventing RA.

## AUTHOR CONTRIBUTIONS

The author contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Klareskog confirms that the author has provided the final approval of the version to be published and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

1. Zaim SR, Savage AK, Gillespie MA, et al. Serum proteomic signatures prior to the diagnosis of rheumatoid arthritis demonstrate evolving biological pathways and identify specific periods of disease development. *Arthritis Rheumatol* 2025;77(9):1166–1178. <https://doi.org/10.1002/art.43175>
2. Kokkonen H, Söderström I, Rocklöv J, et al. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. *Arthritis Rheum* 2010;62(2):383–391.
3. Fredriksson S, Gullberg M, Jarvius J, et al. Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol* 2002;20(5):473–477.
4. Aho K, von Essen R, Kurki P, et al. Antikeratin antibody and antiperinuclear factor as markers for subclinical rheumatoid disease process. *J Rheumatol* 1993;20(8):1278–1281.
5. Rantapää-Dahlqvist S, de Jong BA, Berglin E, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 2003;48(10):2741–2749.
6. Nielsen MM, van Schaardenburg D, Reesink HW, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum* 2004;50(2):380–386.
7. Krischer JP, Liu X, Vehik K, et al; TEDDY Study Group. Predicting islet cell autoimmunity and type 1 diabetes: an 8-year TEDDY study progress report. *Diabetes Care* 2019;42(6):1051–1060.
8. Arbuckle MR, McClain MT, Rubertone MV, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003;349(16):1526–1533.
9. Klareskog L, Stolt P, Lundberg K, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum* 2006;54(1):38–46.
10. Holers VM, Demoruelle MK, Kuhn KA, et al. Rheumatoid arthritis and the mucosal origins hypothesis: protection turns to destruction. *Nat Rev Rheumatol* 2018;14(9):542–557.
11. Hensvold AH, Magnusson PK, Joshua V, et al. Environmental and genetic factors in the development of anticitrullinated protein antibodies (ACPAs) and ACPA-positive rheumatoid arthritis: an epidemiological investigation in twins. *Ann Rheum Dis* 2015;74(2):375–380.
12. Rombouts Y, Willemze A, van Beers JJ, et al. Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. *Ann Rheum Dis* 2016;75(3):578–585.
13. Raposo B, Klareskog L, Robinson WH, et al. The peculiar features, diversity and impact of citrulline-reactive autoantibodies. *Nat Rev Rheumatol* 2024;20(7):399–416.
14. Rech J, Tascilar K, Hagen M, et al. Abatacept inhibits inflammation and onset of rheumatoid arthritis in individuals at high risk (ARIAA): a randomised, international, multicentre, double-blind, placebo-controlled trial. *Lancet* 2024;403(10429):850–859.
15. Cope AP, Jasencova M, Vasconcelos JC, et al; APIPPRA study investigators. Abatacept in individuals at high risk of rheumatoid arthritis (APIPPRA): a randomised, double-blind, multicentre, parallel, placebo-controlled, phase 2b clinical trial. *Lancet* 2024;403(10429):838–849.

## NOTES FROM THE FIELD

## The Inflation Reduction Act and Etanercept

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## Introduction

In August, the Centers for Medicare and Medicaid Services (CMS) announced a negotiated price 67% lower than the manufacturer list price for etanercept (Enbrel; Amgen). This price will go into effect in January 2026. Negotiations with the manufacturer began following passage of the Inflation Reduction Act (IRA) that allows CMS to negotiate prices for up to 20 drugs in 2025.<sup>1</sup> Etanercept was one of the first 10 drugs included for negotiation for Medicare Part D. In February, CMS submitted an initial price offer to the manufacturer of etanercept to begin negotiation. The manufacturer responded with a counteroffer. Then the manufacturer and CMS conducted negotiations. In July, CMS sent a final offer to the manufacturer. In August, an agreed upon Maximum Fair Price (MFP) was reached. In January 2025, CMS released a report on how the final MFP was chosen.<sup>2</sup> We review this report as well as publicly available information related to some redacted elements. We conclude with a look forward to lessons for the future.

The IRA's authorizing legislation established an MFP for drugs marketed for at least seven years, with MFPs varying in part based on the number of years on market. Negotiation was bound by an upper limit—the lesser of the current average net (post-rebate) price paid by Medicare Part D plans or a percentage of non-Federal Average Manufacturer's Prices (non-FAMPs). Because etanercept's initial Food and Drug Administration (FDA) approval was more than 16 years ago, that statutory upper limit set for the MFP was 40% of the non-FAMP. But non-FAMP is lower than the list price, so the statutory ceiling was similar to the ultimately negotiated price.<sup>1</sup>

Section 1194(e) of the IRA requires CMS to consider 10 factors (Table 1).<sup>1</sup> Certain data must be submitted by the

manufacturer, and evidence must be provided about therapeutic alternatives. After entering into negotiations, etanercept's manufacturer provided CMS information on seven factors—research and development costs and recoupment, unit costs of production and distribution, prior federal financial support, patents and exclusivities, regulatory approvals, and market data and revenue and sales volume data. Most are confidential. Under CMS's final guidance, etanercept's manufacturer reported on economic implications of etanercept in settings of diverse populations and on listening communications with 20 selected stakeholder organizations. The CMS administrator stated that weighing the importance of each of these public factors could potentially yield steeper discounts.<sup>3</sup>

For some of the factors that are confidential, complementary publicly available information that is likely to be similar (but not exactly the same) is available from other sources. These include a 2021 House Oversight Committee Staff report on etanercept pricing and revenue; a 2024 Colorado Prescription Drug Advisory Board (PDAB) review on etanercept; and a 2023 report from the Assistant Secretary of Planning and Evaluation (ASPE) report.<sup>4–6</sup>

The House Oversight Committee Staff report noted that between 2003 and 2018, etanercept research and development expenditures were \$2 billion in comparison to \$58.3 billion in US net revenues.<sup>4</sup> The main research and development expenditures would have been incurred pre-1998, when etanercept received its first FDA approval and when the manufacturer purchased etanercept's initial manufacturer for \$16 billion. The House Oversight Committee Staff report noted that etanercept manufacturing costs and production costs declined from 2009 to 2018, while prices rose. The proportion of etanercept revenues accounted for by manufacturing costs was 14%.

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**Table 1.** Ten factors reportedly considered in supporting negotiation of the Maximum Fair Price

| Maximum Fair Price negotiation factors                                                     |
|--------------------------------------------------------------------------------------------|
| Research and development costs (redacted)                                                  |
| Manufacturing production and distribution costs (redacted)                                 |
| Market data and revenue (mostly redacted)                                                  |
| Out-of-pocket costs (redacted)                                                             |
| Wholesale Acquisition Costs (some are redacted) of etanercept and therapeutic alternatives |
| Patents                                                                                    |
| Exclusivities                                                                              |
| Therapeutic alternatives and comparative efficacy and safety                               |
| Special population considerations                                                          |
| Stakeholder considerations                                                                 |

Since FDA approval in 1998, the CMS report notes that the original manufacturer of etanercept has two main patents currently in force. The validity of each patent has been challenged in court and was upheld in district court litigation and affirmed on appeal. The 2021 House Oversight Committee Staff report explained that two competitive FDA-approved biosimilar formulations for etanercept (from Samsung and Siemens) are legally blocked from entering the market until 2028 and 2029.<sup>4</sup> The report noted that etanercept’s manufacturers had applied for 68 secondary patents and received 39 secondary patents that could block introduction of biosimilar competitors for 47.5 years.

Therapeutic alternatives reported by CMS include methotrexate, four tumor necrosis factor inhibitors (TNFis), and 13 non-TNFi biologics or small molecules. Clinically, etanercept use is for rheumatoid arthritis (66%), psoriatic arthritis (17%), and ankylosing spondylitis (7%). For rheumatoid arthritis, the report notes that etanercept is considered a first-line biologic if low disease activity is not achieved. Using a longitudinal registry tracing 6,015 patients between 2012 and 2021, use of TNFis as first-line biologic/targeted synthetic after methotrexate declined from 80% to 66%.<sup>7</sup> Although most other biologic use remained stable over methotrexate, use of disease-modifying antirheumatic drugs (DMARDs) increased to 20%, with one to six different lines of DMARD therapy being used and 43% using at least three different therapy lines. Most persons started a TNFi either as monotherapy or with methotrexate.

The PDAB reported a separate analysis of therapeutic alternatives.<sup>5</sup> The report identified four in-class TNFi therapeutic alternatives for rheumatoid arthritis: adalimumab (initial FDA approval, 2002), certolizumab pegol (initial FDA approval, 2009), golimumab (initial FDA approval, 2009), and infliximab (initial FDA approval, 1999). Overall, among in-class TNFis, etanercept has received FDA approval for six indications, with one FDA approval for a pediatric diagnosis, active juvenile psoriatic arthritis in pediatric patients two years of age and older. In-class TNFi therapeutic competitors had fewer FDA approvals than etanercept. The PDAB reported that when compared to placebo, comparative

studies reported significant improvements for each of etanercept’s six FDA-approved clinical indications—supporting etanercept’s role as a therapeutic advance. For some indications, the PDAB noted that etanercept and its in-class therapeutic alternatives were associated with beneficial treatment effects versus other not-in-class prescription drugs.

The CMS report notes that the Wholesale Acquisition Cost (WAC) of a monthly prescription for etanercept was \$609 in 2018, \$1,293 in 2019, and \$1,762 in 2023. The Federal Supply Schedule Service Price (for the Department of Veterans Affairs) was \$1,550 in 2019 and \$3,183 in 2023. The Big Four Insurers (Humana, Aetna, United Healthcare, and CVS Health) listed WACs of \$1,550 in 2019 and \$4,490 in 2023.

Data on revenue and sales volume were redacted from the CMS report. The 2023 report from the ASPE of the Department of Health and Human Services report includes information on revenue and sales.<sup>6</sup> During 2022, 47,000 Medicare Part D enrollees received etanercept primarily for rheumatoid arthritis, psoriasis, or psoriatic arthritis. Total out-of-pocket spending was \$41 million. Total gross Medicare Part D spending on etanercept was \$2.7 billion, with mean annual drug spending per Medicare Part D enrollee of \$56,639. Mean per Medicare Part D enrollee out-of-pocket cost expenditures were \$921.

The CMS report notes that the manufacturer submitted study findings supporting etanercept’s economic value, with etanercept reportedly having the lowest cost per effectively treated rheumatoid arthritis patient compared to therapeutic alternatives. Comparing clinical costs of etanercept and three therapeutic alternatives (infliximab and adalimumab; each is a TNFi) and abatacept (an inhibitor of the interaction between CD80/CD86 and CD28 on T cells), one submitted study found that etanercept had the greatest effectiveness and lowest cost per effectively treated Medicare patient. That study reported adalimumab as being 114% more costly than etanercept for patients with rheumatoid arthritis on Medicaid. One analysis of US commercial claims found that etanercept had a lower cost per effectively treated patient compared to adalimumab, making it one of the most cost-effective options to conventional DMARDs. In 2026, etanercept will have a lower annual Medicare Part D cost (\$94,821) than those listed currently for other therapeutic TNFis equivalents: abatacept (\$174,090), adalimumab (\$154,540), certolizumab pegol (\$236,743), golimumab (\$140,651), infliximab (\$185,369), and tocilizumab (\$109,351), as well as for JAK inhibitor, tofacitinib (\$130,501). Other analyses reportedly found that persons starting etanercept as their first biologic tended to be more persistent and switched less, suggesting improved outcomes and avoiding additional costs, disruptions, and side effects associated with switching. Among persistent patients and those who switch, patients who started with etanercept had lower one-year total health care costs than patients who started with another biologic. With respect to safety, unlike TNFi competitors adalimumab and infliximab, the manufacturer notes that

etanercept is not known to develop neutralizing antidrug antibodies. Across multiple studies, patients treated with etanercept had stable dosing of 95% compared with 66.4% to 92.2% of patients treated with adalimumab and 40.0% to 83.6% of patients treated with infliximab. This may have led to less dose escalation, with etanercept having lower annual TNFi costs, total rheumatoid arthritis–related medication costs, and total pharmacy costs. The CMS report includes data supporting etanercept as a comparatively effective option for special populations. (The CMS report's citations were redacted).

Written statements were submitted by advocacy networks. The American Association of Retired Persons indicated that the negotiated price should take into consideration high rates of patients' missing etanercept doses because of high out-of-pocket costs. A benefit of the low negotiated price is that reduction in etanercept's Medicare price should decrease out-of-pocket costs and improve compliance. The International Foundation for Autoimmune and Autoinflammatory Arthritis reported that from the patient perspective, patients should not be forced to disrupt continuity care and transfer to therapeutic equivalents for cost reasons. Aimed Alliance, a not-for-profit health policy organization, expressed concern with the CMS patient-focused listening session process, noting that randomly selected persons might not provide a comprehensive perspective. The Arthritis Foundation reported that they had polled a network of patient advocates who noted that etanercept had been very effective, but care became challenging when patients adopted Medicare insurance and were required to switch to another biologic. Chronic Care Policy Alliance stated that CMS should ensure that its negotiation process does not disadvantage any patient with an unmet medical need and should guard against results of negotiations undercutting research or negatively impact development of other products. The National Psoriasis Foundation noted etanercept has been identified as the most effective therapy for psoriasis and that if CMS negotiated price favors etanercept over other TNFis, it may lead to unmet medical needs by not favoring the most effective alternative. The Pharmaceutical Care Management Association requested that CMS clarify information on therapeutic alternatives that Part D plans communicate to enrollees and ensure that CMS selection of therapeutic alternatives are not affected by negotiations.

#### **Do we really know how the negotiations went?**

No. Review of publicly available data for 5 of the 10 factors that CMS reportedly used as well as how CMS weighted the 10 individual data elements to negotiate the 67% discounted list price for etanercept was nontransparent. Overall, our policy consultants and clinical consultants felt that the most important factors determining the final negotiated MFP were the initial statutory ceiling price and cost information on therapeutic alternatives. Rome et al hypothesized that the initial statutory ceiling set for etanercept in 2023 was the most important factor, although their report preceded publication of the 2025 CMS report.<sup>8</sup> Although CMS

probably considered the 10 factors in Table 1, it is possible that the CMS evaluation of data from the manufacturer, patients, and advocacy groups did not merit a price much lower than the initial statutory imposed ceiling.

**What is the future likely to look like?** From 2026 to 2029, pricing for etanercept will be based on the negotiated MFP that will take effect in 2026. This may improve access for Medicare beneficiaries. However, commercial payers will not be bound by the MFP. There may be some competitive downward pressure on etanercept's price borne by commercial payers from adalimumab biosimilars. Etanercept may need to offer greater rebates for commercial payers to maintain formulary position if pharmaceutical benefit management program starts to prefer lower cost adalimumab formulations. Because at least two biosimilar formulations for etanercept will begin marketing in 2029 and will exert downward pricing pressures on etanercept, prices for etanercept paid for by CMS are likely to decrease in 2029.

## **Conclusions**

In response to manufacturer concerns about nontransparent IRA drug price negotiation for etanercept and nine other drugs, CMS issued a statement that it plans to consider “opportunities to bring greater transparency to negotiations,” specifically focusing on stakeholder recommendations on ways to improve the Medicare Drug Price Negotiation Program.<sup>9</sup>

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## **REFERENCES**

1. Inflation Reduction Act of 2022, HR 5376, 117th Cong (2021–2022).
2. Center for Medicare and Medicaid Services. Summary Report for Enbrel. January 2025. Accessed April 8, 2025. <https://www.cms.gov/inflation-reduction-act-and-medicare/medicare-drug-price-negotiation>
3. Staff Report. Committee on Oversight and Reform, US House of Representatives. *Drug Pricing Investigation: Amgen- Enbrel and*

- Sensipar*. Staff Report. October 2020. Accessed October 1, 2024. <https://oversightdemocrats.house.gov/sites/evo-subsites/democrats-oversight.house.gov/files/Amgen%20Staff%20Report%2010-1-20.pdf>
4. Assistant Secretary for Planning and Evaluation. Medicare Drug Price Negotiation Program: Understanding Development and Trends in Utilization and Spending for the Selected Drugs. ASPE; December 14, 2023. Accessed November 16, 2024. <https://aspe.hhs.gov/sites/default/files/documents/4bf549a55308c3aad74b34abcb7a1d1/ira-drug-negotiation-report.pdf>
  5. Colorado Prescription Drug Affordability Board. 2023 *Affordability Review Final Report: Enbrel*. 2024. Accessed April 8, 2025. [https://drive.google.com/drive/folders/1xdHNz\\_KHSB5uL6o2DDSqckOZbCsmRXq2](https://drive.google.com/drive/folders/1xdHNz_KHSB5uL6o2DDSqckOZbCsmRXq2)
  6. Seshamani M. I'm the director of the Center for Medicare. Here's how we executed the first round of drug price negotiation. STAT News. October 29, 2024. Accessed October 31, 2024. [https://www.statnews.com/2024/10/29/drug-price-negotiation-ira-second-round-medicare-director/?utm\\_campaign=rss](https://www.statnews.com/2024/10/29/drug-price-negotiation-ira-second-round-medicare-director/?utm_campaign=rss)
  7. Matsson A, Solomon DH, Crabtree MM, et al. Patterns in the sequential treatment of rheumatoid arthritis in patients starting a b/tsDMARD: 10-year experience from a US-based registry. Research Square Preprint posted online February 27, 2023. doi: [10.21203/rs.3.rs-2624931/v1](https://doi.org/10.21203/rs.3.rs-2624931/v1)
  8. Rome BN, Kesselheim AS, Feldman WB. Medicare's first round of drug price negotiation- measuring success. *N Engl J Med* 2024;391(20): 1865–1868.
  9. CMS Press Release: CMS statement on lowering the cost of prescription drugs. Centers for Medicare and Medicaid Services. January 29, 2025. <https://www.cms.gov/newsroom/press-releases/cms-statement-lowering-cost-prescription-drugs>

## NOTES FROM THE FIELD

# The Present and Future of Genetic Sequencing as Applied to Diagnosis and Management in Rheumatology

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## Introduction

The past decade has witnessed a revolution in applying knowledge gleaned from genetic sequencing to the diagnosis and management of patients with immune-mediated rheumatologic conditions. Increasingly, genetic sequencing is proving useful in the field of rheumatology, but the uptake in clinical practice has been modest relative to the potential benefits of such testing. In this article, we outline the present and future applications of genetic sequencing to rheumatologic care and argue for why this tool should become a consistent item in the “black bag” of the practicing clinician.

## What is the present role of genetic sequencing in rheumatology practice?

Genetic sequencing has become indispensable in diagnosing monogenic diseases in both pediatric and adult patients. Inborn errors of immunity (IEs), caused by germline genetic variants, represent a diverse group of nearly 500 monogenic diseases that can produce a broad spectrum of immune dysregulation, including immunodeficiency, autoimmunity, autoinflammation, allergic conditions, lymphoproliferation, and malignancies.<sup>1</sup> Current classification systems approach IEI with two parallel taxonomies: a clinical/phenotypic system and a molecular/genetic system. This distinction recognizes that many patients with clinical features suggestive of monogenic disease will not receive a molecular diagnosis, and that dysregulation of a single molecular pathway can result in diverse clinical phenotypes. In other words, patients with identical genetic defects can exhibit remarkable clinical heterogeneity in terms of disease manifestation, severity, and age at onset. For instance, in pansclerotic morphea, a rare disfiguring

condition that primarily affects children, adults with pathogenic variants in the *STAT4* gene have been found to exhibit milder disease compared with younger family members with identical gene variants.<sup>2</sup> Identification of IEI or monogenic conditions caused by somatic variants, such as mosaic, gain-of-function variants in the *JAK1*<sup>3</sup> gene as the cause of multiorgan immune dysregulation and loss-of-function variants in *UBA1*<sup>4</sup> as a cause of vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic syndrome, not only helps to yield molecular rather than clinical diagnoses but also enables a targeted therapeutic approach.

To many clinicians, monogenic diseases are rare outliers in clinical practice, such as hyper-IgD syndrome/mevalonate kinase deficiency and tumor necrosis factor receptor-associated periodic syndrome. However, many of the monogenic diseases discovered recently can masquerade as common rheumatologic conditions. Germline loss-of-function variants in the *SOCS1* gene have been shown to result in autoimmune manifestations that fulfill the classification criteria for systemic lupus erythematosus.<sup>5</sup> Similarly, gain-of-function variants in the *IKZF1* gene can result in immune dysregulation that manifests as leukocytoclastic vasculitis, vitiligo, Evans syndrome, and IgG4-related disease.<sup>6</sup> These examples highlight the importance of considering genetic sequencing as a diagnostic tool in the rheumatologic evaluation of patients, many of whom may otherwise go undiagnosed or misdiagnosed based purely on clinical presentation.

## What are the potential future applications of genetic sequencing in rheumatology beyond monogenic diseases?

Common genetic variants, or polymorphisms, contribute to the risk of developing polygenic diseases, which are more

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prevalent in the population. Because of the small effects of each common variant, their translation into clinical practice in rheumatology is currently limited. However, polygenic risk scores (PRSs) address this limitation by estimating genetic risk through calculating the cumulative effects of risk alleles identified across the genome. Outside of rheumatology, the performance of PRSs has improved substantially for many common diseases, driven by large-scale genome-wide association studies (GWASs) and advanced statistical methods. A notable example is venous thromboembolism, where PRS derived from GWAS enables risk prediction comparable to that of monogenic forms of hypercoagulable states (such as factor V Leiden).<sup>7</sup> For certain common diseases, progress in PRS has allowed for practical implementation, such as for cardiovascular risk assessment that integrates both clinical risk factors and PRS in the primary care setting and has been found to be feasible and well accepted in the United Kingdom.<sup>8</sup> Implementation research examining the clinical utility of PRS across 10 common chronic diseases is currently ongoing in US academic medical centers, highlighting the growing potential for translating genetic risk assessment into routine clinical care.<sup>9</sup>

Although the clinical translation of PRS in rheumatology lags behind other specialties, it still holds promise for future applications. For example, a PRS developed for ankylosing spondylitis demonstrated superior diagnostic performance compared with HLA-B27 testing alone in European and East Asian populations. However, validation of this PRS in other ancestral populations is needed before clinical application.<sup>10</sup> Similarly, HLA-B51 is strongly associated with Behçet disease but currently has variable utility for diagnostic purposes; an integrated score incorporating HLA-B51, non-HLA genomic information, and clinical information holds greater promise to improve diagnostic yield. Unlike common diseases, the utility of PRS in rare diseases in screening the general population is limited given a low pretest probability. For instance, with a rare disease having a prevalence of 0.1% in the general population, even an individual with a 10-fold increased risk would have an absolute risk of only 1%, a figure not clinically informative. Nevertheless, PRSs can be valuable in specific clinical contexts. For example, researchers have shown that an extension of the PRS approach can achieve high diagnostic accuracy in populations with confirmed inflammatory arthritis.<sup>11</sup> Beyond disease diagnostics, PRSs could potentially be used for a host of fruitful applications: risk stratification in “undifferentiated connective tissue disease,” facilitating patient selection for clinical trials aimed at preventing disease development in individuals with preclinical autoimmunity, examining the interplay between genetics and environmental factors, and more.

An additional application of genetic sequencing to rheumatology practice may be in the form of testing for somatic variants. Clonal hematopoiesis (CH), characterized by the expansion of hematopoietic stem cell clones and their progeny driven by somatic variants in leukemia-associated genes, represents a common

phenomenon in the general population.<sup>12</sup> The prevalence of CH increases with advancing age and is associated with elevated atherosclerotic cardiovascular risk through persistent proinflammatory states. There is also evidence that CH is associated with higher risk of clinical relapse in giant cell arteritis.<sup>13</sup> Further investigation is warranted to explore how genetic testing could be used beyond disease diagnosis, particularly for risk stratification and prediction of treatment response within the same clinical disease entity.

## What are the challenges and opportunities in incorporating genetic sequencing in clinical care?

From the implementation perspective, incorporating genetic sequencing into clinical care requires a multidisciplinary team with expertise in clinical rheumatology, immunogenetics, and genetic counseling. Such team-based care is essential for candidate patient selection, test selection, variant interpretation, and clinical decision-making. Although single-gene testing may be used in scenarios in which there is high suspicion of a specific monogenic disease, simultaneous testing for multiple genes (including targeted gene panels, whole exome sequencing, and whole genome sequencing) is necessary in most cases given the extensive overlap in clinical manifestations among IELs.<sup>1</sup> The logistics of organizing such teams is challenging and resource-intensive, particularly in situations requiring rapid diagnosis. Although the implementation of rapid genomic sequencing in neonatal and pediatric intensive care unit settings has seen significant progress over the past decade,<sup>14</sup> substantial unmet needs persist for outpatient settings and adult populations. Patient acceptance is another crucial element in the successful implementation of genetic sequencing. When offered for treatment improvement, genetic testing has shown high acceptability among participants from diverse demographic and cultural backgrounds.<sup>15</sup> The strongest predictors influencing willingness for the testing are genetic knowledge and deterministic beliefs.<sup>15</sup> Despite this broader understanding, patient acceptance of genetic sequencing specifically within adult or pediatric rheumatology practice remains understudied.

Regarding variant interpretation, major efforts through the Clinical Genome Resource consortium are systemically curating the pathogenicity of rare variants primarily based on published literature.<sup>16</sup> However, many variants may still be classified as variants of uncertain significance (VUSs) because of limited published evidence. There is a major unmet need to develop and implement functional assays for VUS resolution to address this challenge. For example, in patients with suspected deficiency of adenosine deaminase 2 and VUS in *ADA2*, *ADA2* enzymatic activity can be measured to help with the variant classification.<sup>17</sup> However, such functional assays are not available for most monogenic immune diseases at present in a clinical setting.

From a research perspective, a key priority is expanding our understanding of how monogenic immune disorders manifest across their phenotypic spectrums. Variable expressivity and

reduced penetrance create challenges for elucidating the full phenotypic spectrum from a limited number of families. Besides, there is a massive ancestral disparity in genetic research for both monogenic disorders and studies involving GWASs and PRSs. For variant interpretation in monogenic disease diagnosis, *de facto* benign variants in individuals from less-studied subpopulations often receive VUS classifications because of limited population allele frequency estimates in that population.<sup>18</sup> In GWAS research, differences in causal variants, population allele frequencies, and linkage disequilibrium patterns limit the transferability of PRSs developed from one ancestry to another.<sup>19</sup> Addressing these challenges requires large-scale research involving diverse populations through multicenter registries collecting comprehensive clinical and genotype information.

## Conclusions

In summary, the integration of genetic sequencing into rheumatologic care represents both a remarkable opportunity and a significant challenge. Although genetic testing has already revolutionized the diagnosis and management of certain monogenic diseases, its broader application through PRS and somatic variant testing holds promise for future advances in rheumatology care. Success in implementation requires a coordinated multidisciplinary approach, robust variant interpretation resources, and development of functional assays. Most critically, addressing the substantial ancestral disparities in genetic research through large-scale, diverse population studies will be essential to ensure these advances benefit all patients. As we continue to uncover the genetic basis of immune-mediated diseases, the translation of these discoveries into clinical practice will increasingly shape the future of precision rheumatology.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding authors, Drs Beck and Luo confirm that all authors have provided the final approval of the version to be published and take responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

1. Tangye SG, Al-Herz W, Bousfiha A, et al. Human inborn errors of immunity: 2022 update on the classification from the International Union of Immunological Societies Expert Committee. *J Clin Immunol* 2022;42(7):1473–1507.
2. Baghdassarian H, Blackstone SA, Clay OS, et al. Variant STAT4 and response to ruxolitinib in an autoinflammatory syndrome. *N Engl J Med* 2023;388(24):2241–2252.
3. Gruber CN, Calis JJA, Buta S, et al. Complex autoinflammatory syndrome unveils fundamental principles of JAK1 kinase transcriptional and biochemical function. *Immunity* 2020;53(3):672–684.e11.
4. Beck DB, Ferrada MA, Sikora KA, et al. Somatic mutations in UBA1 and severe adult-onset autoinflammatory disease. *N Engl J Med* 2020;383(27):2628–2638.
5. Hadjadj J, Castro CN, Tusseau M, et al. Early-onset autoimmunity associated with SOCS1 haploinsufficiency. *Nat Commun* 2020;11(1):5341.
6. Hoshino A, Boutboul D, Zhang Y, et al. Gain-of-function IKZF1 variants in humans cause immune dysregulation associated with abnormal T/B cell late differentiation. *Sci Immunol* 2022;7(69):eabi7160.
7. Ghose J, Tragante V, Ahlberg G, et al. Genome-wide meta-analysis identifies 93 risk loci and enables risk prediction equivalent to monogenic forms of venous thromboembolism. *Nat Genet* 2023;55(3):399–409.
8. Fuat A, Adlen E, Monane M, et al. A polygenic risk score added to a QRISK<sup>®</sup>2 cardiovascular disease risk calculator demonstrated robust clinical acceptance and clinical utility in the primary care setting. *Eur J Prev Cardiol* 2024;31(6):716–722.
9. Lennon NJ, Kottyan LC, Kachulis C, et al; GIANT Consortium; All of Us Research Program. Selection, optimization and validation of ten chronic disease polygenic risk scores for clinical implementation in diverse US populations. *Nat Med* 2024;30(2):480–487.
10. Li Z, Wu X, Leo PJ, et al; TCRI AS Group. Polygenic risk scores have high diagnostic capacity in ankylosing spondylitis. *Ann Rheum Dis* 2021;80(9):1168–1174.
11. Knevel R, le Cessie S, Terao CC, et al. Using genetics to prioritize diagnoses for rheumatology outpatients with inflammatory arthritis. *Sci Transl Med* 2020;12(545):eaay1548.
12. Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. *Science* 2019;366(6465):eaan4673.
13. Gutierrez-Rodriguez F, Wells KV, Jones AI, et al. Clonal haematopoiesis across the age spectrum of vasculitis patients with Takayasu's arteritis, ANCA-associated vasculitis and giant cell arteritis. *Ann Rheum Dis* 2024;83(4):508–517.
14. Kingsmore SF, Nofsinger R, Ellsworth K. Rapid genomic sequencing for genetic disease diagnosis and therapy in intensive care units: a review. *NPJ Genom Med* 2024;9(1):17.
15. Likhanov M, Zakharov I, Awofala A, et al. Attitudes towards genetic testing: the role of genetic literacy, motivated cognition, and socio-demographic characteristics. *PLoS One* 2023;18(11):e0293187.
16. Bridges SL Jr, Shapira R, Aksentijevich I, et al; ClinGen Rheumatologic Autoimmune Disease Clinical Domain Working Group. Curating genetic associations with rheumatologic autoimmune diseases to improve patient outcomes. *Arthritis Rheumatol* 2024;76(11):1577–1581.
17. Lee PY, Davidson BA, Abraham RS, et al; DADA2 Foundation. Evaluation and management of deficiency of adenosine deaminase 2: an international consensus statement. *JAMA Netw Open* 2023;6(5):e2315894.
18. Appelbaum PS, Burke W, Parens E, et al. Is there a way to reduce the inequity in variant interpretation on the basis of ancestry? *Am J Hum Genet* 2022;109(6):981–988.
19. Ding Y, Hou K, Xu Z, et al. Polygenic scoring accuracy varies across the genetic ancestry continuum. *Nature* 2023;618(7966):774–781.

# Anti-Peptidylarginine Deiminase 4 Autoantibodies Derived From Patients With Rheumatoid Arthritis Exert Pathogenic Effects by Activating Monocytes and Exacerbating Inflammatory Arthritis

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**Objective.** Autoantibodies targeting peptidylarginine deiminase 4 (PAD4), an enzyme involved in protein citrullination, are found in a subset of patients with rheumatoid arthritis (RA) with severe joint disease. However, the mechanisms by which anti-PAD4 antibodies participate in disease pathogenesis are incompletely defined.

**Methods.** We investigated the role of anti-PAD4 monoclonal antibodies derived from patients with RA using a collagen-induced arthritis (CIA) mouse model and human monocyte in vitro cultures. The cellular targets of anti-PAD4 antibodies were identified using mouse knee joint cells and human peripheral blood mononuclear cells. In addition, PAD4 gene and protein expression was assessed using human fibroblast-like synoviocyte in vitro cultures and a single-cell RNA sequencing data set obtained from patients with RA.

**Results.** We show that anti-PAD4 antibody treatment augmented disease severity in the CIA mouse model, with increased joint damage, myeloid cell infiltration, and synovial fibroblast activation. Arthritic mice administered with anti-PAD4 antibodies had an increased proportion of interleukin-17A (IL-17A), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and interferon- $\gamma$  (IFN $\gamma$ )-producing T cells. Anti-PAD4 antibodies preferentially bound monocytes in both humans and mice, eliciting proinflammatory chemokine production by human monocytes in vitro. T cell cytokines enhanced by anti-PAD4 antibodies in the CIA model (ie, IL-17A, TNF $\alpha$ , and IFN $\gamma$ ) synergized to induce a proinflammatory phenotype in human fibroblast-like synoviocytes.

**Conclusion.** Our findings suggest a model in which anti-PAD4 antibody binding to monocytes triggers an inflammatory cascade that promotes immune cell recruitment to the joint and T cell activation, culminating in synovial fibroblast activation and the development of more severe arthritis.

## INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovial joints

leading to pain, swelling, and irreversible destruction of articular bone and cartilage.<sup>1</sup> A hallmark serologic feature of RA is the generation of autoantibody responses to citrullination-associated antigens.<sup>2</sup> Citrullination is the posttranslational deimination of

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arginine residues to the nonclassic amino acid citrulline by the peptidylarginine deiminase (PAD) enzymes.<sup>3</sup> Antibodies to the citrullinated products of this reaction, termed anti-citrullinated protein antibodies (ACPAs), develop in the preclinical phase, are the most specific biomarkers for the diagnosis of RA, and are found in ~70% of patients.<sup>1</sup> Autoantibodies targeting PAD type 4 (PAD4), one of the five PAD isoforms found in humans and mice, are also present in 25% to 45% of patients with RA and are associated with longer disease duration and severe erosive joint damage.<sup>3–5</sup> Despite the clinical significance of ACPAs and anti-PAD4 autoantibodies in RA, the mechanistic role of autoantibodies in RA pathogenesis remains incompletely defined.

In vitro and in vivo studies using polyclonal and monoclonal ACPAs derived from patients with RA have shown that ACPAs alone are insufficient to induce arthritis but can increase osteoclast formation, bone erosion, proinflammatory cytokine secretion, and pain.<sup>6–8</sup> In vitro studies have also shown that polyclonal ACPAs, in immune complexes with citrullinated fibrinogen, can drive tumor necrosis factor (TNF $\alpha$ ) production by macrophages via coligation of Fc receptors and toll-like receptor 4 (TLR-4).<sup>9</sup> However, a more complex picture has emerged with the identification of ACPA monoclonal antibodies cloned from patients with RA that have the ability to ameliorate inflammatory arthritis in mice.<sup>10,11</sup> This heterogeneity in the effect of ACPAs may be because ACPAs are a diverse group of autoantibodies that target citrullinated motifs and are generally not specific for a single citrullinated protein antigen.<sup>12,13</sup> However, the pathologic effect of anti-PAD4 antibodies, which target a single defined autoantigen, on inflammatory arthritis has not yet been studied in vivo.

Notably, although anti-PAD4 antibodies are detected in 18% of patients before disease diagnosis, their prevalence increases with the duration of established RA and is associated with severe joint damage, suggesting anti-PAD4 antibodies as amplifiers rather than initiators of disease.<sup>3,14</sup> In vitro studies using purified proteins have revealed that a subset of anti-PAD4 antibodies has the capacity to activate the catalytic activity of the enzyme, suggesting that one contribution of anti-PAD4 antibodies to RA pathogenesis is driving increased extracellular citrullination of protein substrates.<sup>2,15</sup> Moreover, although PAD4 has historically been viewed as a nuclear enzyme, a fraction of PAD4 can also be detected on the surface of neutrophils and monocytes, which are abundant PAD4-expressing cell types present in the inflamed RA joint.<sup>16,17</sup> This suggests that anti-PAD4 antibodies may also exert direct cellular effects that amplify proinflammatory processes in RA.

To gain insights into mechanisms by which anti-PAD4 antibodies exacerbate inflammatory arthritis, we performed complementary in vitro and in vivo studies using a panel of anti-PAD4 monoclonal antibodies derived from patients with RA. We observed that anti-PAD4 antibodies augmented arthritis severity, joint damage, and both local and systemic inflammation in the collagen-induced arthritis (CIA) mouse model. Interestingly,

monocytes were the primary cellular target of anti-PAD4 antibodies in humans and mice, and anti-PAD4 antibodies triggered the release of proinflammatory chemokines by human monocytes. Collectively, our data suggest a model in which anti-PAD4 antibody binding to monocytes triggers an inflammatory cascade that promotes immune cell recruitment to the joint, Th1 and Th17 cell skewing, and synovial fibroblast activation, culminating in more severe and erosive arthritis. This work identifies a novel direct cellular mechanism for autoantibodies in RA, which has important implications for defining disease-amplifying pathways and designing autoantigen-specific therapies.

## MATERIALS AND METHODS

**Production of human and chimeric anti-PAD4 antibody clones.** Expression vectors encoding for heavy- and light-chain pairs of five human anti-PAD4 monoclonal antibodies (ie, clones 10, 16, 17, 102, and 104) cloned from peripheral blood of patients with RA and a pair of vectors in which clone 102 somatic hypermutations were reverted to a germline sequence (clone GL102) served as the starting material for this study.<sup>18</sup> Clone GL102 has no demonstrable PAD4-binding ability, being used as a negative isotype control to assess the antigen specificity of antibody binding to human leukocyte populations.<sup>18</sup> Using heavy-chain vectors encoding these six monoclonal antibodies, site-directed mutagenesis was performed to mutate three residues in the Fc $\gamma$  portion of each antibody to residues known to abrogate binding of antibodies to Fc $\gamma$  receptor (triple mutant, ie, L234F, L235E, and P331S).<sup>19</sup> Chimeric anti-PAD4 antibody clone 17 was generated by cloning the variable region of human anti-PAD4 antibody clone 17 heavy chain into pFUSE-mIgG2a-Fc vector (InvivoGen) encoding murine IgG2a heavy chain. Human triple mutant and chimeric anti-PAD4 antibodies were then produced recombinantly using the Expi293 (Thermo Fisher) transient expression system and purified using protein G as previously described.<sup>18</sup> A detailed description of anti-PAD4 antibody clones used in this study is provided in Table S1.

**Validation of human and chimeric anti-PAD4 antibody clones.** To evaluate the binding of human anti-PAD4 antibody clones to human and murine PAD4, 1  $\mu$ g of purified antibody clones 10, 16, 17, 102, and 104 was incubated individually with 1  $\mu$ L of<sup>35</sup>S-methionine-labeled human or mouse PAD4 protein generated using TNT Quick Coupled Transcription/Translation Systems (Promega). Radiolabeled immune complexes were immunoprecipitated with Protein A Agarose (Thermo Fisher) and then washed three times. Bound antigen was eluted using Laemmli buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by autoradiography.

The binding of chimeric anti-PAD4 antibody clone 17 to murine PAD4 was measured using in-house generated anti-

PAD4 enzyme-linked immunosorbent assay (ELISA). Briefly, mouse recombinant PAD4 protein (gift from Celgene) was coated onto each well of a high-binding polystyrene 96-well enzyme immunoassay (EIA) plate (Costar). Chimeric anti-PAD4 clone 17 and mouse IgG2a isotype control (BioXCell) antibodies were serially diluted 1:4-fold from a concentration of 10 µg/mL. The plate was first blocked with 3% milk in phosphate buffered saline (PBS) and then incubated with antibodies, washed, and subsequently incubated for one hour with goat anti-mouse IgG horseradish peroxidase (HRP) secondary antibodies. The signal was read at 450 nm using a Wallac Victor 3 1420 Multilabel Counter plate reader (PerkinElmer).

**Mice and CIA induction.** Wild-type male DBA/1 mice (000670) were purchased from the Jackson Laboratory. Mice were housed in specific pathogen-free animal facilities at Johns Hopkins University. Experiments were conducted with 10- to 12-week-old age-matched mice. For CIA induction, mice were immunized with 100 µg of type II collagen emulsified in complete Freund's adjuvant intradermally on day 0. Anti-PAD4 antibody clone 17 or mouse IgG2a isotype antibody (BioXCell), at 0.33 mg per mouse, was intraperitoneally injected into mice on days 14, 21, and 28 after immunization. All methods and protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

**Cell isolation from knee joint.** Mouse knee joints were minced using a single-edge scalpel and then incubated with tissue digestion enzyme solution for 45 minutes at 37°C with agitation. The digestion enzyme solution was prepared with 0.5 mg/mL Liberase TL (Roche) and 50 U/mL DNase I (Worthington). After incubation with digestion enzymes, tissues were dissociated using gentleMACS Dissociator (Miltenyi). Cells were washed and filtered through 70-µm cell strainers (Falcon).

**Clinical assessment of CIA development.** After CIA induction, the severity of arthritis was scored per limb once a week according to the following gradings: grade 0, no erythema or swelling; grade 1, erythema and mild swelling in one toe or ankle joint; grade 2, erythema and mild swelling in more than one toe or from ankle to toes; grade 3, erythema and moderate swelling of entire foot; and grade 4, erythema and severe swelling of entire foot or ankylosed paw. The sum of scores in four limbs was shown as the clinical score. In addition, we measured the thickness of four paws with a caliper once a week.

**Histologic evaluation of inflammation and complement deposition in CIA joints.** Ankle joints were formalin-fixed and paraffin-embedded (FFPE), cut into 5-µm-thick sections, and stained with hematoxylin and eosin. Arthritis severity was evaluated by microscopic assessment of infiltration, bone and cartilage erosion, pannus formation, and synovial fibroblast

hyperplasia. Each item was graded from 0 to 5. Grading was performed by two independent blinded investigators. Images were acquired on a BX43 microscope (Olympus) with a DS-Fi3 camera (Nikon) using NIS-Elements D Software (Nikon).

Complement deposition was visualized by immunofluorescence. In brief, 10-µm-thick FFPE sections were deparaffinized and rehydrated, permeabilized, and blocked in 5% bovine serum albumin in PBS. Washed slides were then incubated with goat anti-mouse/rat complement component C3d antibody (Biotechne). Washed slides were incubated in Alexa Fluor 488 donkey anti-goat IgG (Life Technologies) before five washes with PBS. Slides were mounted with a coverslip using ProLong Gold antifade reagent with DAPI (Invitrogen) and dried overnight before imaging using a Zeiss Axioskop 50 fluorescent microscope with a Zeiss AxioCam HRC camera. Images were systematically obtained from the ankle joint space for each animal. The amount of C3d deposition within a fixed consistent area was calculated after subtracting background using Image J software, and mean values were reported.<sup>20</sup>

**Imaging of collagen disruption.** On day 35 following CIA induction, mice designated for imaging were intravenously injected with 4 nmol of UV-activated collagen mimetic peptide (CMP) through the tail vein, as previously described.<sup>21</sup> Subsequently, each mouse received an administration of 10 nmol of IRDye800CW BoneTag via the tail vein 72 hours after the CMP injection. After 24 hours, mice were euthanized using cervical dislocation, and their lateral scans were performed using an LI-COR Odyssey imager, capturing images in both a 710-nm channel for CMP and an 800-nm channel for BoneTag.

**Flow cytometry analysis.** Knee joint, spleen, and inguinal lymph node cells were stained with viability marker Live/Dead Fixable Aqua (Thermo Fisher) and then Fc-blocked with anti-CD16/32 monoclonal antibody (Thermo Fisher) and normal rat serum (Thermo Fisher). Next, cells were surface stained with fluorochrome-conjugated antibodies against CD45, CD31, CD140a, NKp46, CD11b, Ly6G, F4/80, Ly6C, CD19, and CD3 (BioLegend, Thermo Fisher, or BD Biosciences). For intracellular cytokine detection in T cells, lymph node cells were stimulated with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL) in the presence of GolgiStop (BD Biosciences) for 4 hours at 37°C before staining. After surface staining, cells were fixed, permeabilized, and stained with antibodies against interleukin-17A (IL-17A), TNFα, and interferon-γ (IFNγ) (BioLegend, Thermo Fisher, or BD Biosciences). For the anti-PAD4 antibody binding test, knee joint and blood immune cells were stained with anti-PAD4 clone 17 or mouse IgG2 isotype control antibodies conjugated with Alexa Fluor 647 (Invitrogen). Sample acquisition was performed on a LSR II or LSRFortessa flow cytometer (BD Biosciences) running FACSDiva (BD Biosciences). Results were analyzed using FlowJo software (BD Biosciences).

### **ELISA for serum cytokines, C-terminal telopeptide of type I collagen, and anti-type II collagen autoantibodies.**

To measure serum levels of proinflammatory cytokines in mice with CIA, mouse serum was isolated from blood by centrifugation and diluted at 1:4 in assay buffer. Murine IFN $\gamma$ , TNF $\alpha$ , IL-6, IL-17A, and IL-12p70 levels were quantified using custom V-PLEX assay composed of components of the Proinflammatory Panel 1 and Th17 Panel 1 Mouse kits (Meso Scale Diagnostics). Degradation of type I collagen in serum samples of mice with CIA was measured using RatLaps (C-terminal telopeptide of type I collagen [CTX-I]) EIA immunoassay kit (Immunodiagnostic Systems). For the quantitation of anti-type II collagen autoantibody levels in the mice with CIA, serum samples were diluted 1:1,000 and 1:100,000, measured using the Mouse Anti-Type II Collagen IgG Antibody IgG1 ELISA kit (Chondrex), and fit to the provided standard curve.

### **Citrullinated protein and PAD4 expression analysis in murine joints.**

To detect the presence of citrullinated proteins and PAD4 in mouse joints, one hind paw per mouse was snap-frozen at  $-80^{\circ}\text{C}$ . Hind paws from three representative mice per group were thawed, the skin was removed, and the hind paws were pulverized using a mortar and pestle prechilled with liquid nitrogen. Two hundred microliters of radioimmunoprecipitation assay buffer was added to the pulverized samples. As controls, lysates from resting DMSO-differentiated HL-60 cells or freshly isolated health donor monocytes treated with 1  $\mu\text{M}$  ionomycin for three hours were included. Fifty micrograms of each lysate was separated by SDS-PAGE and transferred onto nitrocellulose membrane (Invitrogen). Membrane was blocked and incubated with the following specific primary antibodies: anti-peptidyl-citrulline IgM clone F95 (EMD Millipore), rabbit anti-PAD4 polyclonal antibody (Proteintech), and mouse anti- $\beta$ -actin clone AC-15 (Sigma-Aldrich). Blot was subsequently incubated with corresponding HRP-conjugated secondary antibodies, and the signal was developed using SuperSignal West Pico Substrate (Thermo Fisher) and detected using Fluorchem R (ProteinSimple).

**Human participants.** Peripheral blood from healthy donors was obtained from two sources: (1) healthy donors from Johns Hopkins University who signed written informed consent for enrollment in a study of healthy volunteers approved by the institutional review board (IRB00066509) and (2) deidentified leukopaks from healthy donors who donated platelets for medical use at the Anne Arundel Blood Donation Center. Healthy donors from Johns Hopkins University were not pregnant and did not have active tuberculosis, HIV, hepatitis infection or a known history of autoimmune disease or cancer at the time of sample collection. Deidentified leukopaks were from volunteers who met the criteria for leukapheresis, including not having active infection, cancer, or bleeding disorder. Heparinized whole blood was collected from healthy donors and separated using

Ficoll-Paque (GE Healthcare)-based density gradient centrifugation. Monocytes were enriched from the peripheral blood mononuclear cell fraction using Human CD14 Microbeads (Miltenyi Biotec).

### **Human anti-PAD4 antibody binding to blood leukocyte populations.**

Five anti-PAD4 and isotype control (clone GL102) human triple mutant antibodies were labeled with fluorochrome Alexa Fluor 647 using antibody labeling kits (Invitrogen). Whole-blood leukocytes from three different healthy donors were incubated with individual Alexa Fluor 647-labeled antibody clones in combination with antibodies to cell lineage markers (CD3, CD4, CD8, CD19, CD14, CD16, and CD56; from BD Biosciences or BioLegend). Following incubation, cells were washed extensively, and sample acquisition was performed using FACS Aria II (BD Biosciences). Data were analyzed using FCS Express Flow Cytometry Analysis Software (De Novo Software). Normalized percentage binding of each anti-PAD4 antibody to gated cell populations (monocytes, neutrophils, B cells, natural killer [NK] cells, and T cells) was calculated using the following equation and averaged across donors: normalized percentage binding to a cell population = [relative antibody binding to a specific cell population/total relative antibody binding to all cell populations]  $\times$  100, in which “relative antibody binding to a specific cell population” = the number of antibody-bound cells/the total number of cells, and “total relative antibody binding to all cell populations” = the sum of relative binding to all five populations.

### **Multiplex electrochemiluminescence assay for cytokines and chemokines produced by monocytes.**

To determine the effect of human anti-PAD4 antibodies on human monocytes, freshly purified healthy donor monocytes were treated with anti-PAD4 clone 102 or human IgG1 isotype control (Sigma) antibodies. The in-house antibodies were tested for endotoxin, whereas polymyxin B (1 ng/mL) was added in all treatments to eliminate any off-antibody effects. Following 24-hour treatment with antibodies, cell culture media were collected, and levels of IL-1, TNF $\alpha$ , IL-6, IL-8, to monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), MIP- $\beta$ , macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured using a custom U-PLEX assay (Meso Scale Diagnostics). Briefly, each biotinylated capture antibody was added to its unique linker and incubated. The plate was coated with multiplex coating solution prepared by combining the 10 U-PLEX linker-coupled antibody solutions. Sample diluent was added to the plate before the addition of test samples or calibrators. The plate was washed, and detection antibody was added. After washing, read buffer was added to the plate, and data were acquired using a MESO QuickPlex SQ 120MM. Data were analyzed using Discovery Workbench (Meso Scale Diagnostics).

**Human fibroblast-like synoviocyte studies.** Primary human fibroblast-like synoviocytes (HFLS) obtained from a healthy individual and a patient with RA were purchased from Cell Applications. Cells were stimulated with various stimuli for 6 or 24 hours for reverse transcriptase–polymerase chain reaction (RT-PCR) experiments or for 72 hours for immunoblot experiments. To assess PAD4 expression, healthy and RA HFLS were stimulated using 50 ng/mL of recombinant human (rh) IFN $\gamma$  (PeproTech), rhTNF $\alpha$  (BioLegend), and rhIL-17A (R&D Systems) each or in combination. To examine PAD4 expression following TLR agonist stimulation, cells were stimulated with the following:  $1 \times 10^8$  heat-killed *Listeria monocytogenes* per mL (a TLR-2 agonist; InvivoGen), 20  $\mu$ g/mL of poly(I-C) (a TLR-3 agonist; InvivoGen), 100 ng/mL of lipopolysaccharide (a TLR-4 agonist; InvivoGen), 1  $\mu$ g/mL of flagellin (a TLR-5 agonist; InvivoGen), 1% normal human AB serum (Sigma), or 1% human serum heat-aggregated to create immune complexes. Serum starvation was performed by culturing cells in serum-free media for six hours. Cells were also subjected to serum starvation for two hours followed by 1  $\mu$ M ionomycin stimulation for three hours. Whole-cell lysates from monocytes isolated from human peripheral blood incubated with 1  $\mu$ M ionomycin stimulation for three hours served as a positive control for PAD4 expression.

For RT-PCR analysis, total RNA was extracted from HFLS using TRIzol (Invitrogen), and complementary DNA was generated using SuperScript VILO Master Mix (Invitrogen). *PADI4* (probe: Hs00202612\_m1 FAM) and *GAPDH* (probe: Hs99999905\_m1 VIC PL) were amplified using TaqMan gene expression Master Mix (4369016 Applied Biosystems; Thermo Fisher) and detected using QuantStudio 3 System (Thermo Fisher). Expression of *PADI4* was normalized to *GAPDH* expression for each sample and calculated using the  $\Delta\Delta C_t$  method (QuantStudio Design & Analysis Software; Thermo Fisher). Fold change in  $2^{(-\Delta\Delta C_t)}$  was calculated relative to positive control (unstimulated monocytes) and graphed. For immunoblot analysis, 10 to 20  $\mu$ g of lysate per sample was diluted in Laemmli buffer, separated using SDS-PAGE, and immunoblotted using mouse anti-human PAD4 antibody (Abcam for TLR ligand and serum starvation experiments or Sigma-Aldrich for CIA cytokine experiment), mouse anti-human cadherin 11 (R&D Systems), rabbit anti-human cathepsin-K (Abcam), mouse anti-human vascular cell adhesion molecule 1 (VCAM-1) (R&D Systems), and mouse anti-human  $\beta$ -actin (Sigma-Aldrich). After incubation with the respective HRP-conjugated secondary antibodies, the signal was developed using SuperSignal West Pico Substrate (Thermo Fisher) and detected using Fluorchem R (ProteinSimple). The signal was then quantified and normalized to internal  $\beta$ -actin controls using AlphaView software (ProteinSimple).

For flow cytometry analysis, HFLS were harvested after 72 hours of stimulation, blocked using human Fc block (BD Pharmingen), and stained using the antibodies HLA-DR,

CD40, programmed death ligand 1 (PD-L1), CD80, CD83, and CD86 and Live/Dead Blue Fixable Stain (BD Horizon, BioLegend, or Thermo Fisher). Sample acquisition was performed on Cytex Aurora (Cytex Biosciences), and data were analyzed using FCS Express 7 (De Novo Software).

**Analyzing *PADI4* expression in single cells from RA and osteoarthritis synovium.** The Accelerating Medicines Partnership (AMP) RA phase 2 data set interactive site was searched for the “*PADI4*” gene, and corresponding gene expression in cell clusters and the expression summary were exported for stromal cells (fibroblast population) and myeloid cells.<sup>22</sup> Descriptions of fibroblast and myeloid cell subpopulations can be found in the article by Zhang et al.<sup>23</sup>

**Statistics.** GraphPad Prism 9 software was used for statistical analysis. Data were analyzed using Student's *t*-test, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or two-way ANOVA followed by Tukey's multiple comparison test. Statistically significant comparisons were represented as  $P < 0.05$ ,  $P < 0.005$ ,  $P < 0.0005$ , and  $P < 0.0001$ .

## RESULTS

**Anti-PAD4 antibody treatment exacerbates CIA in mice.** To study the effect of anti-PAD4 antibodies in mice, we first evaluated the ability of five anti-PAD4 monoclonal antibodies (termed anti-PAD4ra antibodies), previously cloned from the circulating memory B cells from patients with RA,<sup>18</sup> to bind to murine PAD4. Among these, clones 10, 16, and 17 were clonally related antibodies from a single donor that were found to target a similar epitope on PAD4, whereas clones 102 and 104 were from a second donor and targeted two distinct PAD4 epitopes (Table S1).<sup>18</sup> This analysis revealed strong reactivity of the related clones 10, 16, and 17 to murine PAD4, with negligible binding of clones 102 and 104 (Figure 1A). As such, we selected clone 17 as a representative anti-PAD4 antibody to carry forward into in vivo studies. To enable mouse Fc-dependent effector functions and reduce immunogenicity, we generated a human-mouse chimeric version of clone 17, in which the human heavy-chain constant region was replaced with the mouse IgG2 heavy chain (anti-PAD4ch; Table S1).<sup>18</sup> Importantly, generation of the chimeric antibody did not abrogate antigen binding, with anti-PAD4ch antibody clone 17 demonstrating significant binding to recombinant mouse PAD4 compared to the mouse IgG2 isotype control antibody ( $P < 0.0001$ , two-way ANOVA) (Figure 1B).

We next investigated the pathologic effect of anti-PAD4ch clone 17 antibodies in mice with CIA, an established animal model for RA. DBA/1 mice with CIA were injected with anti-PAD4ch or mouse IgG2 isotype control antibodies on days 14, 21, and 28 after CIA induction (Figure 1C). We found that mice treated with anti-PAD4 antibodies exhibited significantly worsened

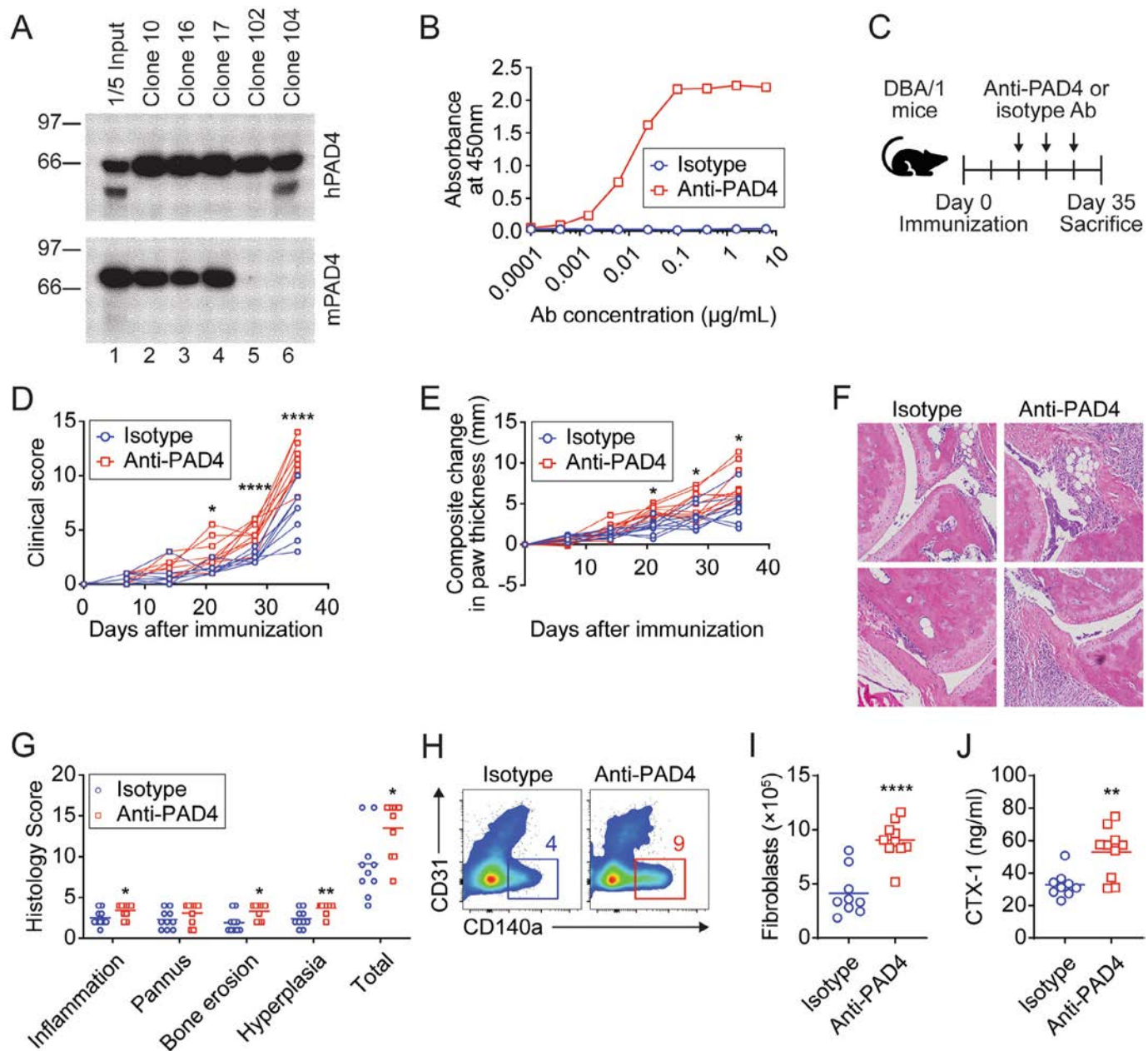


**Figure 1.** Anti-PAD4 antibody treatment worsens arthritis in mice. (A) Binding of anti-PAD4ra antibody clones 10, 16, 17, 102, or 104 to S<sup>35</sup>-labeled in vitro transcribed and translated human or mouse PAD4 protein. (B) Binding capacity of anti-PAD4ch antibody clone 17 to mouse PAD4 protein using ELISA. Mouse IgG2 isotype antibodies served as a control. (C) Experimental timeline for CIA induction and anti-PAD4ch antibody treatment. Male DBA/1 mice were immunized with type II collagen on day 0 and treated with anti-PAD4ch clone 17 or mouse IgG2 isotype control antibodies on days 14, 21, and 28. (D and E) Clinical score (D) and paw thickness change (E) in mice with CIA. Paw thickness change is shown as the sum of changes in four paws. (F and G) Images of H&E staining (F) and histology score (G) on ankle joints from mice treated with anti-PAD4ch or isotype control antibodies. (H and I) Flow cytometry plots (H) and cell number (I) of synovial fibroblasts from mouse knee joints. Fibroblasts were gated on CD45<sup>+</sup>CD31<sup>+</sup>CD140a<sup>+</sup> (Figure S2A). (J) Serum CTX-I level in mice with CIA measured by ELISA. Representative data from three independent experiments with 9 to 10 male mice per group are shown. Two-way ANOVA test (B) or Student's *t*-test (D, E, G, I, and J) was used for statistical analysis. \**P* < 0.05; \*\**P* < 0.005; \*\*\*\**P* < 0.0001. ANOVA, analysis of variance; CIA, collagen-induced arthritis; CTX-I, -terminal telopeptide of type I collagen; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; PAD4, peptidylarginine deiminase 4.

arthritis compared to the isotype control group, starting from day 21 of immunization until the end point at day 35 (Figure 1D). Furthermore, the anti-PAD4 antibody treatment led to an increase in

paw thickness in all four paws compared to the isotype control (Figure 1E and Figure S1A). The histologic analysis confirmed that the treatment with anti-PAD4ch antibody clone 17 worsened

arthritis (Figure 1F). Specifically, the ankle joints of mice treated with anti-PAD4 antibodies exhibited a marked increase in immune cell infiltration, bone and cartilage erosion, and synovial fibroblast hyperplasia compared to the ankle joints of mice in the control group, whereas the severity of pannus formation was comparable between groups (Figure 1G). To assess collagen remodeling in mice with CIA, we used CMP-based probes to label denatured collagen (Figure S1B).<sup>21</sup> The subsequent imaging analysis demonstrated that anti-PAD4 antibody treatment induced elevated collagen disruption in the paws and knees of arthritic mice, with a trend toward significance ( $P = 0.0606$ ) (Figure S1C and D).

Flow cytometry analysis of nonhematopoietic cells demonstrated a significant increase in the number of fibroblasts in the knee joint of mice treated with anti-PAD4 antibodies (Figure 1H and Figure S2A). Additionally, the serum level of cross-linked CTX-I, a biomarker of bone resorption, was elevated in mice treated with anti-PAD4ch antibodies (Figure 1J). To investigate whether the worsening of arthritis due to anti-PAD4 antibody treatment was linked to increased citrullination in the joints, we compared the deposition of citrullinated proteins between the two groups. However, mice treated with anti-PAD4 antibodies exhibited a comparable level of citrullinated proteins and PAD4 protein expression in the joint to that of the isotype control mice (Figure S1E). Given that complement activation is an effector function of IgG1 in humans (the most common anti-PAD4 isotype in RA) and IgG2a in mice, complement component C3d was visualized in the ankle joints of mice injected with anti-PAD4ch or isotype control antibodies by immunofluorescence.<sup>24</sup> This analysis showed that although C3d deposition was highly variable between animals in both the anti-PAD4 and isotype control groups, there was no appreciable difference in C3d deposition between the two groups (Figure S1F and G). In addition, the serum levels of anti-type II collagen antibodies, known as pathogenic autoantibodies in the CIA model, were comparable between the groups (Figure S1H). Collectively, these findings indicate that anti-PAD4 autoantibodies play a pathologic role in inflammatory arthritis by exacerbating inflammation and tissue damage in the joint and that this process involves mechanisms other than increasing citrullination or anticollagen antibodies as well as complement activation.

**Anti-PAD4 antibody treatment increases local and systemic inflammation in mice with CIA.** In our histologic analysis, treatment with anti-PAD4ch antibodies resulted in increased infiltration of inflammatory cells into the joints of arthritic mice compared to the isotype control group (Figure 1F and G). To further investigate the specific inflammatory cell populations that were increased following anti-PAD4 antibody treatment, we profiled the immune cells present in the knee joint using flow cytometry (Figure S2A). The number of total CD45<sup>+</sup> cells in the joints of anti-PAD4ch antibody-treated mice was significantly higher than

that of the isotype control group (Figure 2A), which was consistent with the histologic findings (Figure 1F and G).

Among immune cell populations, myeloid cells, including neutrophils, monocytes, and macrophages, were the major contributors to the severe joint infiltration observed in arthritic mice treated with anti-PAD4 antibodies (Figure 2B). Additionally, we found an increase in the number of neutrophils and monocytes in the spleen of anti-PAD4 antibody-treated mice (Figure 2C). In contrast, the number of lymphoid compartment cells, such as T cells and B cells, in the knee joint was comparable between the anti-PAD4 antibody and isotype control groups (Figure S2B). However, we found that a greater percentage of CD4<sup>+</sup> T cells from the draining lymph node of arthritic mice treated with anti-PAD4ch antibody clone 17 produced TNF $\alpha$  and IL-17A compared to that from the isotype control group (Figure 2D and E). Similarly, a greater percentage of CD8<sup>+</sup> T cells in the lymph nodes of the anti-PAD4 antibody treatment group produced TNF $\alpha$  and IFN $\gamma$  compared to that in the control group (Figure 2D and F). Supporting the Th17 skewing in CD4<sup>+</sup> T cells following anti-PAD4ch antibody treatment, we also observed significantly elevated serum levels of IL-17A in mice treated with these antibodies, whereas other cytokine levels remained comparable with those in the isotype control group (Figure 2G and Figure S2C). These data suggest that anti-PAD4 autoantibodies enhance both innate and adaptive immune responses in the context of inflammatory arthritis.

**Anti-PAD4 antibodies preferentially bind to murine monocytes in the joint and blood.** Given that increased myeloid cell infiltration into the joints and spleens was observed in anti-PAD4ch antibody-treated CIA mice and the recent report that PAD4 is expressed on the surface of neutrophils and monocytes,<sup>17,25</sup> we next examined whether anti-PAD4 antibodies could bind directly to murine leukocytes. Knee joint cells were isolated from DBA/1 mice with CIA or under naive conditions and stained with anti-PAD4ch clone 17 or mouse IgG2 control antibodies *ex vivo* (Figure 3A). Interestingly, monocytes were the primary cellular target of anti-PAD4 antibodies in the joints of arthritic or naive mice (Figure 3A and B and Figure S3A). Other immune cell populations, such as neutrophils, macrophages, T cells, B cells, and NK cells, exhibited minimal binding by these antibodies (Figure 3A and B and Figure S3A). Monocytes present in the mouse knee joint with CIA showed a higher level of anti-PAD4 antibody binding than monocytes in the naive healthy joint (Figure 3B). Additionally, to investigate whether anti-PAD4 antibodies bind to synovial fibroblasts, potentially leading to increased hyperplasia shown in the CIA mouse model (Figure 1F–I), we stained fibroblasts isolated from arthritic or naive mouse knee joints with anti-PAD4ch clone 17 or isotype control antibodies (Figure 3A and B and Figure S3A). There was minimal anti-PAD4 antibody binding to synovial fibroblasts, with no differences between CIA and naive conditions.

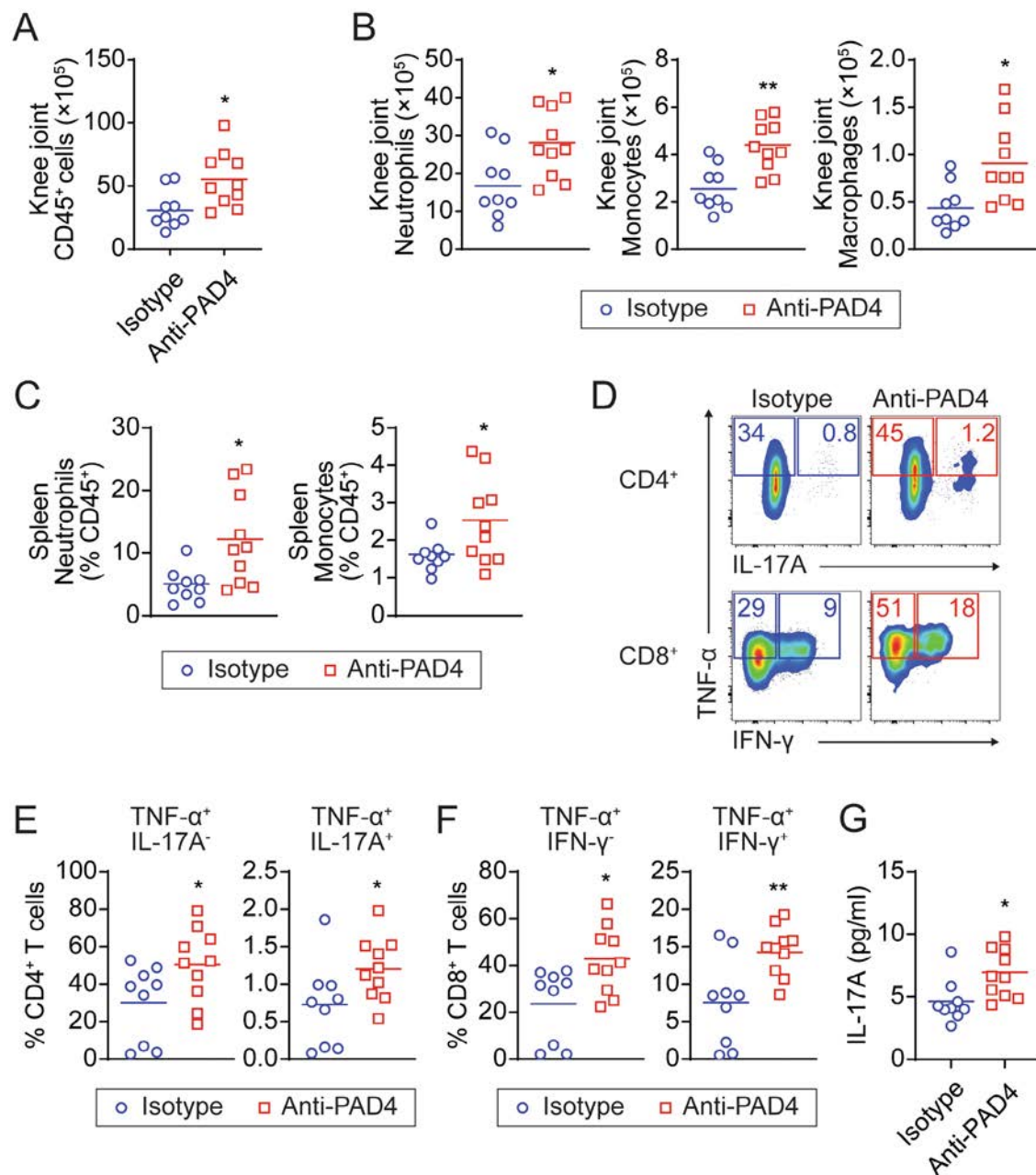


**Figure 2.** Anti-PAD4 antibody treatment leads to severe synovial inflammation in mice with arthritis. Male DBA/1 mice were immunized with type II collagen on day 0 and treated with anti-PAD4ch clone 17 or mouse IgG2 isotype control antibodies on days 14, 21, and 28. (A) CD45<sup>+</sup> cell number in mouse knee joints with CIA. (B) The number of knee joint neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>), monocytes (CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>+</sup>), and macrophages (CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>+</sup>) in mice with arthritis. The gating strategy used for flow cytometry analysis is illustrated in Figure S2A. (C) The number of spleen neutrophils and monocytes in arthritic mice treated with anti-PAD4ch or isotype control antibodies. (D–F) Flow cytometry plots (D) and frequencies of cytokine-producing cells in CD4<sup>+</sup> (E) and CD8<sup>+</sup> (F) T cells from draining lymph nodes. Intracellular TNF $\alpha$  and IL-17A were stained in CD4<sup>+</sup> T cells. TNF $\alpha$  and IFN $\gamma$  were stained in CD8<sup>+</sup> T cells. (G) Serum IL-17A level in mice measured using electrochemiluminescence assay. Representative data from three independent experiments with 9 to 10 male mice per group are shown. Student's *t*-test was used for statistical analysis. \**P* < 0.05; \*\**P* < 0.005. CIA, collagen-induced arthritis; IFN $\gamma$ , interferon- $\gamma$ ; IL-17A, interleukin-17A; PAD4, peptidylarginine deiminase 4; TNF $\alpha$ , tumor necrosis factor  $\alpha$ . Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43168/abstract>.

To determine whether anti-PAD4 antibodies bind to circulating mouse monocytes as well as those in the joint, we tested antibody binding to blood leukocytes. We again found that anti-PAD4ch antibody clone 17 targeted monocytes in the blood of

naive mice (Figure 3C and D and Figure S3B). Unlike in the knee joint, circulating neutrophils were also bound by these antibodies, although the binding level was lower than that of monocytes (Figure 3C and D and Figure S3B). These findings suggest that

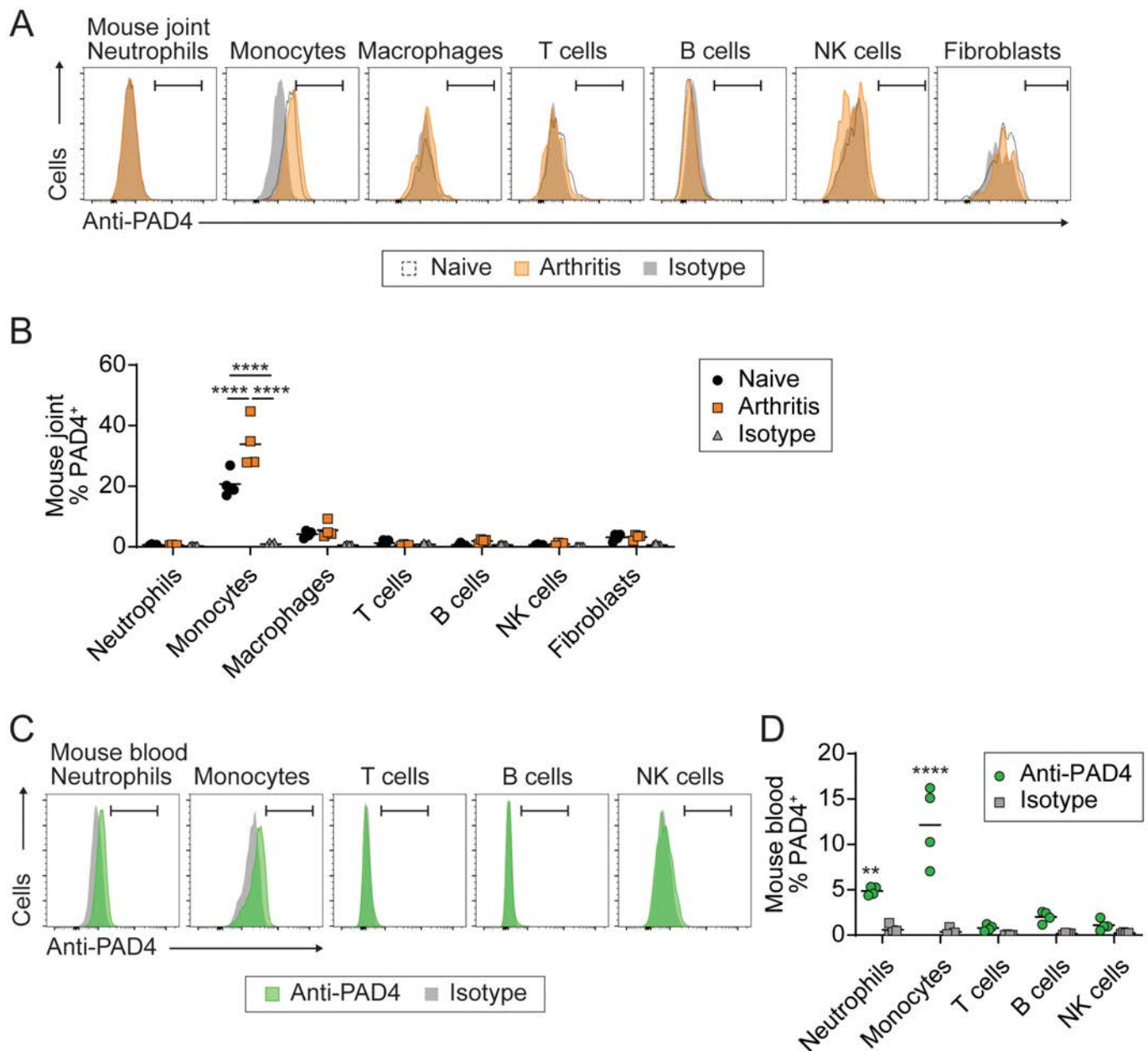


**Figure 3.** Anti-PAD4 antibodies bind to monocytes in mouse joints and blood. (A and B) Histograms (A) and frequencies (B) of anti-PAD4ch antibody clone 17-bound cells in knee joint neutrophils, monocytes, macrophages, T cells, B cells, NK cells, and fibroblasts from DBA/1 mice with CIA or naive mice. Male mice were immunized with type II collagen on day 0 to induce CIA and killed on day 35. Mouse IgG2 isotype antibodies served as a control. (C and D) Histograms (C) and frequencies (D) of anti-PAD4ch antibody clone 17-bound cells in blood neutrophils, monocytes, T cells, B cells, and NK cells from naive mice. Mouse IgG2 isotype antibodies served as a control. Representative data from three independent experiments with four to five male mice per group are shown. Two-way ANOVA followed by post hoc Tukey's multiple comparison test was used for statistical analysis. \*\* $P < 0.005$ ; \*\*\*\* $P < 0.0001$ . ANOVA, analysis of variance; CIA, collagen-induced arthritis; NK, natural killer; PAD4, peptidylarginine deiminase 4. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43168/abstract>.

anti-PAD4 antibodies primarily target murine monocytes in both blood and joints under arthritic or homeostatic conditions.

**Anti-PAD4 antibodies bind to and activate human monocytes.** Given the striking preference of anti-PAD4 antibodies for murine monocytes in blood and joints, we sought to identify

the cellular targets of anti-PAD4 antibodies in human peripheral blood using the complete panel of RA-derived anti-PAD4 monoclonal antibodies (clones 10, 16, 17, 102, and 104). Importantly, we used two approaches to address whether antibody binding to target cells was antigen specific (ie, dependent on the variable region). First, the Fc domains of the monoclonal antibodies were

mutated to abrogate the nonspecific binding of the antibody to leukocyte Fc $\gamma$  receptors (termed triple mutant [tm] antibodies) (Table S1).<sup>19</sup> Second, because clone 102 requires somatic hypermutations for PAD4 binding,<sup>18</sup> the somatic hypermutations were reverted to the germline sequence and the Fc portion mutated, resulting in the clone GL102tm, an isotype control antibody, which should bind to neither PAD4 nor cellular Fc $\gamma$  receptors.

We found that, as observed with mouse cells, anti-PAD4 antibodies preferentially bound to human monocytes compared to other circulating leukocyte populations (Figure 4A and B). Four of the five clones showed significant binding to monocytes compared to the isotype control GL102tm antibody. The binding of anti-PAD4tm monoclonal antibodies to human neutrophils was highly variable, ranging from 8% to 40% depending on the donor, and did not demonstrate significant binding over the GL102tm isotype control (Figure 4A). Some anti-PAD4 antibody clones showed variable but significant binding to B and NK cells, whereas negligible binding was observed in T cells for all clones (Figure 4A and Figure S4A). In addition to the heterogeneity

among donors, the difference in antibody binding is likely explained because the antibodies target different epitopes on PAD4.<sup>18</sup>

Given the preference of anti-PAD4 antibodies for monocytes, we next investigated the effect of anti-PAD4 antibody binding to monocytes using the human anti-PAD4ra antibody clone 102, which exhibited the highest affinity for human PAD4 in our previous study<sup>18</sup> and also robustly bound to monocytes in our flow cytometry analysis (Figure 4A). For this evaluation, monocytes isolated from three healthy human donors were incubated with anti-PAD4ra clone 102 or human IgG1 isotype control antibodies, and the production of myeloid cell cytokines and chemokines was measured using a multiplex electrochemiluminescence assay (Figure 4C). Our results demonstrated that anti-PAD4 antibodies enhanced the secretion of IL-8, MCP-1, and MIP-1 $\beta$  in human monocytes compared to the isotype control but did not induce IL-1 $\beta$ , IL-6, IL-12p70, TNF $\alpha$ , GM-CSF, M-CSF, or MIP-1 $\alpha$  production (Figure 4C and Figure S4B). Therefore, our data indicate that anti-PAD4 antibodies primarily bind to human

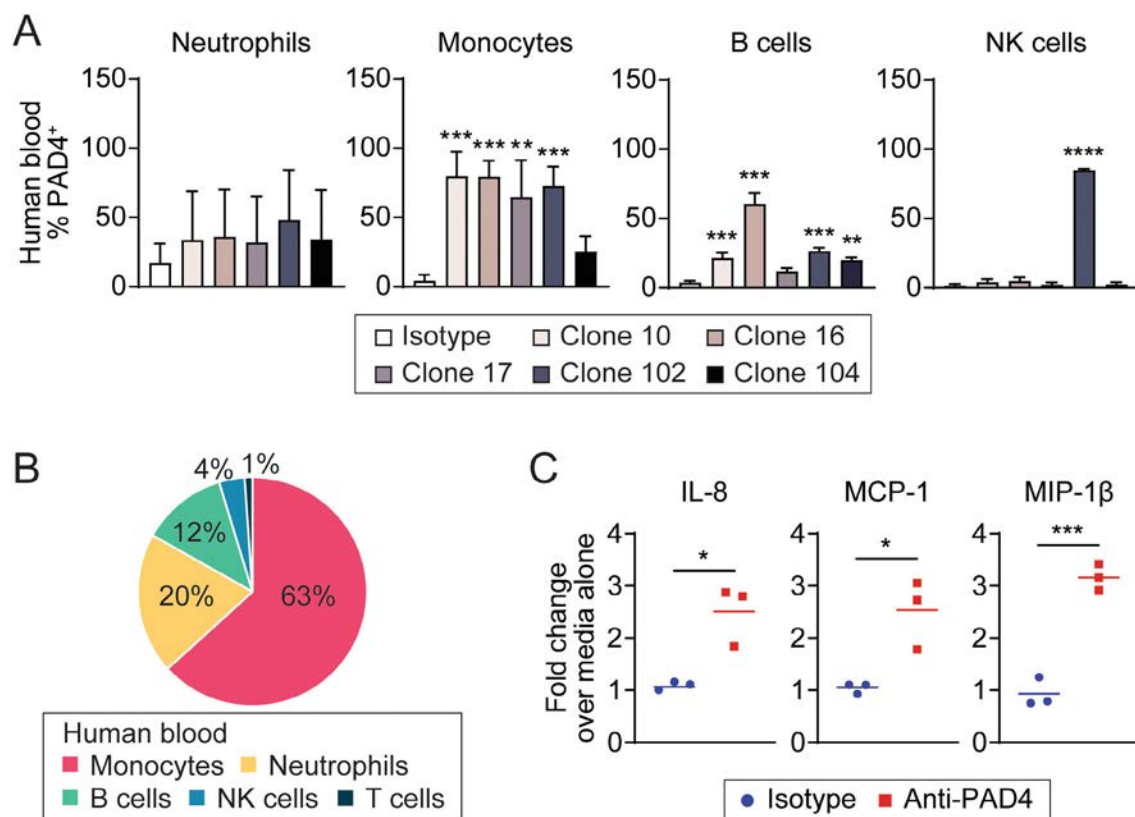


**Figure 4.** Anti-PAD4 antibodies target and activate human monocytes. Human blood leukocytes were obtained from three healthy individuals. (A and B) Frequencies (A) and pie chart (B) of anti-PAD4 antibody-bound cells in human blood leukocytes. Cells were stained with Alexa Fluor 647-labeled anti-PAD4tm antibody clones 10, 16, 17, 102, and 104 and isotype antibody clone GL102tm. (C) Chemokine production by human monocytes stimulated with anti-PAD4 or isotype antibodies. Cells were incubated with anti-PAD4ra clone 102 or human IgG1 isotype antibodies for 24 hours, and their chemokine production was measured using multiplex electrochemiluminescence assay. One-way ANOVA followed by post hoc Tukey's multiple comparison test (A) or Student's *t*-test (C) was used for statistical analysis. \**P* < 0.05; \*\**P* < 0.005; \*\*\**P* < 0.0005; \*\*\*\**P* < 0.0001. ANOVA, analysis of variance; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein 1; MIP-1 $\beta$ , macrophage inflammatory protein 1 $\beta$ ; NK, natural killer; PAD4, peptidylarginine deiminase 4. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43168/abstract>.

monocytes and promote the production of proinflammatory chemokines.

**Anti-PAD4 antibodies can indirectly activate synovial fibroblasts through the cytokines produced by T cells.** In addition to the effects on leukocyte populations, our findings in the CIA model also revealed an effect of anti-PAD4 antibodies on mouse synovial fibroblasts, with more severe synovial hyperplasia in mice treated with anti-PAD4 antibodies compared to the control group (Figure 1F–I). However, in our ex vivo experiment, direct binding of anti-PAD4 antibodies to mouse fibroblasts was negligible, with a significantly lower binding level compared to monocytes (Figure 3A and B and Figure S3A). Despite our findings, it is unclear from the current literature whether human fibroblasts express PAD4 under physiologic or pathologic conditions, with studies showing conflicting data about the presence of PAD4 messenger RNA (mRNA) and protein in HFLS.<sup>26–28</sup> Expression of PAD4 by synovial fibroblasts could render them direct cellular targets of anti-PAD4 antibodies, whereas the lack of expression would suggest an indirect mechanism of fibroblast activation.

To first examine whether human fibroblasts express PAD4, we measured PAD4 expression by qPCR and immunoblotting in HFLS derived from a healthy donor and an individual with RA at baseline and in the presence of IFN $\gamma$ , TNF $\alpha$ , and IL-17A, the proinflammatory environment present in the CIA joint (Figure 2D–G). Although some negligible bands were detected in unstimulated healthy HFLS by immunoblotting, but not in RA HFLS, PAD4 mRNA expression was not confirmed in either healthy or RA HFLS regardless of the stimuli (Figure 5A and Figure S5A). Because these cytokines are not the only potential inducers of PAD4 expression relevant to RA pathogenesis, we also tested the ability of a variety of environmental stimuli, including TLR ligands and serum starvation, to induce PAD4 protein expression and found that none of these were able to induce PAD4 protein expression in RA or healthy HFLS (Figure S5B). As an additional unbiased search for PAD4 expression in cells within the RA synovium, we interrogated *PADI4* expression (the gene that encodes PAD4) in a single-cell RNA sequencing data set of synovial tissue cells from 70 patients with RA and 9 patients with osteoarthritis, compiled for the AMP RA phase 2 database.<sup>23</sup> As expected, *PADI4* mRNA was detected in a monocyte subset (identified as the M-7 subpopulation) thought to consist of infiltrating proinflammatory monocytes and, interestingly, in dendritic cells (a DC1 subset identified as the M-12 cluster and a plasmacytoid dendritic cell population identified as the M-13 cluster; Figure S5C). However, *PADI4* mRNA was undetectable in all fibroblast cell clusters (Figure S5C), providing additional evidence that synovial fibroblasts are likely not primary cellular targets of anti-PAD4 antibodies.

To examine the possibility that anti-PAD4 antibodies exert their effects on synovial fibroblasts, via an indirect mechanism,

we next investigated whether T cell cytokines enhanced in anti-PAD4 antibody-treated mice could have indirect effects on synovial fibroblast activation (Figure 2D–G). In HFLS derived from a patient with RA, we found that a combination of IFN $\gamma$ , TNF $\alpha$ , and IL-17A effectively induced the expression of markers associated with activated and invasive fibroblasts, including HLA-DR, PD-L1, CD40, cadherin 11, and VCAM-1 (Figure 5B–D). The treatment with IFN $\gamma$  alone was sufficient to significantly induce the expression of HLA-DR, PD-L1, and cadherin 11 in HFLS, whereas TNF $\alpha$  treatment alone induced weak expression of VCAM-1, and IL-17A treatment alone did not induce any of the markers tested. HFLS derived from a healthy individual showed a similar response to T cell cytokine stimulation as observed in HFLS from the patient with RA (Figure S6A–C). Taken together, these findings suggest that anti-PAD4 antibodies induce indirect activation of synovial fibroblasts by promoting a proinflammatory cytokine milieu in the arthritic joint, which may synergize to drive the histopathologic features of joint destruction and synovial fibroblast activation illustrated in our model shown in Figure 6.

## DISCUSSION

In this study, we investigated the cellular targets and pathogenic role of anti-PAD4 autoantibodies derived from patients with RA in mouse and in vitro models.<sup>18</sup> Our findings demonstrated that anti-PAD4 autoantibodies significantly exacerbated the severity of CIA in mice, with an increase in histopathologic findings, including immune cell infiltration and synovial tissue damage, with evidence of increased myeloid cell recruitment, Th1 and Th17 cell activation, and invasive fibroblast proliferation. Monocytes were found to be the primary cellular targets of anti-PAD4 antibodies in both mice and humans, with anti-PAD4 binding leading to proinflammatory activation of human monocytes, indicating that monocytes may be a principal and crucial target of these antibodies in the pathogenesis of RA. Proinflammatory T cell cytokines augmented by anti-PAD4 antibodies in the CIA model stimulated HFLS activation in vitro, suggesting anti-PAD4 antibody binding to monocytes as a key upstream feature driving disease-amplifying pathways. Although it was well known that anti-PAD4 autoantibodies and ACPAs are associated with disease progression in RA, the underlying mechanism remained incompletely understood.<sup>6–8</sup> Our studies suggest a novel mechanism whereby anti-PAD4 autoantibodies directly activate monocytes, triggering an inflammatory cascade that culminates in adaptive immune cell recruitment to the synovial tissue in RA, leading to synovial fibroblast activation (Figure 6).

We hypothesize that the increased production of proinflammatory chemokines by monocytes treated with anti-PAD4 antibodies, as observed in vitro, is responsible for driving the observed notable infiltration of myeloid cells into the joints of arthritic mice treated with anti-PAD4 antibodies (Figure 6A–C). IL-8, also known as CXCL8, is one of the most potent

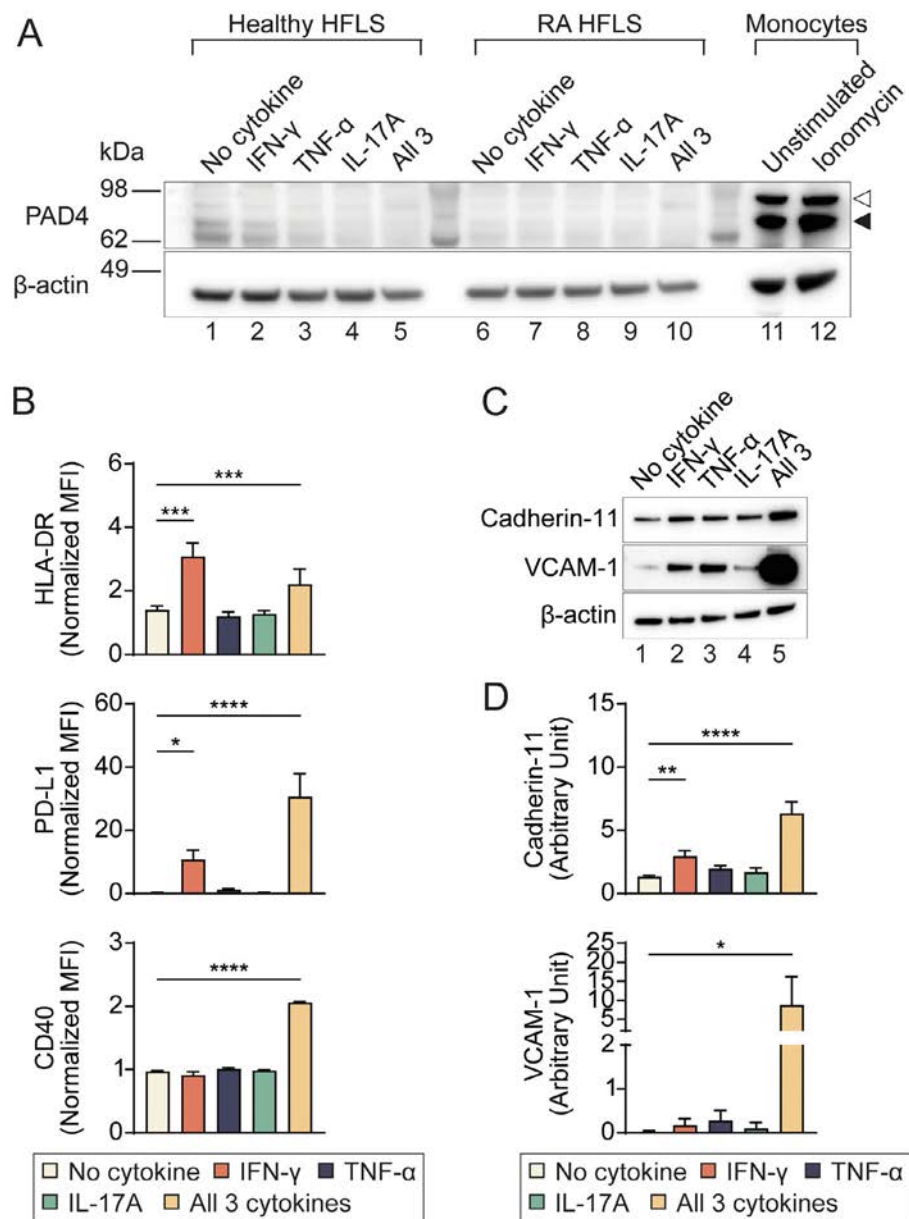


**Figure 5.** T cell cytokines stimulate synovial fibroblast activation. Primary HFLS obtained from a healthy individual or a patient with RA were stimulated with IFN $\gamma$ , TNF $\alpha$ , IL-17A, or a combination of all three cytokines for 72 hours. (A) PAD4 protein expression (closed arrowhead) in HFLS from a healthy individual (healthy HFLS) or a patient with RA (RA HFLS). Human blood monocytes with or without ionomycin stimulation served as a positive control. Nonspecific bands are indicated with the open arrowhead. (B) Surface expression of activation markers in HFLS from a patient with RA determined by flow cytometry analysis. (C and D) Protein expression of activation markers in HFLS from a patient with RA measured by Western blot analysis. Representative data from three independent experiments are shown. One-way ANOVA followed by post hoc Tukey's multiple comparison test was used for statistical analysis. \* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0005$ ; \*\*\*\* $P < 0.0001$ . ANOVA, analysis of variance; HFLS, human fibroblast-like synoviocytes; IFN $\gamma$ , interferon- $\gamma$ ; IL-17A, interleukin-17A; MFI, mean fluorescence intensity; PAD4, peptidylarginine deiminase 4; PD-L1, programmed death ligand 1; RA, rheumatoid arthritis; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; VCAM-1, vascular cell adhesion molecule 1. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43168/abstract>.

chemokines responsible for attracting neutrophils to sites of damage or infection and promoting their proinflammatory activities. Another chemokine, MCP-1 or CCL2, contributes to the migration of proinflammatory monocytes from the bone marrow to infected or injured organs. Additionally, MIP-1 $\beta$ , also known as CCL4, is involved in recruiting and activating various immune cell

populations, including monocytes and lymphocytes. Consistent with our findings, several studies have reported elevated levels of proinflammatory chemokines, such as IL-8, MCP-1, and MIP-1 $\beta$ , in the synovial fluid of patients with RA.<sup>29,30</sup> Particularly, increased IL-8 levels in the synovial fluid have been associated with enhanced neutrophil recruitment and a high titer of ACPAs.<sup>30</sup>

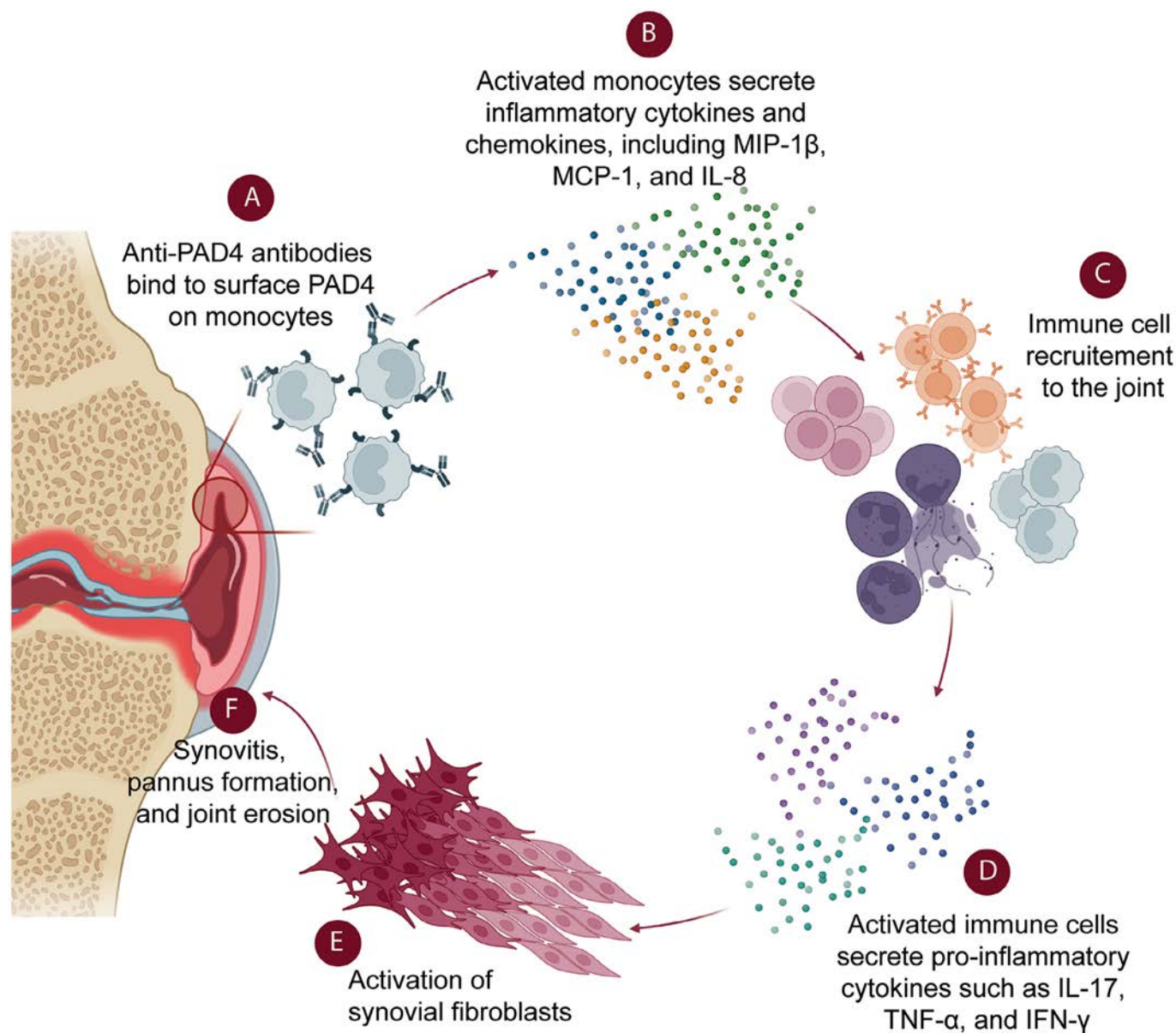


**Figure 6.** Proposed mechanism of disease exacerbation by anti-PAD4 antibodies. This model is based on our complementary in vitro and in vivo data showing that (A) anti-PAD4 antibodies preferentially bind monocytes in mice and humans, (B) stimulate the release of proinflammatory chemokines by human monocytes, and (C) result in increased immune cell infiltration into arthritic joints in CIA mice, (D) including an increased proportion of T cells producing IL-17A, TNF $\alpha$ , and IFN $\gamma$ , (E and F) which can induce an activated and invasive synovial fibroblast phenotype. CIA, collagen-induced arthritis; IFN $\gamma$ , interferon- $\gamma$ ; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1 $\beta$ , macrophage inflammatory protein 1 $\beta$ ; PAD4, peptidylarginine deiminase 4; TNF $\alpha$ , tumor necrosis factor  $\alpha$ . Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43168/abstract>.

Furthermore, these chemokines not only influence the recruitment and activation of immune cells but also directly impact bone remodeling in RA. CCL2-deficient mice exhibited higher bone mass, lower CTX-I levels, and a decreased number of osteoclasts compared to wild-type controls.<sup>31</sup> An in vitro study supported this by demonstrating that MCP-1 treatment directly promoted the recruitment and differentiation of osteoclast progenitors.<sup>29</sup> Similarly, IL-8 and MIP-1 $\beta$  were found to be involved in the differentiation and migration of osteoclasts,

respectively.<sup>32,33</sup> Therefore, the up-regulated production of these chemokines in monocytes due to anti-PAD4 antibody binding may cause severe infiltration and bone resorption within the RA joint.

In our in vivo study, we discovered the enhanced activity of T cells following treatment with anti-PAD4 autoantibodies, supported by elevated IL-17A levels in the serum and an increase in TNF $\alpha$ -producing lymph node CD4<sup>+</sup> or CD8<sup>+</sup> cells, including subsets that produced IL-17A and/or IFN $\gamma$ , respectively (Figure 6D).

The role of T cells in the pathogenesis of CIA is well established, with recognition of collagen peptides as the predominant mechanism.<sup>34</sup> In patients with RA, the role of T cells is more multifaceted, with accumulation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets in the inflamed synovium.<sup>35</sup> In addition to the classic role of CD4<sup>+</sup> T cells in supporting B cell activation and antibody production, T cells in RA are also thought to contribute to synovial tissue remodeling and angiogenesis through contact-dependent and cytokine-mediated activation of stromal cells, fibroblasts, and osteoclasts. In our study, we observed up-regulation of IL-17A, TNF $\alpha$ , and IFN $\gamma$  in the lymph node T cells of arthritic mice treated with anti-PAD4 antibodies, and all cytokines have been linked to RA pathogenesis.

Previous reports have demonstrated the presence of IL-17A-producing Th17 cells in the synovial tissue of patients with RA, and IL-17A is known to stimulate synovial fibroblasts to produce proinflammatory cytokines and chemokines, as well as enhance the differentiation of mesenchymal stem cells into osteoclasts.<sup>36–38</sup> TNF $\alpha$ , a well-known proinflammatory cytokine produced by T cells and other leukocyte populations, drives synovial inflammation and bone degradation in RA, and TNF $\alpha$  inhibitors are widely clinically used for the treatment of RA.<sup>39</sup> Although the role of IFN $\gamma$  in CIA has been hard to define, with conflicting reports showing protective and disease-promoting effects, patients with RA have higher levels of IFN $\gamma$  levels than healthy controls, which decrease with methotrexate treatment, and both IFN $\gamma$ -positive cells and up-regulation of IFN-induced genes can be found in the synovium.<sup>40–43</sup> Importantly, our findings revealed that the anti-PAD4 antibodies did not bind to T cells in humans or mice, suggesting that the activation of T cells is an indirect effect of anti-PAD4 antibodies on monocyte-driven immune responses in the synovial tissue. In this regard, in addition to promoting T cell chemotaxis to the site of inflammation, there are several reports demonstrating that the proinflammatory chemokines produced by monocytes in response to anti-PAD4 antibodies (ie, IL-8, MCP-1, and MIP-1 $\beta$ ) can exert direct effects on T cells, including down-regulation of IL-4, increased proliferation, and higher levels of IFN $\gamma$  production upon antigen challenge.<sup>44,45</sup>

In arthritic mice treated with anti-PAD4 antibodies, increased synovial fibroblast hyperplasia was observed as one of the pathologic features, alongside severe inflammation. However, our *in vivo* and *in vitro* studies indicated that fibroblasts are not a primary target of anti-PAD4 antibodies, supported by the finding that PAD4 is not expressed in synovial fibroblasts at either the mRNA or protein level, even after stimulation with proinflammatory cytokines such as IL-17A, TNF $\alpha$ , and IFN $\gamma$ . This finding is consistent with some findings that show a lack of PAD4 expression in human HFLS cells.<sup>28</sup> However, given the conflicting literature on this topic, we cannot exclude the possibility that, under some conditions, PAD4 expression could be induced in synovial fibroblasts in the RA joint.<sup>26–28</sup> Although our current working hypothesis, based on our data and consistent with the current

literature, is that chronic inflammation may contribute to the progression of synovial hyperplasia, additional work is needed to define mechanisms underlying synovial fibroblast hyperplasia in RA.<sup>46</sup> Our *in vitro* study suggests that this chronic inflammation may be driven, at least in part, by the T cell cytokines, such as TNF $\alpha$ , IFN $\gamma$ , and IL-17A, produced in the setting of anti-PAD4 antibodies. These cytokines alone and in combination may instruct HFLS to become activated, proliferate, and adopt an invasive phenotype, as supported by previous literature.<sup>47–49</sup> It is important to note that although the HFLS from the one healthy donor and one RA donor that we tested behaved similarly in this assay, future study of a larger number of donors is warranted to understand donor heterogeneity and reveal if subtle differences between healthy and RA HFLS responses may exist. Taken together, these findings suggest that anti-PAD4 antibodies facilitate the activation and proliferation of synovial fibroblasts during RA via an indirect mechanism that promotes the production of inflammatory T cell cytokines, downstream of initial monocyte activation (Figure 6E and F).

Although our study demonstrated that the binding of anti-PAD4 antibody clones to monocytes exacerbated CIA and triggered the release of proinflammatory chemokines, the precise mechanism by which these antibodies activate monocytes remains unclear. Recently, PAD4 has been found on the surface of monocytes and in traditional secretory compartments.<sup>17,50</sup> Coupled with our current findings, these studies suggest that anti-PAD4 autoantibody binding to PAD4 present on the monocyte surface may stimulate a signaling cascade that culminates in monocyte activation. Understanding the mechanism for how ligation of PAD4, which lacks transmembrane or signaling domains, by anti-PAD4 antibodies transmits intracellular signals is an important area of future study. In addition to direct cellular effects, anti-PAD4 antibodies may also activate PAD4 catalytic activity on the monocyte surface and exacerbate arthritis because of the increased generation of extracellular citrullinated antigens. Previous studies have revealed that genetic deletion of PAD4 or treatment of PAD4 inhibitor led to disease improvement in various arthritis mouse models, with reduced serum levels of proinflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$ , IL-6, and GM-CSF.<sup>51–53</sup> In addition, anti-PAD4 antibodies may recognize monocytes undergoing extracellular trap formation.<sup>54</sup>

Although the discovery of agonistic PAD4 autoantibodies suggests that this mechanism may be operative in patients with RA,<sup>15</sup> there were no differences in the deposition of citrullinated proteins in CIA mice between the anti-PAD4 antibody and isotype control treatment groups. This suggests that augmentation of citrullination is not the only mechanism whereby anti-PAD4 antibodies may amplify disease and that anti-PAD4 antibodies can also stimulate direct cellular effects on monocytes, independent of citrullination. An important caveat to our study is that all our experiments were performed with engineered anti-PAD4 monoclonal antibodies, whose variable region sequences were cloned

from circulating memory B cells isolated from patients with RA. Although these were sequences naturally arising in patients with RA, they represent binding to single PAD4 epitopes and may not wholly recapitulate the effects of the polyclonal anti-PAD4 antibody response known to occur in patients with RA.<sup>5</sup> Future studies with human polyclonal anti-PAD4 antibodies and combinations of anti-PAD4 monoclonal antibodies will be helpful in defining the combined effects of targeting multiple PAD4 epitopes on RA severity.

This study provides evidence for the pathogenic role of anti-PAD4 autoantibodies in RA and suggests that anti-PAD4 antibodies primarily target monocytes, resulting in the production of proinflammatory chemokines, which recruit and activate various immune cell populations to the joint, including Th1 and Th17 cells, creating a proinflammatory milieu that activates synovial fibroblasts (Figure 6). These data are consistent with the notion that anti-PAD4 antibodies can act as amplifiers of RA severity, as supported by human RA data demonstrating that anti-PAD4 antibodies are associated with RA duration and destructive joint disease. Our study suggests that anti-PAD4 autoantibodies are a potential therapeutic target for the treatment of RA and lays the foundation for further studies aimed at elucidating the mechanistic role of citrullination-associated autoantibodies in RA pathogenesis.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Drs Čiháková and Darrah confirm that all authors have provided the final approval of the version to be published and take responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## ROLE OF THE STUDY SPONSOR

Bristol Myers Squibb had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by Bristol Myers Squibb.

## REFERENCES

- Arend WP, Firestein GS. Pre-rheumatoid arthritis: predisposition and transition to clinical synovitis. *Nat Rev Rheumatol* 2012;8(10):573–586.
- Darrah E, Andrade F. Rheumatoid arthritis and citrullination. *Curr Opin Rheumatol* 2018;30(1):72–78.
- Curran AM, Naik P, Giles JT, et al. PAD enzymes in rheumatoid arthritis: pathogenic effectors and autoimmune targets. *Nat Rev Rheumatol* 2020;16(6):301–315.
- Halvorsen EH, Pollmann S, Gilboe IM, et al. Serum IgG antibodies to peptidylarginine deiminase 4 in rheumatoid arthritis and associations with disease severity. *Ann Rheum Dis* 2008;67(3):414–417.
- Harris ML, Darrah E, Lam GK, et al. Association of autoimmunity to peptidyl arginine deiminase type 4 with genotype and disease severity in rheumatoid arthritis. *Arthritis Rheum* 2008;58(7):1958–1967.
- Harre U, Georgess D, Bang H, et al. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *J Clin Invest* 2012;122(5):1791–1802.
- Krishnamurthy A, Circiumaru A, Sun J, et al. Combination of two monoclonal anti-citrullinated protein antibodies induced tenosynovitis, pain, and bone loss in mice in a peptidyl arginine deiminase-4-dependent manner. *Arthritis Rheumatol* 2023;75(2):164–170.
- Wigerblad G, Bas DB, Fernandes-Cerqueira C, et al. Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. *Ann Rheum Dis* 2016;75(4):730–738.
- Sokolove J, Zhao X, Chandra PE, et al. Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcγ receptor. *Arthritis Rheum* 2011;63(1):53–62.
- He Y, Ge C, Moreno-Giró À, et al. A subset of antibodies targeting citrullinated proteins confers protection from rheumatoid arthritis. *Nat Commun* 2023;14(1):691.
- Raposo B, Afonso M, Israelsson L, et al. Divergent and dominant anti-inflammatory effects of patient-derived anticitrullinated protein antibodies (ACPA) in arthritis development. *Ann Rheum Dis* 2023;82(5):724–726.
- Sahlström P, Hansson M, Steen J, et al. Different hierarchies of anti-modified protein autoantibody reactivities in rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(10):1643–1657.
- Steen J, Forsström B, Sahlström P, et al. Recognition of amino acid motifs, rather than specific proteins, by human plasma cell-derived monoclonal antibodies to posttranslationally modified proteins in rheumatoid arthritis. *Arthritis Rheumatol* 2019;71(2):196–209.
- Kolfenbach JR, Deane KD, Derber LA, et al. Autoimmunity to peptidyl arginine deiminase type 4 precedes clinical onset of rheumatoid arthritis. *Arthritis Rheum* 2010;62(9):2633–2639.
- Darrah E, Giles JT, Ols ML, et al. Erosive rheumatoid arthritis is associated with antibodies that activate PAD4 by increasing calcium sensitivity. *Sci Transl Med* 2013;5(186):186ra65.
- Vossenaar ER, Radstake TR, van der Heijden A, et al. Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. *Ann Rheum Dis* 2004;63(4):373–381.
- Zhou Y, Chen B, Mittereder N, et al. Spontaneous secretion of the citrullination enzyme PAD2 and cell surface exposure of PAD4 by neutrophils. *Front Immunol* 2017;8:1200.
- Shi J, Darrah E, Sims GP, et al. Affinity maturation shapes the function of agonistic antibodies to peptidylarginine deiminase type 4 in rheumatoid arthritis. *Ann Rheum Dis* 2018;77(1):141–148.
- Oganesyan V, Gao C, Shirinian L, et al. Structural characterization of a human Fc fragment engineered for lack of effector functions. *Acta Crystallogr D Biol Crystallogr* 2008;64(Pt 6):700–704.
- Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9(7):676–682.
- Li Y, Foss CA, Summerfield DD, et al. Targeting collagen strands by photo-triggered triple-helix hybridization. *Proc Natl Acad Sci USA* 2012;109(37):14767–14772.
- Accelerating Medicines Partnership (AMP) RA phase II. <https://immunogenomics.io/ampra2/app/?ds=fibroblast&gene=PADI4&groupby=none>
- Zhang F, Jonsson AH, Nathan A, et al; Accelerating Medicines Partnership: RA/SLE Network. Deconstruction of rheumatoid arthritis synovium defines inflammatory subtypes. *Nature* 2023;623(7987):616–624.

24. Gómez-Bañuelos E, Shi J, Wang H, et al. Heavy chain constant region usage in antibodies to peptidylarginine deiminase 4 as a marker of disease subsets in rheumatoid arthritis. *Arthritis Rheumatol* 2022;74(11):1746–1754.
25. Thomas MA, Naik P, Wang H, et al. The monocyte cell surface is a unique site of autoantigen generation in rheumatoid arthritis. *Proc Natl Acad Sci USA* 2024;121(17):e2304199121.
26. Yu R, Li C, Sun L, et al. Hypoxia induces production of citrullinated proteins in human fibroblast-like synoviocytes through regulating HIF1 $\alpha$ . *Scand J Immunol* 2018;87(4):e12654.
27. Chang X, Yamada R, Suzuki A, et al. Localization of peptidylarginine deiminase 4 (PAD4) and citrullinated protein in synovial tissue of rheumatoid arthritis. *Rheumatology (Oxford)* 2005;44(1):40–50.
28. Carmona-Rivera C, Carlucci PM, Goel RR, et al. Neutrophil extracellular traps mediate articular cartilage damage and enhance cartilage component immunogenicity in rheumatoid arthritis. *JCI Insight* 2020;5(13):e139388.
29. Sucur A, Jajic Z, Artukovic M, et al. Chemokine signals are crucial for enhanced homing and differentiation of circulating osteoclast progenitor cells. *Arthritis Res Ther* 2017;19(1):142.
30. Gorlino CV, Dave MN, Blas R, et al. Association between levels of synovial anti-citrullinated peptide antibodies and neutrophil response in patients with rheumatoid arthritis. *Eur J Immunol* 2018;48(9):1563–1572.
31. Sul OJ, Ke K, Kim WK, et al. Absence of MCP-1 leads to elevated bone mass via impaired actin ring formation. *J Cell Physiol* 2012;227(4):1619–1627.
32. Bendre M, Gaddy D, Nicholas RW, et al. Breast cancer metastasis to bone: it is not all about PTHrP. *Clin Orthop Relat Res* 2003;415(suppl):S39–S45.
33. Xuan W, Feng X, Qian C, et al. Osteoclast differentiation gene expression profiling reveals chemokine CCL4 mediates RANKL-induced osteoclast migration and invasion via PI3K pathway. *Cell Biochem Funct* 2017;35(3):171–177.
34. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. *Nat Protoc* 2007;2(5):1269–1275.
35. Weyand CM, Goronzy JJ. T-cell-targeted therapies in rheumatoid arthritis. *Nat Clin Pract Rheumatol* 2006;2(4):201–210.
36. Chabaud M, Durand JM, Buchs N, et al. Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum* 1999;42(5):963–970.
37. Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 1996;183(6):2593–2603.
38. Chabaud M, Garnerio P, Dayer JM, et al. Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis. *Cytokine* 2000;12(7):1092–1099.
39. Scott DL, Kingsley GH. Tumor necrosis factor inhibitors for rheumatoid arthritis. *N Engl J Med* 2006;355(7):704–712.
40. Lee J, Lee J, Park MK, et al. Interferon gamma suppresses collagen-induced arthritis by regulation of Th17 through the induction of indoleamine-2,3-deoxygenase. *PLoS One* 2013;8(4):e60900.
41. Boissier MC, Chiochia G, Bessis N, et al. Biphasic effect of interferon- $\gamma$  in murine collagen-induced arthritis. *Eur J Immunol* 1995;25(5):1184–1190.
42. Lama M, Sarkar R, Ghosh B. Serum cytokine profiles in patients with rheumatoid arthritis before and after treatment with methotrexate. *J Interferon Cytokine Res* 2023;43(8):344–350.
43. Kato M. New insights into IFN- $\gamma$  in rheumatoid arthritis: role in the era of JAK inhibitors. *Immunol Med* 2020;43(2):72–78.
44. Gesser B, Lund M, Lohse N, et al. IL-8 induces T cell chemotaxis, suppresses IL-4, and up-regulates IL-8 production by CD4+ T cells. *J Leukoc Biol* 1996;59(3):407–411.
45. Lillard JW Jr, Singh UP, Boyaka PN, et al. MIP-1 $\alpha$  and MIP-1 $\beta$  differentially mediate mucosal and systemic adaptive immunity. *Blood* 2003;101(3):807–814.
46. Falconer J, Murphy AN, Young SP, et al. Review: synovial cell metabolism and chronic inflammation in rheumatoid arthritis. *Arthritis Rheumatol* 2018;70(7):984–999.
47. Hwang SY, Kim JY, Kim KW, et al. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF- $\kappa$ B- and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther* 2004;6(2):R120–R128.
48. Lee A, Qiao Y, Grigoriev G, et al. Tumor necrosis factor  $\alpha$  induces sustained signaling and a prolonged and unremitting inflammatory response in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2013;65(4):928–938.
49. Lowin T, Anssar TM, Bäuml M, et al. Positive and negative cooperativity of TNF and interferon- $\gamma$  in regulating synovial fibroblast function and B cell survival in fibroblast/B cell co-cultures. *Sci Rep* 2020;10(1):780.
50. Thomas MA, Kim SY, Curran AM, et al. An unbiased proteomic analysis of PAD4 in human monocytes: novel substrates, binding partners and subcellular localizations. *Philos Trans R Soc Lond B Biol Sci* 2023;378(1890):20220477.
51. Shelef MA, Sokolove J, Lahey LJ, et al. Peptidylarginine deiminase 4 contributes to tumor necrosis factor  $\alpha$ -induced inflammatory arthritis. *Arthritis Rheumatol* 2014;66(6):1482–1491.
52. Suzuki A, Kochi Y, Shoda H, et al. Decreased severity of experimental autoimmune arthritis in peptidylarginine deiminase type 4 knockout mice. *BMC Musculoskelet Disord* 2016;17(1):205.
53. Willis VC, Banda NK, Cordova KN, et al. Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis. *Clin Exp Immunol* 2017;188(2):263–274.
54. Okamoto Y, Ghosh T, Okamoto T, et al. Subjects at-risk for future development of rheumatoid arthritis demonstrate a PAD4-and TLR-dependent enhanced histone H3 citrullination and proinflammatory cytokine production in CD14<sup>hi</sup> monocytes. *J Autoimmun* 2021;117:102581.

# Serum Proteomic Signatures Before the Diagnosis of Rheumatoid Arthritis: Evolving Biologic Pathways and Specific Periods of Disease Development

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**Objective.** This longitudinal case-control study evaluated serum proteomics before a clinical diagnosis of rheumatoid arthritis (RA) (ie, pre-RA) to evaluate biologic pathways of disease development and inform prediction of timing of onset of future disease.

**Methods.** Patients (n = 213) meeting the 1987 American College of Rheumatology classification criteria for RA and matched controls without RA (n = 215) were identified in the Department of Defense Serum Repository. Serum samples from patients before and after RA diagnosis and controls were tested for RA-related autoantibodies (anti-cyclic citrullinated peptide-3 [anti-CCP3] and rheumatoid factor [RF] isotypes IgM and IgA) and 197 proteins using a commercial platform (Olink). We applied linear mixed effect models to identify biomarkers distinguishing patients from controls before RA diagnosis and analyzed longitudinal patterns of enriched pathways; in addition, models were developed to classify the time of a sample in relationship to the time of RA diagnosis.

**Results.** Levels of anti-CCP3, RFIgA, and RFIgM demonstrated the greatest differences between patients and controls ≤5 years before RA diagnosis. Longitudinal analyses identified 104 proteins that were differentially expressed between patients and controls; 60 proteins were differentially expressed ≤5 years before diagnosis, 42 proteins were differentially expressed within and before five years of diagnosis, and 2 proteins were differentially expressed >5 years before diagnosis. Kyoto Encyclopedia of Genes and Genomes analyses identified that these proteins were associated with 32 pathways, including 21 pathways that were enriched ≤5 years before diagnosis. Within the anti-citrullinated protein antibody-positive samples from before RA diagnosis and controls, a set of features classified if that sample was from a period <3 years before RA diagnosis, with an area under the receiver operating characteristic (ROC) curve of 0.78 (95% confidence interval 0.67–0.89) in a training set and 0.80 (0.68–0.92) in a validation set.

**Conclusion.** Autoantibodies and protein signatures evolve in distinct stages before a diagnosis of RA. Furthermore, protein biomarkers may identify biologic pathways relevant to specific stages. These can be further explored to potentially improve prediction of disease onset and identify stage-specific biologic pathways to target with preventive interventions.

## INTRODUCTION

It is now well established that there is a stage of rheumatoid arthritis (RA) development that can be identified through

elevations of serum autoantibodies, including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) in the absence of inflammatory arthritis (IA), which is the hallmark of a clinical diagnosis of RA.<sup>1,2</sup> Although imperfect, autoantibodies

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and other features provide sufficient ability to identify “at-risk” populations and support the conduct of multiple clinical trials that have intervened in the at-risk stage of disease in an attempt to delay, prevent, or modulate the future onset of clinical RA.<sup>3–5</sup> These trials have used agents including glucocorticoids, rituximab, atorvastatin, methotrexate, hydroxychloroquine, and abatacept that are already approved for, or are used in, established clinical RA.<sup>3,4,6–10</sup> Although most of these agents did not prevent RA, in two trials (and compared to placebo), abatacept was associated with decreased rates of progression to clinical RA during the drug-treatment phases; furthermore, the rates of clinical RA remained lower in the abatacept arms at the end of a drug-free follow-up period.<sup>3,4</sup> In addition, the overall rates of development of clinical RA in these trials have ranged from 18–57% but typically been less than 40%.<sup>3,4,6–10</sup> As such, additional understanding of the biology of the at-risk stage and features that are associated with progression to clinical RA may lead to more effective targets for prevention and improved prediction of future RA in individuals who are at risk for future clinical RA.

Studies using proteomics methods<sup>11–13</sup> have demonstrated the potential for identifying protein signatures in the pre-RA stage that may inform the pathophysiology of RA development and improve prediction of future RA. Therefore, we evaluated autoantibodies and protein biomarkers before and after RA diagnosis using a unique sample set of individuals with RA and controls obtained from the Department of Defense Serum Repository (DoDSR).

## PATIENTS AND METHODS

**Study design, participants and ethical considerations.** This longitudinal case-control observational study included 213 individuals diagnosed with RA (patients) who had clinical data and serum samples available from before (ie, pre-RA) and after their diagnosis of clinical RA and serial samples from 215 individuals without RA (controls) who were matched to the patients by age, sex, race and ethnicity, and duration of sample storage. Details of the case-control selection have been published previously,<sup>14</sup> but in brief, data and serum samples were obtained from the DoDSR, which houses serum samples collected from the United States Uniformed Services personnel; these samples are collected and initially processed at local collection facilities, then transported for long-term storage at a central location at –30° centigrade.<sup>15,16</sup> Additionally, clinical data were obtained from the Armed Forces Health Longitudinal Technology

Application. Of the 213 patients, 212 patients (>99%) met the 1987 American College of Rheumatology classification criteria for RA at or after their diagnosis of clinical RA,<sup>17</sup> and the remaining patient was diagnosed with RA by a board-certified rheumatologist. Clinical data on study participants included age, sex, race and ethnicity, and relative days from the time of sample collection to RA diagnosis. All study activities were approved by institutional review boards at the participating institutions. All data were anonymized, and therefore specific written consent for participating in this study was not required. However, all participants in the DoDSR are consented for data and biospecimen collection.

Of note, although we have previously published studies of combinations of autoantibody and cytokine and chemokine testing in a separate DoDSR<sup>18–21</sup> cohort, the work and analyses presented herein includes a separate and distinct cohort with no overlap in participants with those previous studies. Furthermore, we have previously published results of autoantibody and serum calprotectin testing on the same individuals studied herein<sup>14,22,23</sup>; however, the results of autoantibody and calprotectin testing that we report are on a new platform, the results have not been presented previously, and the proteomic results presented herein are novel.

**Autoantibodies.** All samples were tested for ACPA with the anti-cyclic citrullinated peptide-3 (anti-CCP3) (IgG) assay (Inova Diagnostics, Inc., San Diego, CA) and RF IgA and IgM on the BIO-FLASH (Chemiluminescence) platform (Inova Diagnostics, Inc). Positive levels for each autoantibody were set using the manufacturer’s suggested cutoffs to define subgroups by autoantibody positivity, and their absolute and/or log-transformed values were used in longitudinal and classification analyses.

**Proteomics.** All samples were tested for 197 protein analytes. This testing was conducted using proximity extension immunoassay provided by Olink (part of Thermo Fisher Scientific, Waltham, MA) using the Immune Response, Inflammation, and Cell Regulation panels, which capture a variety of markers that may be related to the development of autoimmune and inflammatory diseases (additional details of this testing are in the Supplementary Extended Methods).<sup>24–28</sup> The relative levels of proteins were reported on an arbitrary log2-based normalized protein expression (NPX) scale. All samples were also tested for serum calprotectin on the BIO-FLASH (Chemiluminescence) platform (Inova Diagnostics, Inc).

**Statistical analyses.** Fisher’s exact test was used to compare descriptive or categorical variables between patients and

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controls. The Mann-Whitney U-test was used to compare patients and controls for continuous variables. Multiple comparisons were adjusted using the Benjamini Hochberg procedure. All statistical analyses were done in R (version 4.1.0 [2021-05-18]). The area under the receiver operating characteristic curve (AUC) was calculated using the pROC R package, whereas sensitivity, specificity, and positive predictive value (PPV) were calculated using the *epiR* R package.

**Longitudinal protein modeling.** We fit linear mixed models (LMMs) on the centered and scaled NPX of patients and controls using age, sex, racial and ethnic background, and time-to-disease onset as fixed effects, and participant-specific random intercepts and slopes. We used the LMMs to predict NPX for each participant at each year and tested for the corresponding differences between patients and controls for  $y = 0, \dots, -15$  years before RA diagnosis. Proteins with false discovery rate (FDR)  $< 0.05$  were deemed statistically significant. We estimated log2 fold change (patient/control) by subtracting the mean difference in log2 (NPX) between patients and controls. Additional details are presented in the Supplementary Extended Methods.

**Pathway enrichment analysis.** Pathway enrichment analysis was conducted on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database using WebGestaltR.<sup>29</sup> We used the set of differential proteins for each year,  $P_y$ , to identify enriched KEGG pathways (FDR  $< 0.1$ ).

**Analyses within autoantibody-positive subgroups.** Primary proteomic analyses focused on all patients with RA and controls regardless of autoantibody positivity. In addition, we evaluated proteomics in the following six subgroups of cases (and in comparison to all controls): (1) ACPA<sup>+</sup>, (2) ACPA<sup>-</sup>, (3) ACPA<sup>+</sup>/RF<sup>+</sup>, (4) ACPA<sup>+</sup>/RF<sup>-</sup>, (5) ACPA<sup>-</sup>/RF<sup>+</sup>, and (6) ACPA<sup>-</sup>/RF<sup>-</sup>. ACPA or RF positivity was defined if an individual was positive for ACPA or any RF at any point before or after RA diagnosis; negativity was defined if an individual tested negative at all time points.

**Classifier development.** To develop a model that classifies when a sample will be in the time course of pre-RA (ie,  $< 3$  years from RA), we randomly divided patients with pre-RA ACPA<sup>+</sup> samples and controls with ACPA<sup>+</sup> samples into a training set ( $n = 69$  participants) and a test set ( $n = 55$  participants), and defined five different binary (yes or no) outcome variables indicating whether the sample was within one to five years of onset. Because the ACPA<sup>+</sup> controls ( $n = 5$ ) did not have a diagnosis of RA, their samples were treated as being  $> 5$  years prior to diagnosis. We then used these ACPA<sup>+</sup> participants to develop penalized logistic regression models (pLRMs) for RA onset within one to five years, selecting features based on their

predictive ability using bootstrapping and cross-validation. We further refined the three-year pLRM to ensure features were statistically meaningful in predicting onset within three years. The pLRMs were assessed on the first recorded ACPA<sup>+</sup> visit using the AUC, sensitivity, specificity, and PPV, using the pre-RA (or control) samples that were first ACPA<sup>+</sup>. Additional details are presented in the Supplementary Extended Methods.

## RESULTS

A description of the participants and a visualization of the study design are provided in Table 1 and Figure 1A. The patients were a mean of 34.9 years old at the time of RA diagnosis, 54% were women, and 48% were White. The controls were a mean of 34.8 years old at a time point matched to the patients' age at RA diagnosis, 55% were women, and 48% were White. Between two and four samples were collected for each of the 213 patients over a period of  $> 20$  years, with a mean of 2.85 samples per participant ( $\approx 2$  samples from before RA diagnosis [ie, pre-RA] and one sample after RA diagnosis). Samples from controls had a similar time span of collection compared to the patients.

In the post-RA diagnosis time point, 69%, 47%, and 59% of the patients were positive for CCP3IgG, RFlgA, and RFlgM, respectively. In the latest sample (ie, most recently collected), 2.8%, 3.3%, and 3.7% of controls were positive for CCP3IgG, RFlgA, and RFlgM, respectively. There were elevated levels of autoantibodies in cases relative to controls  $> 10$  years before diagnosis (Figure 1B), with a visual inflection point at five years at which the mean levels of anti-CCP3 markedly increase. These results suggest that initial breaks in tolerance and generation of autoantibodies may occur  $> 10$  years before diagnosis but that a time point of  $\leq 5$  years before RA diagnosis may be important in terms of expansion of autoimmune dysregulation and resulting increases in autoantibody levels.

**Longitudinal protein expression.** We first confirmed that there were no substantial changes in protein levels in relationship to time of storage of serum sample (see Supplementary Figure 1 and additional results below). We then applied LMMs (Supplementary Figure 2A and B) to model protein expression across  $> 20$  years of samples (Supplementary Figure 2C). In comparing protein expression between all cases and controls at year 0 (time of RA diagnosis), we identified 99 differentially expressed proteins (DEPs) (FDR  $< 0.05$ ) between patients and controls (Figure 1C). The four proteins with largest differences in expression at this time point were lymphocyte activation gene (LAG3), interleukin-10 (IL-10), glial cell line-derived neurotrophic factor family receptor  $\alpha 2$  (GFRA2), and signaling threshold-regulating transmembrane adapter 1 (SIT1) (Figure 1D). Of these, LAG3 and IL-10 had increased expression in patients, and GFRA2 and SIT1 were decreased.

**Table 1.** Participant characteristics\*

| Characteristics                                        | Patients with RA | Controls   | P value                 |
|--------------------------------------------------------|------------------|------------|-------------------------|
| Participants, n (samples)                              | 213 (608)        | 215 (614)  | –                       |
| Samples per participant, mean (SD)                     | 2.85 (0.72)      | 2.86 (0.7) | 0.782 <sup>a</sup>      |
| Samples per participant before RA diagnosis, mean (SD) | 1.88 (0.73)      | –          | –                       |
| Sex, n (%)                                             |                  |            | >0.99 <sup>b</sup>      |
| Female                                                 | 116 (54)         | 118 (55)   | –                       |
| Male                                                   | 97 (46)          | 97 (45)    | –                       |
| Age, mean (SD), yr                                     |                  |            |                         |
| Age at first visit                                     | 26.5 (7.1)       | 25 (6.9)   | 0.015 <sup>a</sup>      |
| Age at RA diagnosis                                    | 34.9 (8.1)       | –          | –                       |
| Age at last visit                                      | 35.8 (8.1)       | 34.8 (7.8) | 0.285 <sup>a</sup>      |
| Race and ethnicity, n                                  |                  |            | >0.99 <sup>b</sup>      |
| Asian                                                  | 8                | 8          | –                       |
| African American                                       | 60               | 60         | –                       |
| White                                                  | 102              | 104        | –                       |
| Hispanic or Latino                                     | 27               | 27         | –                       |
| American Indian                                        | 3                | 3          | –                       |
| Other                                                  | 13               | 13         | –                       |
| Autoantibody levels and positivity at first visit      |                  |            |                         |
| CCP3 IgG, mean (SD), CU                                | 220.8 (606.4)    | 7.8 (7.2)  | $1.73 \times 10^{-9a}$  |
| >20 U/mL, n (%)                                        | 69 (32.39)       | 5 (2.33)   | $2.28 \times 10^{-18b}$ |
| RfIgA, mean (SD), CU                                   | 21.8 (63.8)      | 8.2 (61.3) | $2.2 \times 10^{-6a}$   |
| >20 IU/mL, n (%)                                       | 39 (18.31)       | 6 (2.79)   | $6.65 \times 10^{-8b}$  |
| RfIgM, mean (SD), U/mL                                 | 14.9 (60.3)      | 1 (1.6)    | $9.85 \times 10^{-9a}$  |
| >5 IU/mL, n (%)                                        | 58 (27.23)       | 5 (2.33)   | $1.55 \times 10^{-14b}$ |
| Autoantibody levels and positivity at last visit       |                  |            |                         |
| CCP3 IgG, mean (SD), CU                                | 751.4 (987.7)    | 6.5 (4.6)  | $2.38 \times 10^{-43a}$ |
| >20 U/mL, n (%)                                        | 146 (68.54)      | 6 (2.79)   | $1.01 \times 10^{-52b}$ |
| RfIgA, mean (SD), CU                                   | 70.1 (149)       | 6.3 (27.2) | $1.09 \times 10^{-35a}$ |
| >20 IU/mL, n (%)                                       | 101 (47.42)      | 7 (3.26)   | $5.45 \times 10^{-29b}$ |
| RfIgM, mean (SD), U/mL                                 | 37.3 (70.7)      | 7.2 (90)   | $6.96 \times 10^{-37a}$ |
| >5 IU/mL, n (%)                                        | 126 (59.15)      | 8 (3.72)   | $1.67 \times 10^{-39b}$ |

\* Summary statistics are provided for patients with RA and controls. Continuous variables were tested using Mann-Whitney test, and categorical variables were tested using Fisher's exact test. anti-CCP3, anti-cyclic citrullinated peptide-3; RA, rheumatoid arthritis; RF, rheumatoid factor.

<sup>a</sup> Mann-Whitney U-test.

<sup>b</sup> Fisher's exact test.

We further evaluated longitudinal protein expression over the 15-year period before RA diagnosis in patients, and commensurate time period in controls (Figure 2A and B). We identified 104 DEPs (FDR < 0.05) at any point between 0 and 15 years before diagnosis (full summary available in Supplementary Table 1 and Supplementary Figure 3). Furthermore, we observed that 60 proteins were exclusively differentially expressed within five years of RA diagnosis (eg, IL-10, nuclear factor of activated T cells 3, SLAM (signaling lymphocytic activation molecule) family member 8), which aligns with the period that the increase in autoantibodies (ie, inflection point) was seen (Figure 1B). In addition, 42 proteins (eg, galectin 7, CXC motif chemokine ligand 11) were differentially expressed between patients and controls both before and after the five-year inflection point, and 2 proteins (calsyntenin 3 and methionine aminopeptidase) showed differences in expression only in the period >5 years before diagnosis. These protein clusters are shown in Supplementary Table 2, and the trajectories reveal potential coordination of protein expression, which may suggest loss of regulation at different stages during disease

progression but with increasing changes during a period ≤5 years before diagnosis of clinical RA. Using time-to-symptom-onset as an outcome resulted in no major differences in DEP analyses (correlations in P values and effect sizes >0.9, Supplementary Figure 4A), as time-to-symptom onset was <1 year before disease onset for >85% of patients (Supplementary Figure 4B).

As mentioned above, to assess the impact of sample storage duration, we compared protein expression from patients three years before RA diagnosis grouping samples collected between 1991 and 2006 (earliest 50%) versus those between 2007 and 2012 (most recent 50%). In these analyses, 3 of 197 proteins were significantly different (Supplementary Figure 1), suggesting minimal differences due to sample storage duration.

**Pathway enrichment analysis.** Thirty-two distinct KEGG pathways were identified as enriched (FDR < 0.1) in different patterns between 0 and 15 years before RA diagnosis (Figure 2C, Supplementary Table 3).<sup>30</sup> Furthermore, 21 KEGG pathways showed enrichment ≤5 years of RA diagnosis, including

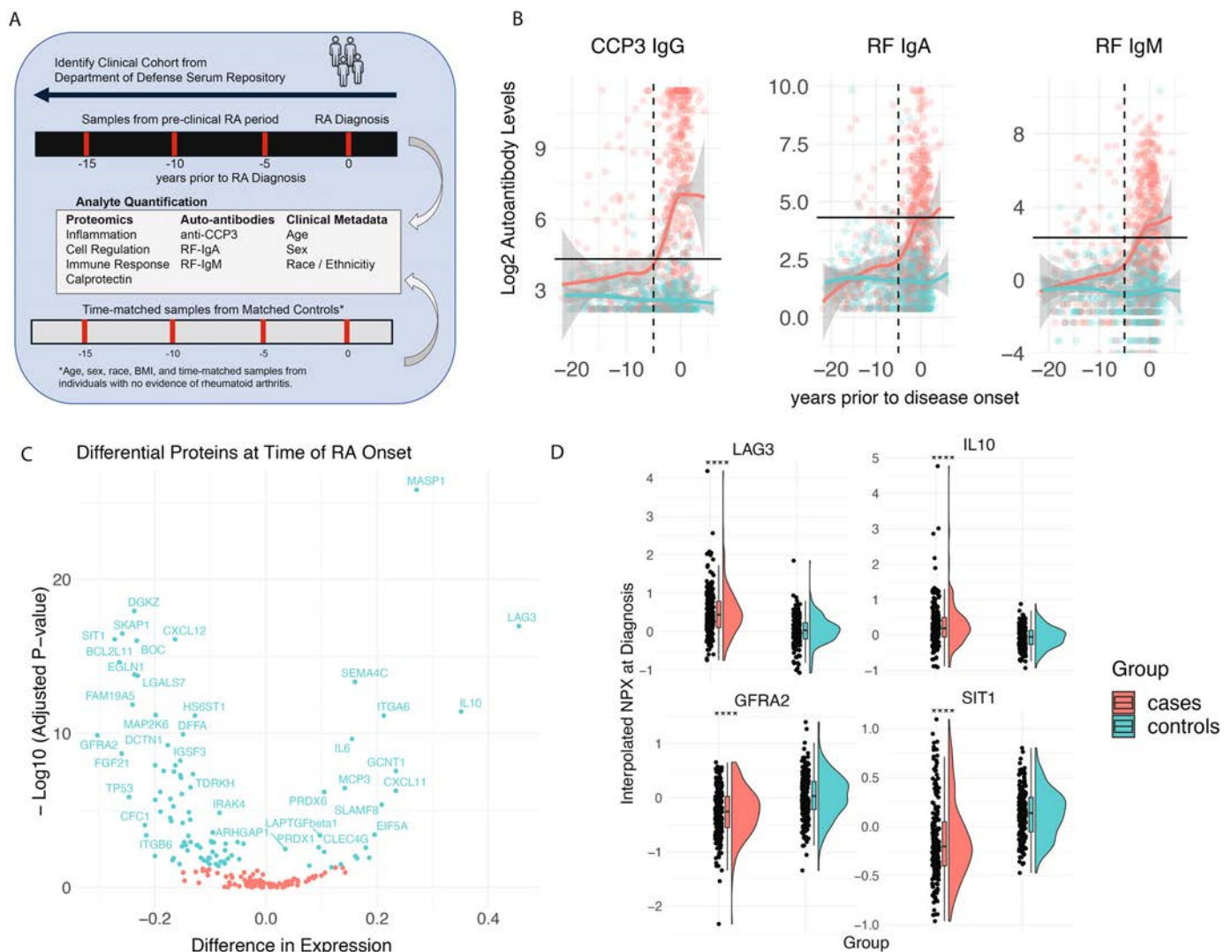


**Figure 1.** Longitudinal biomarkers study. (A) Study design and overview. (B) Longitudinal log2 autoantibody counts for CCP3IgG, RF IgA, and RF IgM are shown for individuals who developed RA (red), and matched controls (blue). Each dot corresponds to a sample, and lowess curves with their 95% confidence intervals were overlaid to show trend lines for RA and healthy controls. Data span approximately 20 years before disease onset through five years before diagnosis and the black horizontal lines represent clinical positivity cutoffs (CCP3 = 20 U/mL, RF IgA = 20 IU/mL, RF IgM = 5 IU/mL). Vertical dashed lines were overlaid at five years before diagnosis. (C) A volcano plot showing the log2 fold change (case/control) on the x-axis and  $-\log_{10}$  (Benjamini Hochberg-corrected  $P$  values) on the y-axis. Light blue dots denote proteins with FDR < 0.05, and red dots correspond to proteins with FDR > 0.05. A subset of the differential proteins (light blue) was labeled with their abbreviated protein names. (D) Raincloud plots for protein expression at disease onset for LAG3, IL-10, GFRA2, and SIT1. Each dot corresponds to a participant, and the asterisks denote statistical significance in expression between patients and controls. Figures were generated using Adobe Illustrator. BMI, body mass index; CCP3, cyclic citrullinated peptide-3; FDR, false discovery rate; GFRA2, glial cell line-derived neurotrophic factor family receptor  $\alpha 2$ ; IL-10, interleukin-10; LAG3, lymphocyte activation gene; RA, rheumatoid arthritis; RF, rheumatoid factor; SIT1, signaling threshold-regulating transmembrane adapter 1.

pathways related to disease or infection (eg, inflammatory bowel disease, Epstein-Barr virus, malaria) and adaptive immunity (eg, T cell and B cell receptor signaling pathways). There were an additional 11 pathways that were enriched either before and/or after the five-year inflection point (eg, intestinal immune network for IgA production).

To further explore which proteins were driving pathway enrichment, we constructed a bipartite graph of DEPs that were

enriched in >3 pathways. This analysis demonstrated that 16 DEPs were most significantly associated with pathway enrichment (Figure 2D). Notably, IL-6, transforming growth factor  $\beta 1$ , and IL-10 were associated with the most pathways (21, 18, and 12 KEGG pathways, respectively), whereas integrins  $\alpha 6$ ,  $\beta 6$ , and  $\alpha 11$  were associated with 5, 4, and 4 pathways, respectively. Because these markers are associated with immune activation or suppression, in aggregate, these findings suggest that both

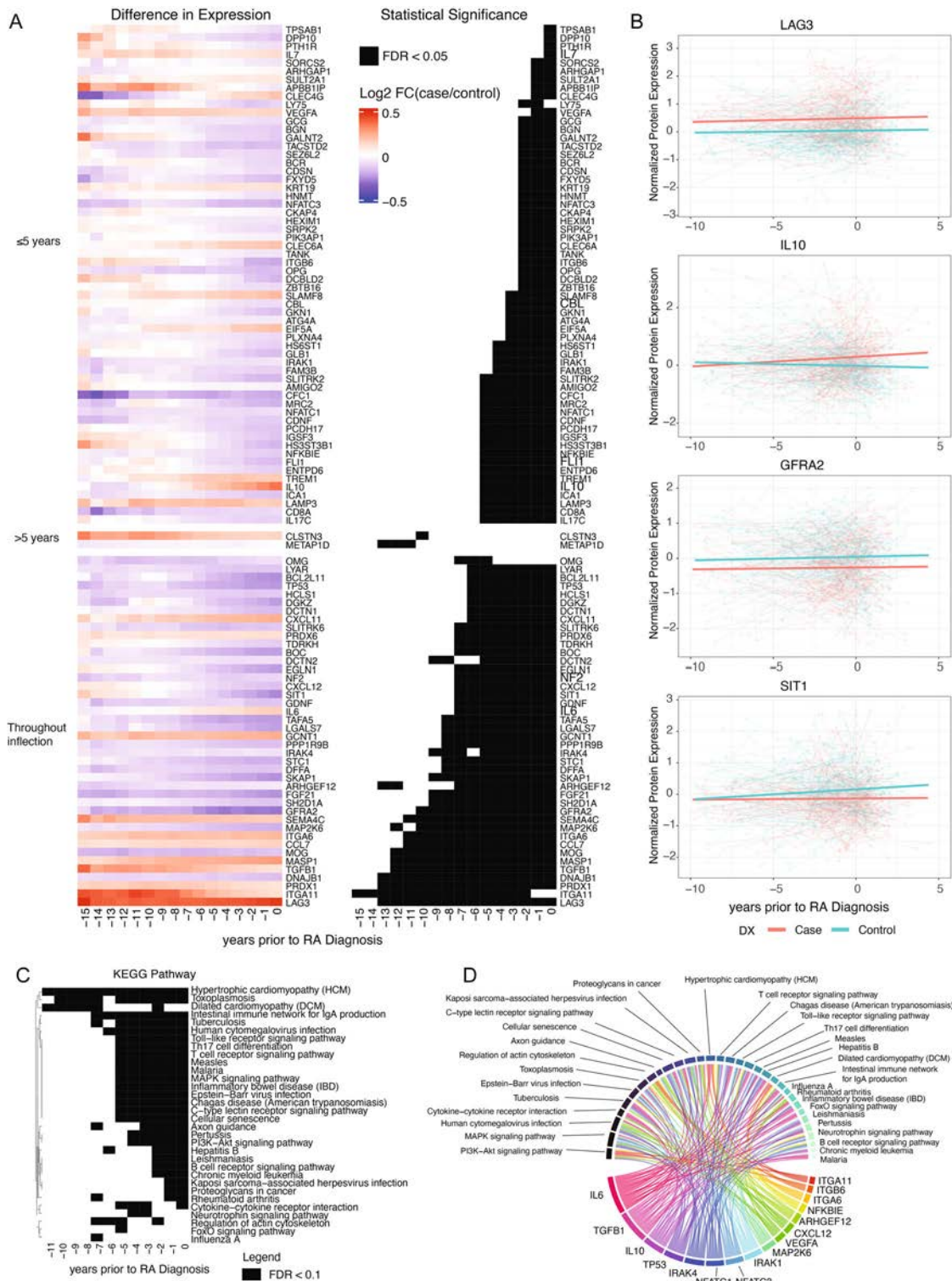


**Figure 2.** Global protein and pathway enrichment analysis. (A) Heatmap showing longitudinal signature of 104 proteins that were differentially expressed between patients and matched controls. Left: Each cell denotes the protein expression differences between patients and controls (red = higher in pre-RA, blue = higher in controls). Right: Cells denote statistical significance (FDR < 0.05) for each protein at each time point. (B) KEGG pathway enrichment in the years before RA diagnosis. The analyses were conducted between 0 and 15 years before diagnosis; however, no pathways were enriched between 11 and 15 years and were thus omitted from the graph. (C) Bipartite graph demonstrating the differential proteins enriching the KEGG pathways. Figures were generated using Adobe Illustrator. DX, disease group; FDR, false discovery rate; GFRA2, glial cell line-derived neurotrophic factor family receptor  $\alpha 2$ ; IL-10, interleukin-10; KEGG, Kyoto Encyclopedia of Genes and Genomes; LAG3, lymphocyte activation gene; RA, rheumatoid arthritis; SIT1, signaling threshold-regulating transmembrane adapter 1.

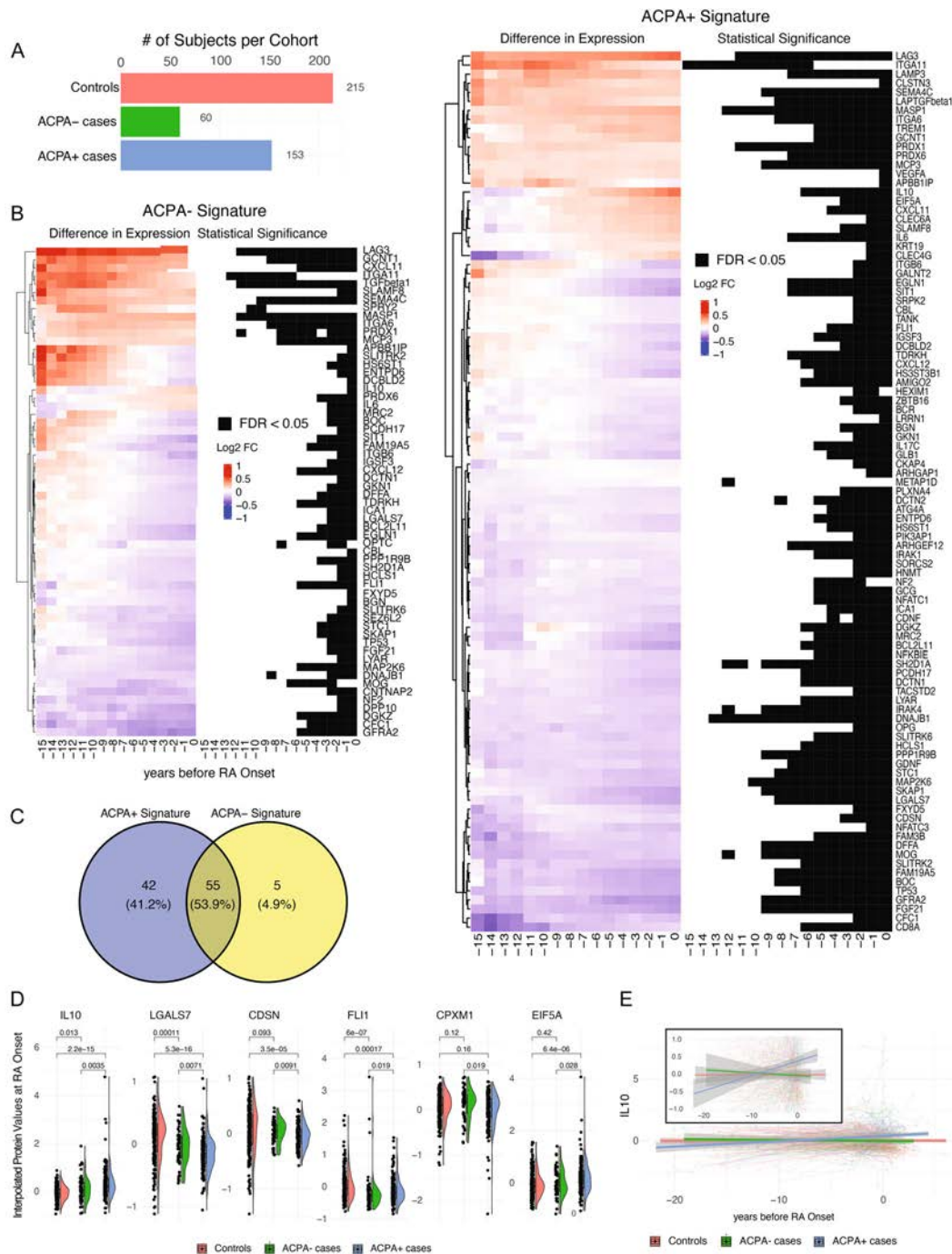


**Figure 3.** Subgroup comparisons within ACPA (positive) and ACPA (negative) participants with RA as determined by anti-CCP3IgG testing. (A) Barplot illustrates the number of ACPA-positive patients, ACPA-negative patients, and control participants in the study. (B) Left: Heatmaps showing longitudinal signature of differential protein expression for ACPA-positive cases showing log2 FC (case/control) and statistical significance. Right: Heatmaps showing longitudinal signature of differential protein expression for ACPA-negative cases showing log2 FC (case/control) and statistical significance. (C) Venn Diagram showing overlap in protein signatures between ACPA-positive and ACPA-negative cases. (D) Raincloud plots of normalized protein expression for IL-10, LGALS7, CDSN, FLI1, and EIF5a for ACPA-positive cases, ACPA-negative cases, and matched controls. Nominal Mann-Whitney U-test test *P* values show pairwise differences in protein expression between the three comparisons. (E) Longitudinal normalized protein expression for IL-10 for ACPA-positive cases (blue), ACPA-negative cases (green), and matched controls (red). The x-axis contains years before disease onset for cases and equivalent time for controls. The y-axis contains normalized protein expression for IL-10. Trend lines and 95% confidence intervals illustrate population trajectories over time. Insert: close-up of IL-10 trajectories in expression values (range -1 to 1) to highlight differences across subgroups. Figures were generated using Adobe Illustrator. ACPA, anti-citrullinated protein antibody; anti-CCP3IgG, anti-cyclic citrullinated protein antibody-3 immunoglobulin G; CDSN, cornodesmosin; CPXM1, carboxypeptidase X, M14 family member 1; EIF5a, eukaryotic translation initiation factor 5A-1; FC, fold change; FDR, false discovery rate; FLI1, friend leukemia integration 1 transcription factor; IL-10, interleukin-10; LGALS7, galectin-7; RA, rheumatoid arthritis; SIT1, signaling threshold-regulating transmembrane adapter 1.

immune-promoting and immune-suppressing pathways might play a role at certain time points in the transition from pre-RA to clinical RA.

### Proteomic signatures in autoantibody subgroups.

We further evaluated proteomic signatures based on autoantibody positivity to assess potential longitudinal differences<sup>31–33</sup> by autoantibody subgroups (description in Supplementary Table 4). Of these, 153 patients were anti-CCP3<sup>+</sup> at least one time point before or after RA diagnosis, whereas 60 patients were anti-CCP3<sup>−</sup> at all time points (Figure 3A). In longitudinal comparisons, we observed 97 DEPs in anti-CCP3<sup>+</sup> cases compared to controls (Figure 3B, Supplementary Table 5), and 60 DEPs were identified in anti-CCP3<sup>−</sup> cases compared to controls (Figure 3B, Supplementary Table 6). Furthermore, 53 of 97 DEPs were observed within anti-CCP3<sup>+</sup> cases  $\leq 5$  years before disease onset. Notably, in anti-CCP3<sup>−</sup> cases, 30 of 60 DEPs were observed within two years of RA diagnosis, signaling potentially different inflection points between anti-CCP3<sup>+</sup>/− subgroups.

A Venn diagram comparing the longitudinal signatures (Figure 3C) of anti-CCP3<sup>+</sup> cases ( $n = 97$  DEPs relative to controls) and anti-CCP3<sup>−</sup> cases ( $n = 60$  DEPs) illustrates that 42 of 97 proteins were unique to anti-CCP3<sup>+</sup> cases, whereas 5 of 60 proteins were unique to anti-CCP3<sup>−</sup> cases, and 55 proteins were shared. In a direct comparison between anti-CCP3<sup>+</sup> and anti-CCP3<sup>−</sup> cases at the time of diagnosis (year 0), we observed six DEPs at a  $P$  value  $< 0.05$  (Figure 3D) but none after FDR correction. IL-10 was the top DEP between anti-CCP3<sup>+</sup> and anti-CCP3<sup>−</sup> cases at the time of diagnosis. Furthermore, the longitudinal trajectories demonstrate elevated values of IL-10 in anti-CCP3<sup>+</sup> cases relative to both anti-CCP3<sup>−</sup> cases and controls in the period  $\leq 5$  years before RA diagnosis (Figure 3E).

We additionally evaluated differential protein expression between the following subgroups: anti-CCP3<sup>+</sup>/RF<sup>+</sup> ( $n = 128$ ), anti-CCP3<sup>+</sup>/RF<sup>−</sup> ( $n = 25$ ), anti-CCP3<sup>−</sup>/RF<sup>+</sup> ( $n = 21$ ), and anti-CCP3<sup>−</sup>/RF<sup>−</sup> ( $n = 39$ ) against controls ( $n = 215$ ), (Supplementary Figure 5A). We restricted the analyses to 0 to 10 years given the sparsity of observations and the impact of sample size in the subgroups analyses. DEPs and enriched pathways for anti-CCP3<sup>+</sup>, anti-CCP3<sup>−</sup>, and anti-CCP3<sup>+</sup>/RF<sup>+</sup> and anti-CCP3<sup>−</sup>/RF<sup>−</sup> are shown in Supplementary Figure 5B and E and Supplementary Figure 6, respectively, with larger differences observed in the autoantibody-positive subgroups relative to controls.

**Clinical and biomarker features to estimate the time to diagnosis of clinical RA.** Following previous work,<sup>19,20</sup> we evaluated the role of combinations of clinical variables, autoantibody counts (log2-centered and scaled), and protein biomarkers to determine the temporal location of a sample in the pre-RA period to identify individuals who are at risk for imminent onset of clinical RA (Figure 4A). We first split our data into training ( $n = 69$  participants) and validation ( $n = 55$  participants)

sets. The percentage of participants with a sample within one to five years of RA diagnosis (ie, outcome variable) ranged between 17% and 64% in the training set and were statistically comparable across training and validation (Supplementary Figure 7A and B). Furthermore, we evaluated our model using the first recorded anti-CCP3<sup>+</sup> sample for each participant to estimate time to RA onset after first testing anti-CCP3<sup>+</sup>. We trained our models selecting protein, clinical, and autoantibody features based on their ability to predict for a participant's sample being present for different intervals (eg,  $\leq 5$  years,  $\leq 4$  years) and compared this “full” model to autoantibody-only models. In the full models, training and validation AUCs ranged from 0.71 to 0.79 and 0.68 to 0.78, respectively, whereas in the autoantibody models, the AUCs ranged from 0.58 to 0.66 (Supplementary Figure 8). All but one of the full models were significant ( $P < 0.05$ ), whereas no autoantibody-only model was statistically significant (Supplementary Figure 8).

In particular, because many clinical trials in RA prevention have been conducted over two to three years, we further focused on optimizing the three-year classification model (see cohort characteristics, Supplementary Figure 9A) and present the significant features in the final model (Figure 4B, Table 2), which can be used to classify whether an anti-CCP3<sup>+</sup> sample is  $\leq 3$  years before RA diagnosis. We accounted for sex differences in our cohort and included CCP3 given its predictive power (Supplementary Figure 9B), excluding additional nonsignificant clinical features (Supplementary Figure 9C). The refined  $\leq 3$ -year model yielded an AUC of 0.80 (95% confidence interval 0.68–0.92; Figure 4C, left and middle), with an AUC 0.15 points greater than the three-year CCP3 with sex model (DeLong  $P < 0.1$ , Figure 4C, right).<sup>34</sup> Longitudinally, the model predictions were higher closer to disease onset (Supplementary Figure 9D). These findings suggest that models that use combinations of clinical features, autoantibodies, and protein biomarkers can be used in anti-CCP3<sup>+</sup> individuals to more accurately estimate a time to diagnosis of RA than models that include only autoantibodies.

## DISCUSSION

In this study, we have identified several key findings related to the natural history and development of RA. First, although autoantibodies may be elevated in individuals in the pre-RA state for  $> 10$  years before diagnosis, there appears to be an “inflection point” of increase in levels approximately 5 years before diagnosis. Second, there are evolving differences in protein expression across the pre-RA period, suggesting that different pathways are active at distinct time points in RA development, with the greatest changes observed approximately five years before RA diagnosis. In the context of autoantibody levels, this suggests the five-year period before RA diagnosis is an important period in which there are multiple dysregulated processes leading to expansion of autoimmunity and a transition to clinical RA. Third, the proteomic profile suggests certain infection-related biologic

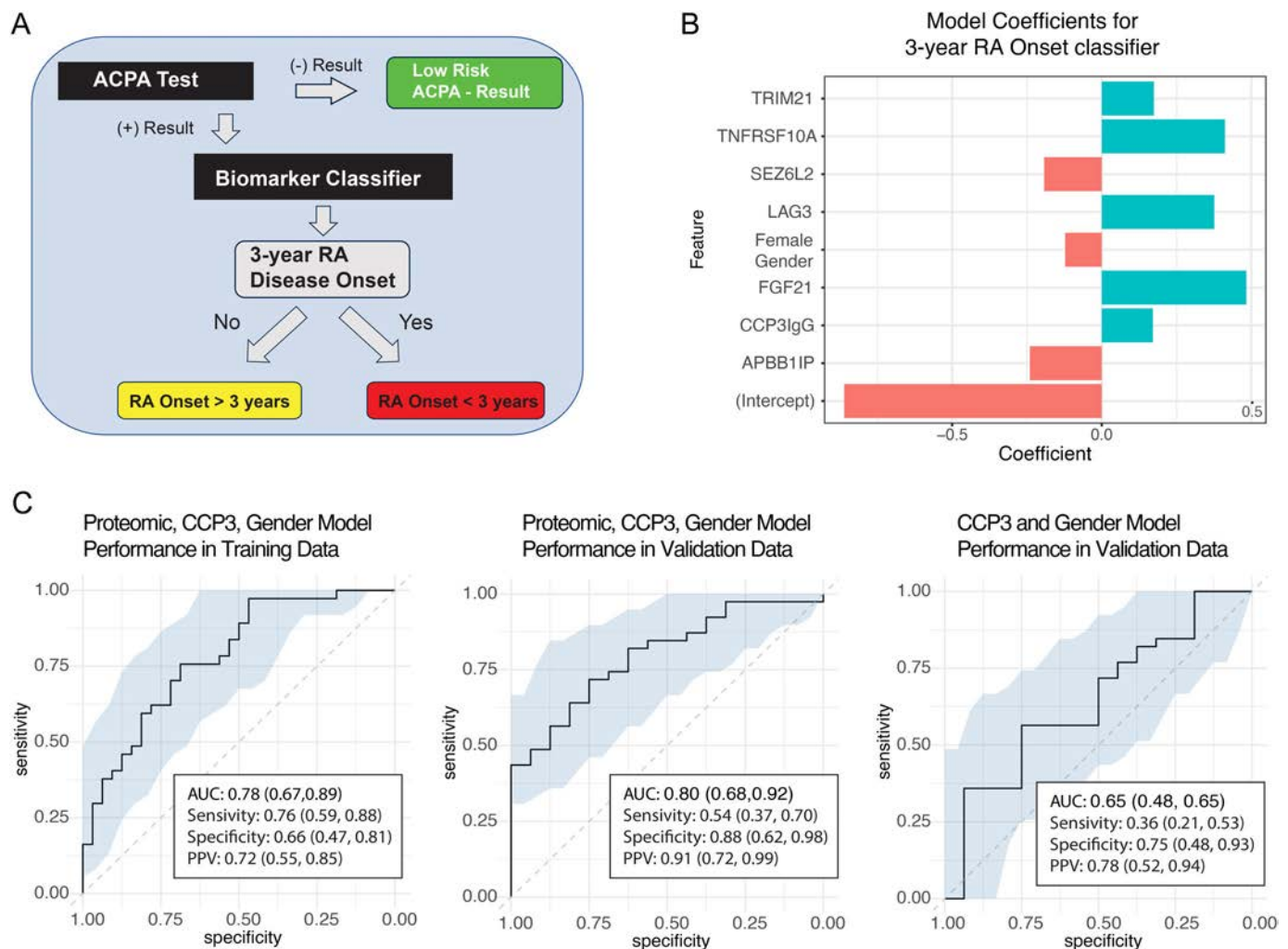


**Figure 4.** Predictive biomarkers. (A) Predictive algorithm overview: An individual first undergoes testing for ACPA positivity (herein assayed with anti-CCP3IgG). Individuals who test negative are deemed low risk; those who test positive are then further tested using predictive biomarkers to assess the potential timing of future development of RA. (B) The heatmap shows biomarkers and covariates used in the predictive model. The y-axis contains each of the biomarkers, and the x-axis represents a distinct model predicting 1:5 years before disease onset. Each cell contains the coefficient value for the biomarker in their corresponding model. Colors denote whether each marker predicts the likelihood (red = higher, blue = lower) of conversion for each year before disease onset. (C) Receiver operating characteristics curves and their 95% confidence intervals demonstrate the performance of the predictive algorithm in (left) the training set and (right) the validation set. Model performance metrics and 95% confidence intervals are shown in the insert. Figures were generated using Adobe Illustrator. ACPA, anti-citrullinated protein antibody; AUC, area under the receiver operating curve; (anti-) CCP3, cyclic citrullinated peptide-3; LAG3, lymphocyte activation gene; PPV, positive predictive value; RA, rheumatoid arthritis.

(KEGG) pathways with several specific markers including IL-10, IL-6, and LAG3 playing prominent roles. Fourth, there are differences in immune signatures between anti-CCP3+ and anti-CCP3- RA. Finally, combinations of clinical, autoantibody and proteomic factors can be used to classify when an individual may be in the time course of disease development and potentially identify anti-CCP3+ individuals within three years of clinical RA onset.

The proteomic findings in this study are particularly intriguing. Notably, certain proteins may differentiate patients from controls more than 10 years before disease onset, whereas other proteins only differentiate patients from controls within 5 years before disease onset. These proteomic expression differences may point

to different biologic processes that are specific for certain stages of disease development. In particular, the roles of LAG3,<sup>35–37</sup> integrins,<sup>38–40</sup> and<sup>19,41–44</sup> IL-10 have been previously studied in RA in human and mouse studies. Given the potential immunomodulatory functions<sup>45</sup> of IL-10, it is interesting to note the elevated circulating levels of IL-10 in progression to RA. We and others have previously observed elevated IL-10 in the peripheral circulation or synovial fluid of pre-RA and individuals with RA.<sup>19,46,47</sup> However, in other autoimmune diseases, low IL-10 expression is associated with disease.<sup>48,49</sup> Our findings herein could indicate that IL-10 is failing to adequately regulate immunity, or that it may have nonimmunosuppressive functions. For example, LAG3-positive Treg cells secrete IL-10, which impairs antigen

**Table 2.** Coefficients for each variable that was significantly associated with a sample being present  $\leq 3$  years before RA diagnosis versus  $> 3$  years\*

| Clinical and biomarker features included in the model | E-net coefficient <sup>a</sup> | Bootstrapped GLM Z score <sup>b</sup> | Bootstrapped P value <sup>c</sup> |
|-------------------------------------------------------|--------------------------------|---------------------------------------|-----------------------------------|
| (Intercept)                                           | -0.860862182                   | -3.631810317                          | 0.0001                            |
| Female = 1                                            | -0.1222891684                  | -0.2560945989                         | 0.3989                            |
| Log2 (CCP3IgG) <sup>d</sup>                           | 0.1708286178                   | 3.617776656                           | 0.0001                            |
| TRIM21                                                | 0.1743369218                   | 1.876469788                           | 0.0303                            |
| TNFRSF10A                                             | 0.4117755252                   | 3.346933334                           | 0.0004                            |
| FGF21                                                 | 0.4832916159                   | 2.786857425                           | 0.0027                            |
| LAG3                                                  | 0.3761205546                   | 2.025988912                           | 0.0214                            |
| APBB1IP                                               | -0.2403581322                  | -1.7802846                            | 0.0375                            |
| SEZ6L2                                                | -0.1924052943                  | -2.559670529                          | 0.0052                            |

\* Use example: To identify individuals with imminent risk to RA, we apply these coefficients to obtain the probability of anti-CCP3 positive pre-RA sample being from  $\leq 3$  years from the time of RA diagnosis. For example, for a man with a relative expression of 0 across all biomarkers, the probability of being within  $\leq 3$  years of conversion would approximately be  $\text{logistic}(-0.8609) = 0.297$ . The second example, for a woman with  $\text{log}_2(\text{anti-CCP3 levels}) = 7$  and relative expression of 1 across all remaining biomarkers, the probability of being within  $\leq 3$  years of conversion would approximately be  $\text{logistic}(1.22541) = 0.773$ . Using a Youden index-based threshold of 0.61, the first case sample would be classified as  $> 3$  years of conversion, whereas the second case sample would be classified as being within  $\leq 3$  years of conversion. APBB1IP, amyloid beta precursor protein binding family B member 1 interacting protein; CCP3, cyclic citrullinated peptide-3; FGF21, fibroblast growth factor 21; GLM, generalized linear model; LAG3, lymphocyte activation gene; RA, rheumatoid arthritis; SEZ6L2, seizure-related 6 homolog like 2; TNFRSF10A, tumor necrosis factor receptor superfamily 10a; TRIM21, tripartite motif-containing protein 21.

<sup>a</sup> E-net coefficient = variable coefficient from the Elastic Net model.

<sup>b</sup> Bootstrapped Z score = mean (GLM coefficient)/SD(GLM coefficient) from 5,000 iterations of unpenalized logistic regression model.

<sup>c</sup> Bootstrapped P value = empirical P value from bootstrapped Z score. The absolute |Z| was used to determine the significance to account for positive and negative features. Sex was not significant; however it is a known risk factor for RA, so we kept it in the model.

<sup>d</sup> CCP3IgG values were  $\text{log}_2$  transformed and then centered and scaled relative to controls.

presentation and costimulation of naive T cells, and perhaps our finding indicates that this role of IL-10 is insufficient to control transition to RA.<sup>50–52</sup> In contrast, T peripheral helper cells from RA synovial tissue express increased IL-10, and therefore our findings herein could represent IL-10 secretion from cells that are driving disease.<sup>53</sup> Future studies will need to explore the role of IL-10, and these other markers, in the evolution of RA.

It is also intriguing to identify increasing levels of autoantibodies and DEPs starting approximately five years before a diagnosis of RA. There are certainly immune abnormalities earlier in the pre-RA period, but this may indicate that this five-year period represents a critical period in which multiple pathways of immune dysregulation act to facilitate a transition from pre-RA to clinical RA. In particular, the differential expressions of IL-10, IL-6, and LAG3 may indicate that these are key markers of disease processes in pre-RA. Furthermore, although KEGG pathway analyses may overattribute biomarkers to certain pathways, they suggest several mucosal and/or infection-related pathways (eg, inflammatory bowel disease, Epstein-Barr virus) are active in this period highlighting important factors in transition and aligning with hypotheses that mucosal and/or microbial factors may play a key role in the development of RA<sup>54,55</sup> and will need to be explored in future prospective studies.

To contextualize our findings herein to prediction of future RA, previous studies have shown that autoantibodies are predictors of future RA.<sup>14</sup> Additionally, previous studies have observed

that proteomics (and in some cases genetic factors)<sup>56</sup> can be used to discriminate between at-risk individuals who do or do not progress to clinical RA<sup>11,57</sup> and in the context of prevention of clinical RA, emerging data that the number of positive autoantibodies may identify which at-risk individuals may respond best to abatacept.<sup>12,58</sup> These new findings have important implications as they expand on the understanding that protein biomarkers can be used along with clinical and autoantibody features to provide clinical utility. Furthermore, these findings demonstrate that proteomics can define specific pathways that may guide a precision-medicine approach for the selection of specific preventive interventions may be most appropriate for a given stage of immune dysregulation in the pre-RA period. An important caveat is that, given the case-control design of this study, the ability to develop prediction models is not as robust as could be done using prospective studies in which, for example, not all individuals who are at risk for RA develop clinical RA.<sup>59</sup> However, the findings provide support for further explorations of combined clinical, autoantibody, and proteomic models to both identify individuals who are at an increased risk for future RA as well as estimate their potential time to diagnosis and identify specific biologic pathways within individuals that may be targeted for prevention.

We also note in our analyses that there are differences in biomarker profiles between anti-CCP3+ and anti-CCP3- cases. We noted that proteomic profiles had an inflection point closer to a diagnosis of RA in anti-CCP- compared to anti-CCP3+ cases.

This is in-line with findings from a number of published studies that have demonstrated that different biologic pathways may be dysregulated in seropositive and seronegative RA, potentially due to differences in genetic and/or environmental factors that drive disease.<sup>31–33</sup> This needs to be explored further in future studies because it may provide greater insights into prediction and preventive targets in seronegative RA, especially given there are a growing number of studies that suggest that anti-CCP- pre-clinical state may be identified through assessment of symptom complexes and imaging.<sup>10,60</sup>

There are several limitations to this study. First, our cohort was derived retrospectively from a serum repository (DoDSR) that may have potential biases based on participant and sample selection and may introduce some bias in levels of biomarkers due to age of sample; however, although the initial sample processing across multiple DoDDR sites system<sup>15</sup> may impact protein levels, our analyses suggest no systemic biases due to sample storage time. Second, although this study contains >20 years of data before disease onset, patients have, on average, three samples, with an average of two pre-RA samples. This resulted in both sparse and heterogeneous trajectories across individuals and consequently introduced some challenges in modeling both participant and population longitudinal trajectories. Specifically, models may be more robust using resampling from a larger number of samples to capture more granular changes in pre-RA signatures. Additionally, because there was greater sparsity in the period >10 years before RA diagnosis, some participants were excluded in time points before their earliest visits, and our analyses have a sampling bias closer to disease onset. Third, Olink provides relative measures of circulating protein abundance in serum, which limits direct comparisons of protein quantities; furthermore, circulating proteins do not provide direct insights into specific cell populations that may have known implications in autoimmunity and RA (eg, T cells and B cells).

Fourth, although this study presents a large and diverse cohort of individuals with RA, because of the nature of the United States Uniformed Services, the DoDSR-derived RA cohort is younger and has a higher prevalence of men than other RA cohorts, and that may limit, to some extent, the generalizability of our findings. Fifth, published models for prediction of future RA in at-risk individuals incorporate multiple factors including symptoms, examination findings, and biomarkers and imaging.<sup>59,61</sup> However, because of the retrospective nature of our data, we have limited data related to symptom onset before a diagnosis of RA. Our analyses yielded similar results using time-to-symptom versus time-to-disease onsets; however, future prospective studies may further evaluate the relationships among biomarkers, symptoms, and the first appearance of IA. Finally, we performed autoantibody tests using two systems (RF and anti-CCP3) that are commonly available for RA, and a set of protein biomarkers contained in the three Olink panels Inflammation, Immune Response, and Cell Regulation. We believe that these

markers provide us with good insight into the biology of pre-RA. However, we acknowledge that there are additional serum biomarkers that could be tested, including different commercial anti-CCP assays (eg, anti-CCP2), ACPA arrays, antibodies to carbamylated proteins, antibodies to peptidyl arginine deiminase, and other antimodified protein antigens such as acetylated proteins and glycosylation of autoantibodies, as well as additional protein and cellular biomarkers. These all may be important assays to improve our understanding of the biology of RA development and should be explored in future<sup>62–66</sup> studies.

In conclusion, we have observed that autoantibodies and protein signatures evolve in distinct stages before a diagnosis of RA. Furthermore, protein biomarkers may identify biologic pathways relevant to specific stages. These can be further explored to potentially improve prediction of disease onset and identify stage-specific biologic pathways to target with preventive interventions.

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## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding authors, Drs Li and Deane confirm that all authors have provided the final approval of the version to be published and take responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

1. Gravallese EM, Firestein GS, Koscal N, et al. What is rheumatoid arthritis? *N Engl J Med* 2024;390(13):e32.
2. Deane KD, Holers VM. Rheumatoid arthritis pathogenesis, prediction, and prevention: an emerging paradigm shift. *Arthritis Rheumatol* 2021;73(2):181–193.
3. Cope AP, Jasencova M, Vasconcelos JC, et al; APIPPRA study investigators. Abatacept in individuals at high risk of rheumatoid arthritis (APIPPRA): a randomised, double-blind, multicentre, parallel, placebo-controlled, phase 2b clinical trial. *Lancet* 2024;403(10429):838–849.
4. Rech J, Tascilar K, Hagen M, et al. Abatacept inhibits inflammation and onset of rheumatoid arthritis in individuals at high risk (ARIAA): a randomised, international, multicentre, double-blind, placebo-controlled trial. *Lancet* 2024;403(10429):850–859.
5. Deane KD, et al. Therapeutic interception in individuals at risk of rheumatoid arthritis to prevent clinically impactful disease. *Ann Rheum Dis* 2025;84(1):14–28.

6. Bos WH, Dijkman BAC, Boers M, et al. Effect of dexamethasone on autoantibody levels and arthritis development in patients with arthralgia: a randomised trial. *Ann Rheum Dis* 2010;69(3):571–574.
7. Gerlag DM, Safy M, Majer KI, et al. Effects of B-cell directed therapy on the preclinical stage of rheumatoid arthritis: the PRAIRI study. *Ann Rheum Dis* 2019;78(2):179–185.
8. van Boheemen L, Turk S, Beers-Tas MV, et al. Atorvastatin is unlikely to prevent rheumatoid arthritis in high risk individuals: results from the prematurely stopped STATins to Prevent Rheumatoid Arthritis (STAPRA) trial. *RMD Open* 2021;7(1):e001591.
9. Deane KD, Striebich C, Feser M, et al. Hydroxychloroquine does not prevent the future development of rheumatoid arthritis in a population with baseline high levels of antibodies to citrullinated protein antigens and absence of inflammatory arthritis: interim analysis of the StopRA trial [abstract]. *Arthritis Rheumatol* 2022;74(suppl 9). <https://acrabstracts.org/abstract/hydroxychloroquine-does-not-prevent-the-future-development-of-rheumatoid-arthritis-in-a-population-with-baseline-high-levels-of-antibodies-to-citrullinated-protein-antigens-and-absence-of-inflammatory/>.
10. Krijbolder DI, Verstappen M, van Dijk BT, et al. Intervention with methotrexate in patients with arthralgia at risk of rheumatoid arthritis to reduce the development of persistent arthritis and its disease burden (TREAT EARLIER): a randomised, double-blind, placebo-controlled, proof-of-concept trial. *Lancet* 2022;400(10348):283–294.
11. O'Neil LJ, Spicer V, Smolik I, et al. Association of a serum protein signature with rheumatoid arthritis development. *Arthritis Rheumatol* 2021;73(1):78–88.
12. Qin Q, Song R, Du P, et al. Systemic proteomic analysis reveals distinct exosomal protein profiles in rheumatoid arthritis. *J Immunol Res* 2021;2021:9421720.
13. Kovács OT, Tóth E, Ozohanics O, et al. Proteomic changes of osteoclast differentiation in rheumatoid and psoriatic arthritis reveal functional differences. *Front Immunol* 2022;13:892970.
14. Kelmenson LB, Wagner BD, McNair BK, et al. Timing of elevations of autoantibody isotypes prior to diagnosis of rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(2):251–261.
15. Perdue CL, Eick-Cost AA, Rubertone MV. A brief description of the operation of the DoD serum repository. *Mil Med* 2015;180(10 Suppl):10–12.
16. Rubertone MV, Brundage JF. The Defense Medical Surveillance System and the Department of Defense Serum Repository: glimpses of the future of public health surveillance. *Am J Public Health* 2002;92(12):1900–1904.
17. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31(3):315–324.
18. Majka DS, Deane KD, Parrish LA, et al. Duration of preclinical rheumatoid arthritis-related autoantibody positivity increases in subjects with older age at time of disease diagnosis. *Ann Rheum Dis* 2008;67(6):801–807.
19. Deane KD, O'Donnell CI, Hueber W, et al. The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner. *Arthritis Rheum* 2010;62(11):3161–3172.
20. Sokolove J, Bromberg R, Deane KD, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. *PLoS One* 2012;7(5):e35296.
21. Lingampalli N, Sokolove J, Lahey LJ, et al. Combination of anti-citrullinated protein antibodies and rheumatoid factor is associated with increased systemic inflammatory mediators and more rapid progression from preclinical to clinical rheumatoid arthritis. *Clin Immunol* 2018;195:119–126.
22. Bettner LF, Peterson RA, Bergstedt DT, et al. Combinations of anti-cyclic citrullinated protein antibody, rheumatoid factor, and serum calprotectin positivity are associated with the diagnosis of rheumatoid arthritis within 3 years. *ACR Open Rheumatol* 2021;3(10):684–689.
23. Bergstedt DT, Tarter WJ, Peterson RA, et al. Antibodies to citrullinated protein antigens, rheumatoid factor isotypes and the shared epitope and the near-term development of clinically-apparent rheumatoid arthritis. *Front Immunol* 2022;13:916277.
24. Bergemalm D, Andersson E, Hultdin J, et al; IBD Character Consortium. Systemic inflammation in preclinical ulcerative colitis. *Gastroenterology* 2021;161(5):1526–1539.e9.
25. Consiglio CR, Cotugno N, Sardh F, et al; CACTUS Study Team. The immunology of multisystem inflammatory syndrome in children with COVID-19. *Cell* 2020;183(4):968–981.e7.
26. Camargo MC, Song M, Ito H, et al. Associations of circulating mediators of inflammation, cell regulation and immune response with esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol* 2021;147(10):2885–2892.
27. Wik L, Nordberg N, Broberg J, et al. Proximity extension assay in combination with next-generation sequencing for high-throughput proteome-wide analysis. *Mol Cell Proteomics* 2021;20:100168.
28. Assarsson E, Lundberg M, Holmquist G, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One* 2014;9(4):e95192.
29. Liao Y, Wang J, Jaehnig EJ, et al. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Res* 2019;47(W1):W199–W205.
30. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 2000;28(1):27–30.
31. Aho K, Kurki P. Seropositive versus seronegative rheumatoid arthritis—time for a new definition. *J Rheumatol* 1994;21(3):388–390.
32. Kisten Y, Arnaud L, Levitsky A, et al. Distinct fluorescence optical imaging patient clusters emerge for seropositive and seronegative rheumatoid arthritis. *ACR Open Rheumatol* 2023;5(9):474–480.
33. Barra L, Pope JE, Orav JE, et al; CATCH Investigators. Prognosis of seronegative patients in a large prospective cohort of patients with early inflammatory arthritis. *J Rheumatol* 2014;41(12):2361–2369.
34. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988;44(3):837–845.
35. Nakachi S, Sumitomo S, Tsuchida Y, et al. Interleukin-10-producing LAG3<sup>+</sup> regulatory T cells are associated with disease activity and abatacept treatment in rheumatoid arthritis. *Arthritis Res Ther* 2017;19(1):97.
36. Hu S, Liu X, Li T, et al. LAG3 (CD223) and autoimmunity: emerging evidence. *J Autoimmun* 2020;112:102504.
37. Pedersen JM, Hansen AS, Skejød C, et al. Lymphocyte activation gene 3 is increased and affects cytokine production in rheumatoid arthritis. *Arthritis Res Ther* 2023;25(1):97.
38. Lowin T, Straub RH. Integrins and their ligands in rheumatoid arthritis. *Arthritis Res Ther* 2011;13(5):244.
39. Wilder RL. Integrin alpha V beta 3 as a target for treatment of rheumatoid arthritis and related rheumatic diseases. *Ann Rheum Dis* 2002;61 Suppl 2(suppl 2):ii96–ii99.
40. De Giuseppe A, Deichsel A, Rauwolf K, et al. AB0089 integrin  $\alpha 11\beta 1$  deficiency affects the course of disease in the arthritic hTNFtg mouse. *Ann Rheum Dis* 2022;81:1176.
41. Padyukov L, Hytönen AM, Smolnikova M, et al. Polymorphism in promoter region of IL10 gene is associated with rheumatoid arthritis in women. *J Rheumatol* 2004;31(3):422–425.
42. Hernández-Bello J, Oregón-Romero E, Vázquez-Villamar M, et al. Aberrant expression of interleukin-10 in rheumatoid arthritis:

- relationship with IL10 haplotypes and autoantibodies. *Cytokine* 2017; 95:88–96.
43. Heo YJ, Joo YB, Oh HJ, et al. IL-10 suppresses Th17 cells and promotes regulatory T cells in the CD4+ T cell population of rheumatoid arthritis patients. *Immunol Lett* 2010;127(2):150–156.
  44. Keystone E, Wherry J, Grint P. IL-10 as a therapeutic strategy in the treatment of rheumatoid arthritis. *Rheum Dis Clin North Am* 1998; 24(3):629–639.
  45. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol* 2012; 32(1):23–63.
  46. Kokkonen H, Söderström I, Rocklöv J, et al. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. *Arthritis Rheum* 2010;62(2):383–391.
  47. Cush JJ, Splawski JB, Thomas R, et al. Elevated interleukin-10 levels in patients with rheumatoid arthritis. *Arthritis Rheum* 1995;38(1): 96–104.
  48. Cao Y, Goods BA, Raddassi K, et al. Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci Transl Med* 2015;7(287):287ra74.
  49. Blair PA, Noreña LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. *Immunity* 2010;32(1):129–140.
  50. Möttönen M, Isomäki P, Saario R, et al. Interleukin-10 inhibits the capacity of synovial macrophages to function as antigen-presenting cells. *Br J Rheumatol* 1998;37(11):1207–1214.
  51. Buelens C, Willems F, Delvaux A, et al. Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur J Immunol* 1995;25(9):2668–2672.
  52. Matsumoto M, Baba A, Yokota T, et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity* 2014;41(6):1040–1051.
  53. Rao DA, Gurish MF, Marshall JL, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* 2017;542(7639):110–114.
  54. Holers VM, Demoruelle MK, Kuhn KA, et al. Rheumatoid arthritis and the mucosal origins hypothesis: protection turns to destruction. *Nat Rev Rheumatol* 2018;14(9):542–557.
  55. Holers VM, Demoruelle KM, Buckner JH, et al. Distinct mucosal endotypes as initiators and drivers of rheumatoid arthritis. *Nat Rev Rheumatol* 2024;20(10):601–613.
  56. Scott IC, Seegobin SD, Steer S, et al. Predicting the risk of rheumatoid arthritis and its age of onset through modelling genetic risk variants with smoking. *PLoS Genet* 2013;9(9):e1003808.
  57. O'Neil LJ, Meng X, Mcfadyen C, et al. Serum proteomic networks associate with pre-clinical rheumatoid arthritis autoantibodies and longitudinal outcomes. *Front Immunol* 2022;13:958145.
  58. Cope A, Jasenecova M, Vasconcelos J, et al. OP0130 abatacept in individuals at risk of developing rheumatoid arthritis: results from the arthritis prevention in the pre-clinical phase of RA with abatacept (APIPPRA) trial [abstract]. *Ann Rheum Dis* 2023;82:86.
  59. Duquenne L, Hensor EM, Wilson M, et al. Predicting inflammatory arthritis in at-risk persons: development of scores for risk stratification. *Ann Intern Med* 2023;176(8):1027–1036.
  60. van Steenbergen HW, Aletaha D, Beaart-van de Voorde LJ, et al. EULAR definition of arthralgia suspicious for progression to rheumatoid arthritis. *Ann Rheum Dis* 2017;76(3):491–496.
  61. van de Stadt LA, Witte BI, Bos WH, et al. A prediction rule for the development of arthritis in seropositive arthralgia patients. *Ann Rheum Dis* 2013;72(12):1920–1926.
  62. Kissel T, Reijm S, Slot LM, et al. Antibodies and B cells recognising citrullinated proteins display a broad cross-reactivity towards other post-translational modifications. *Ann Rheum Dis* 2020;79(4): 472–480.
  63. Hafkenscheid L, de Moel E, Smolik I, et al. N-linked glycans in the variable domain of IgG anti-citrullinated protein antibodies predict the development of rheumatoid arthritis. *Arthritis Rheumatol* 2019; 71(10):1626–1633.
  64. Di Matteo A, Mankia K, Duquenne L, et al. Third-generation anti-cyclic citrullinated peptide antibodies improve prediction of clinical arthritis in individuals at risk of rheumatoid arthritis. *Arthritis Rheumatol* 2020; 72(11):1820–1828.
  65. Kolfenbach JR, Deane KD, Derber LA, et al. Autoimmunity to peptidyl arginine deiminase type 4 precedes clinical onset of rheumatoid arthritis. *Arthritis Rheum* 2010;62(9):2633–2639.
  66. Gan RW, Trouw LA, Shi J, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. *J Rheumatol* 2015;42(4):572–579.

# Recognition of Glycine Versus Nonglycine Citrulline Motifs Dictating the HLA Class II Association of Anticitrullinated Protein Antibodies: Insights From Autoantibody Profiling of 6,900 Scandinavian Patients With Rheumatoid Arthritis

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**Objective.** Rheumatoid arthritis (RA)–associated anticitrullinated protein antibodies (ACPAs) target various antigens by binding short citrulline amino acid motifs, resulting in heterogeneous ACPA profiles among patients. Here we analyzed ACPA patterns by recognized citrulline motifs in relation to the RA risk factors HLA-DRB1 shared epitope (SE) alleles and history of smoking.

**Methods.** Rheumatoid factor (RF) and anticyclic citrullinated peptide (CCP2) isotypes, 15 anti-Cit- and four anti-Carb/Acet-peptide-IgG, were centrally measured in 6,907 patients from five Scandinavian RA cohorts using fluoroenzyme immunoassay and a custom-made multiplex solid-phase microarray. HLA-DRB1 SE alleles were imputed from single-nucleotide polymorphism genotyping data.

**Results.** Single-citrulline peptides derived from four multicitrulline peptides (Cit Fib $\alpha_{36-50}$ , Cit Fib $\beta_{60-74}$ , Cit TNC5, and Cit Vim $_{60-75}$ ) showed differential binding patterns, supporting recognition of citrulline motifs rather than long peptides. Four citrulline peptides (Cit Fib $\beta_{36-52}$ , Cit Fib $\beta_{60-74}$ -Cit3, Cit Fil $_{307-324}$ , and Cit Vim $_{60-75}$ -Cit1) captured 97% of IgG anti-CCP2+ patients. Patient subsets based on ACPA, anti-Carb/Acet, and RF displayed differences in ACPA composition and disease activity but not comorbidities. Different ACPAs overlapped, but when dichotomizing patients based on high reactivity to peptide citrulline motifs, only ACPA to nonglycine citrulline motif associated with HLA SE alleles. In IgG anti-CCP2+ patients, 90% of those with only high nonglycine ACPA were HLA SE allele carriers compared with 67% in the group with glycine motif–only ACPA (odds ratio 4.5). Smoking status associated with IgA and glycine motif ACPA.

**Conclusion.** Although citrulline-glycine motifs are prevalent ACPA targets, our data reveal that HLA SE alleles are primarily associated with ACPA to nonglycine citrulline motifs, providing insight in ACPA T cell dependence. Yet, the etiologic significance of ACPA targeting different protein structures remains unknown.

## INTRODUCTION

More than two-thirds of all patients diagnosed with rheumatoid arthritis (RA) have “seropositive” disease as defined by the

presence of RA autoantibodies, primarily rheumatoid factor (RF) reactivity (anti-Fcy) and/or anticitrullinated protein autoantibodies (ACPAs).<sup>1</sup> Notably, seropositive RA has been linked to worse disease progression with more erosive disease compared

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with “seronegative” disease.<sup>2</sup> Important risk factors associated with seropositive RA include exposure to cigarette smoking and certain HLA-DRB1 variants denoted shared epitope (SE) alleles.<sup>3</sup> Interestingly, although the HLA-DR association places the adaptive immune response centrally in RA pathogenesis, and there is a strong correlation between SE and ACPA positivity, there are also indications that SE alleles are a stronger predictor of clinical arthritis than the presence of ACPA alone.<sup>4</sup>

ACPAs are highly specific for RA and commonly measured with cyclic citrullinated peptide (CCP2) assays using commercial synthetic peptides designed for high diagnostic accuracy. However, serum ACPAs have been found to recognize many different citrullinated proteins and peptides.<sup>5</sup> Proteins with disordered structures and regions enriched for glycine, serine, and threonine have been suggested to be particularly targeted.<sup>6</sup> Multiplex antigen panels have been developed to capture these ACPA fine specificity variations between patients with RA.<sup>7–10</sup> The clinical relevance of interpatient heterogeneity of ACPA peptide targets is, however, still controversial. Despite previous efforts, it has been difficult to demonstrate any clear association with disease progression or treatment outcome. Furthermore, adding to the complexity, studies using patient single cell–derived monoclonal antibodies show that individual ACPA IgG molecules can bind a multitude of different cit-peptides and that the peptide interaction is determined by only a couple of additional residues besides the citrulline (ie, cit-motifs).<sup>11</sup> This means that serological fine specificity patterns are not necessarily reflecting parallel ACPA clones but also include multireactive clones that create a direct dependence between different ACPA measurements.

Patients with RA, especially those positive for ACPA, can also have other IgG antimodified protein autoantibodies (AMPA) targeting additional posttranslational modifications of proteins, primarily carbamylation and acetylation.<sup>7,12–14</sup> AMPAs correlate with citrulline reactivity in antibody serology; certain monoclonal CCP2+ ACPAs have also been found to directly cross-react with carbamylated and acetylated peptides.<sup>11</sup> Moreover, although RF may not be as specific for RA as ACPA, RF is postulated to play important roles in amplifying inflammatory response. For instance, there is an additive effect of ACPA and RF positivity on the risk of bone erosions.<sup>15</sup> Furthermore, in patients with early RA, the presence of RF is associated with systemic inflammation in an ACPA-dependent manner.<sup>16</sup> In clinical practice, seropositivity is often determined based on only CCP2 IgG and RF IgM measurements, yet other isotypes or autoantibody reactivities can capture additional seropositive patients who display similar clinical risk factor associations as those defined based on CCP2 IgG and/or

RF IgM.<sup>17</sup> Moreover, IgA ACPA and RF have been proposed to reflect a link with mucosal triggering, but the role in pathogenesis is not well understood.

To further delineate the (co-)occurrence of RF, ACPAs, other AMPAs, and their association with RA risk factors (here: smoking and HLA-DRB1 SE alleles), we investigated RA autoantibody profiles in >6,900 patients with RA from five different Scandinavian RA cohorts using new optimized assays. To do so, we first validated the array's capacity to capture distinct ACPA cit-peptide binding based on citrulline motifs and assessed the overall frequency and dependence of different ACPA and AMPA fine specificities in the patients with RA. We hypothesized that ACPA fine specificities targeting glycine-containing citrulline motif in proteins may have different immunologic origin and clinical relevance than nonglycine ACPA. To explore this hypothesis further, we investigated two key topics: (1) do different ACPA and autoantibody compositions associate with different clinical subsets of patients with RA? (2) Do ACPA fine specificities targeting certain citrulline amino acid motifs (with and without glycine) associate differently with the main RA risk factors of HLA-DRB1 SE alleles and smoking?

## MATERIALS AND METHODS

**Study populations with RA.** Our study population was made up of patients with RA from five Scandinavian cohorts<sup>18</sup> (summarized in Supplementary Figure 1 and Supplementary Table 1): The Swedish Rheumatology Quality registry biobank (SRQb; N = 3,895),<sup>19</sup> the Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen (ARCTIC; N = 212),<sup>20</sup> the Danish Nationwide Clinical Rheumatology Registry (DANBIO) and corresponding samples within the Danish Rheumatologic Biobank (DRB; N = 2,324),<sup>21</sup> the Norwegian Antirheumatic Drug Register<sup>22</sup> (NOR-DMARD, N = 302), and the Ultrasound in Rheumatoid Arthritis patients starting Biologic Treatment (ULRABIT; N = 174) cohort.<sup>23</sup> The study was approved by the regional ethics review boards and followed the Declaration of Helsinki, and all patients gave informed consent. Inclusion in ARCTIC, NOR-DMARD, and ULRABIT required fulfillment of the American College of Rheumatology (ACR)/EULAR classification criteria for RA.<sup>1</sup> Because SRQb and DANBIO/DRB are clinical routine care RA registers, fulfillment of ACR/EULAR classifications is not a formal criterion for inclusion (a clinical RA diagnosis assigned by the treating rheumatologist is sufficient) but is fulfilled in most cases.

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**Blood samples and other information.** One stored plasma sample per patient ( $n = 6,907$ ) was sent for centralized antibody analysis. Depending on the source cohort, this sample was taken at different points during the RA disease course permitting the stratification into early versus established RA. In an initial screening, we split the statistical analyses into (1) patients with early RA ( $n = 597$ ) consisting of disease-modifying antirheumatic drug (DMARD)-naïve patients with RA from the ARCTIC cohort ( $n = 212$ ; baseline time point, average  $<7$  months disease duration) and SRQb ( $n = 385$ ;  $<90$  days from time of diagnosis at the time of sampling) and (2) a cross-sectional validation dataset with blood samples taken at any later timepoint during the RA disease, consisting of 3,510 patients from the Swedish SRQb (excluding the early RA samples) and 2,800 patients from the Danish and Norwegian RA cohorts.

Smoking status was available for 3,212 patients from the SRQb cohort and 2,316 patients from the DANBIO/DRB cohort; individuals were categorized as “ever smokers” (including current and former smokers) or “never smokers” based on self-report. Clinical measurements close to the time of blood sampling ( $\pm 14$  days) were retrieved from the SRQ register and were in this time window available for 2,185 of the patients from the SRQb cohort. Through linkage of SRQb to the Swedish National Patient Register and the Prescribed Drug Register, we also retrieved data on comorbid conditions during the 5 years preceding donation of blood for the study. Details of the comorbidity definitions can be found elsewhere.<sup>24</sup>

For SRQb, *HLA-DRB1 SE alleles* were imputed using SNP2HLA<sup>25</sup> from genetic data for 2,652 patients, genotyped on the Illumina global screening array (deCODE Genetics, Reykjavik, Iceland).<sup>19</sup> Patients were determined to be SE allele positive if carrying *HLA-DRB1\*01* (except *\*0103*), *\*04*, *\*10*, or *\*14:02*. For 2,735 patients from the Danish and Norwegian cohorts (DANBIO/DRB, ARCTIC, NOR-DMARD, and ULRABIT) *HLA-DRB1 SE alleles* were imputed by SNP2HLA<sup>25</sup> from genetic data for 28 single-nucleotide polymorphisms (SNPs) from the HLA locus from a customized genotyping effort by AmpliSeq on Ion-Torrent 5S XL covering 375 SNPs in collaboration with Thermo Fisher Scientific.

**Monoclonal antibodies.** CCP2+ human monoclonal antibodies were generated by cloning Igs from single cells from patients with RA as previously described.<sup>26–29</sup> They originated from different patients, tissues, and B cell compartments (Supplementary Table 2). All clones were expressed as recombinant human IgG1 in Hek293 Expi cells (Thermo Fisher Scientific).

**Autoantibody measurements.** IgM, IgG, IgA RF and IgG, and IgA anti-CCP2 analyses were performed by fully automated commercial fluoroenzyme immunoassay for in vitro diagnostics (EliA, Phadia AB, Uppsala, Sweden). ACPA fine specificities were screened using a new optimized version of the

previously reported custom-made multiplex solid-phase microarray platform (Thermo Fisher Scientific, ImmunoDiagnostics). The array contained four original peptides with multiple citrulline sites and 15 single-citrulline peptides derived from human fibrinogen (alpha and beta chain), vimentin, tenascin C, and filaggrin; two acetylated histone 4 peptides with naturally occurring sites; and two synthetic filaggrin (cfc1) peptides in which the arginine/citrulline site in the original peptide was replaced by either homocitrulline or acetyl-lysine as well as arginine control peptides. On the array, biotinylated peptides are immobilized by binding to streptavidin (see Supplementary Table 2 for peptide design). For autoantibody serology screenings, fluorescent intensity was normalized to arbitrary units. For each array batch, the cut-off for positivity for each antigen was determined by the 98th percentile for plasma from 235 to 570 healthy controls (Thermo Fisher Scientific). High signal was defined as three times the cut-off for positivity (Supplementary Table 3). In total, 712 healthy controls were analyzed on the array.

**Statistical methods.** Spearman analyses were used for investigation of direct correlations of continuous values. Differences in autoantibody levels between groups were evaluated with Mann-Whitney or Kruskal-Wallis analysis with Dunn correction for multiple comparisons. Differences in frequencies of positive tests were assessed with Fisher exact tests. Logistic regression was used for further multivariate analysis. Statistical analysis was performed using JMP 16 (SAS Institute) and Prism 9 (Graphpad).  $P$  values  $<0.05$  were considered statistically significant. Unsupervised hierarchical clustering was performed using the Ward method.

## RESULTS

**ACPA binding is determined by recognition of single-citrulline motifs.** We developed an optimized antigen array including a panel of single-citrulline peptides for improved capture of different ACPA specificities based on cit-motifs. The array was first validated using a panel of 33 previously published patient-derived CCP2+ monoclonal ACPAs (Supplementary Table 2). Most of the monoclonals bound to several cit-peptides on the array. Certain clones also bound to carbamylated (Carb) and acetylated (Acet) peptides (Supplementary Figure 2). Except for two clones, the few CCP2+ clones that were negative on the array were found to bind other cit-peptides by enzyme-linked immunosorbent assay (Supplementary Figure 2). Importantly, when studying the four peptide families of an original multi-cit peptide and corresponding single-cit derivatives (TNC5, Vim<sub>60–75</sub>, Fib $\alpha$ <sub>36–50</sub>, and Fib $\beta$ <sub>60–74</sub>), the binding preferences for the clones could be determined (Supplementary Figure 2B–E). In most cases, clones that bound to the original peptides recognized only one of the single-cit peptides or only peptides with similar cit-motifs.

Next, we investigated the plasma IgG binding patterns in cross-sectional patients with RA ( $n = 6,907$ ; cohort description in Supplementary Figure 1 and Supplementary Table 1). Binding was specific for citrulline peptides and low/no binding was detected to the arginine (Arg) version of the peptides (Supplementary Table 4). When analyzing patients positive for any citrulline peptides in the families TNC5 ( $n = 3,114$ ; 45%), Vim<sub>60-75</sub> ( $n = 4,591$ ; 66%), Fib $\alpha_{36-50}$  ( $n = 2,645$ ; 38%), or Fib $\beta_{60-74}$  ( $N = 4,474$ ; 65%), we observed patient-specific binding patterns (Figure 1A–D). For example, when detecting binding to the original TNC5 peptide in a patient, a preferential strong binding could be seen for one of the four cit-sites but typically not for all. Interestingly, for the peptide families Vim<sub>60-75</sub> and Fib $\beta_{60-74}$  we could detect a clear dominance of one cit-site, Cit Vim<sub>60-75-cit1</sub> and Cit Fib $\beta_{60-74-cit3}$ . On the other hand, for the TNC5 and Fib $\alpha_{36-50}$  peptide families binding was seen to all sites but differently in different patients.

Several single-cit peptide reactivities displayed significant direct correlation with each other, but this correlation was typically stronger outside of the peptide families. Surprisingly, the strongest correlation was seen by the two peptides with unrelated sequences: Cit Fib $\alpha_{36-50-cit1}$  and Cit Fib $\beta_{60-74-cit2}$  (Supplementary Figure 3). Notably, in several cases one of the single-cit peptides captured significantly more patients than the original multi-cit peptide (ie, Cit Fib $\alpha_{36-50-cit1}$  vs Cit Fib $\alpha_{36-50}$ ; Cit Fib $\beta_{60-74-cit3}$  vs Cit Fib $\beta_{60-74}$ ; and Cit Vim<sub>60-75-cit1</sub> vs Cit Vim<sub>60-75</sub>) (Figure 1 and Supplementary Figure 2B–D).

**Frequencies and overlap between different autoantibody classes and isotypes.** Four single cit-peptides stood out with a high frequency of positivity: Cit Fib $\beta_{36-52}$  (51%), Cit Fib $\beta_{60-74-cit3}$  (59%), Cit Fil<sub>307-324</sub> (54%), and Cit Vim<sub>60-75-cit1</sub> (63%) (Table 1; Figure 2A). When we combined these four peptides that we denote “group A” peptides, they captured 97% of IgG CCP2+ patients and 22% of IgG CCP2–. The other 11 peptides (“group B” peptides) varied in frequency of positivity. Moreover, the ACPA patterns were relatively consistent between RA cohorts included in the study, as seen by dichotomizing patients into early DMARD-naïve patients with RA ( $n = 597$ ) and two cross-sectional replication cohorts as (1) the cross-sectional part of the Swedish cohort from the SRQb ( $n = 3,510$ ) and (2) the cross-sectional part from the Norwegian and Danish cohorts ( $n = 2,800$ ) (Table 1).

Notably, we also observed that 56% of IgG anti-CCP2+ patients (2,646 of 4,723) had antibody reactivity to carbamylated or acetylated peptide epitopes (Table 1). There was a positive correlation between Acet/Carb positivity and the number of detectable ACPA fine specificities (Supplementary Figure 4). Using the Fil<sub>307-324</sub> peptide and replacing the arginine/citrulline residue with acetyl-lysine or homocitrulline further illustrated the overlap between reactivities, with a large proportion of Cit Fil<sub>307-324</sub>–positive patients also being positive for Carb Fil<sub>307-324</sub> or triple

positive for the Cit, Carb, and Acet versions (Supplementary Figure 5). Reactivity to acetylated histone 4, Acet His<sub>41-18 K5</sub>, and Acet His<sub>41-18 K16</sub> was less frequent but substantially overlapped with Carb/Acet flaggrin positivity (Supplementary Figure 6). Although cit-reactivity alone was common, only a few patients was uniquely positive for Carb or Acet without Cit-positivity.

When evaluating the total seropositivity, 76% of the patients with RA were seropositive based on CCP2 IgG and/or RF IgM. Including IgA CCP2 and RF isotypes further increased the size of the seropositive group to 83% ( $n = 5,759$ ). Furthermore, a few additional seropositive individuals were captured when including IgG to Acet/Carb, resulting in the final proportion of 84% ( $n = 5,805$ ) of patients positive for at least one of the investigated markers, which reduced the seronegative group to 16% of all included patients (Figure 3A; Supplementary Table 6). Of CCP2 IgG negative patients, 11% were positive for at least one of the ACPA fine specificities. The cit-reactivity levels in these patients were not necessarily low or just above cut-off. However, it should be emphasized that including multiple peptide tests significantly reduced the specificity as seen by high combined positivity in the analyzed healthy controls, despite high specificity for each individual test ( $n = 712$ ; 22% cit-peptide positivity; Supplementary Table 4). Hence, a more appropriate approach in testing may be to use the high cut-off, which maintained specificity (4.5% positivity in controls) but increased the seropositivity to 79% in combination with CCP2 and/or RF ( $n = 5,460$ ; Supplementary Tables 4 and 6).

In general, there was a large overlap between different ACPA fine specificities. A total of 91% of all patients with RA who were positive for any ACPA fine specificity were positive for two or more peptides. Yet, among patients positive for only one peptide ( $n = 493$ ), the same four group A peptides dominated: Cit Fib $\beta_{36-52}$ , Cit Fib $\beta_{60-74-cit3}$ , Cit Fil<sub>307-324</sub>, and Cit Vim<sub>60-75-cit1</sub> (9%, 13%, 14%, and 15% positives, respectively; Supplementary Figure 7). Notably, many individuals displayed high signals (three times the cut-off) to both group A and group B peptides (Figure 3B–C; Supplementary Table 5). Only a smaller subset (5% of all patients with RA) had high reactivity restricted to the less frequent (group B) peptides (Figure 3C).

Many cit-peptides that are commonly used as antigens for detecting ACPA fine specificities display glycine (gly) residues next to the citrulline, either in the +1 (cit-gly) or –1 (gly-cit) position. Of the 15 single-cit peptides, 4 peptides contained cit-gly or gly-cit motifs and 11 did not (Table 1). Importantly, of the dominating (group A) peptides, two contained gly motifs (Cit Fib $\beta_{36-52}$  and Cit Fil<sub>307-324</sub>) and two did not (Cit Fib $\beta_{60-74-cit3}$  and Cit Vim<sub>60-75-cit1</sub>). There was still a large overlap between these reactivities among patients: 49% had high signal for both gly-motif peptides and non-gly motif peptides. Nevertheless, 5% had high reactivity only to gly-motif peptides, and 15% of all patients had high reactivity to only peptides with non-gly motifs (Figure 3D). Interestingly, the patient group with only gly-peptide ACPAs had significantly

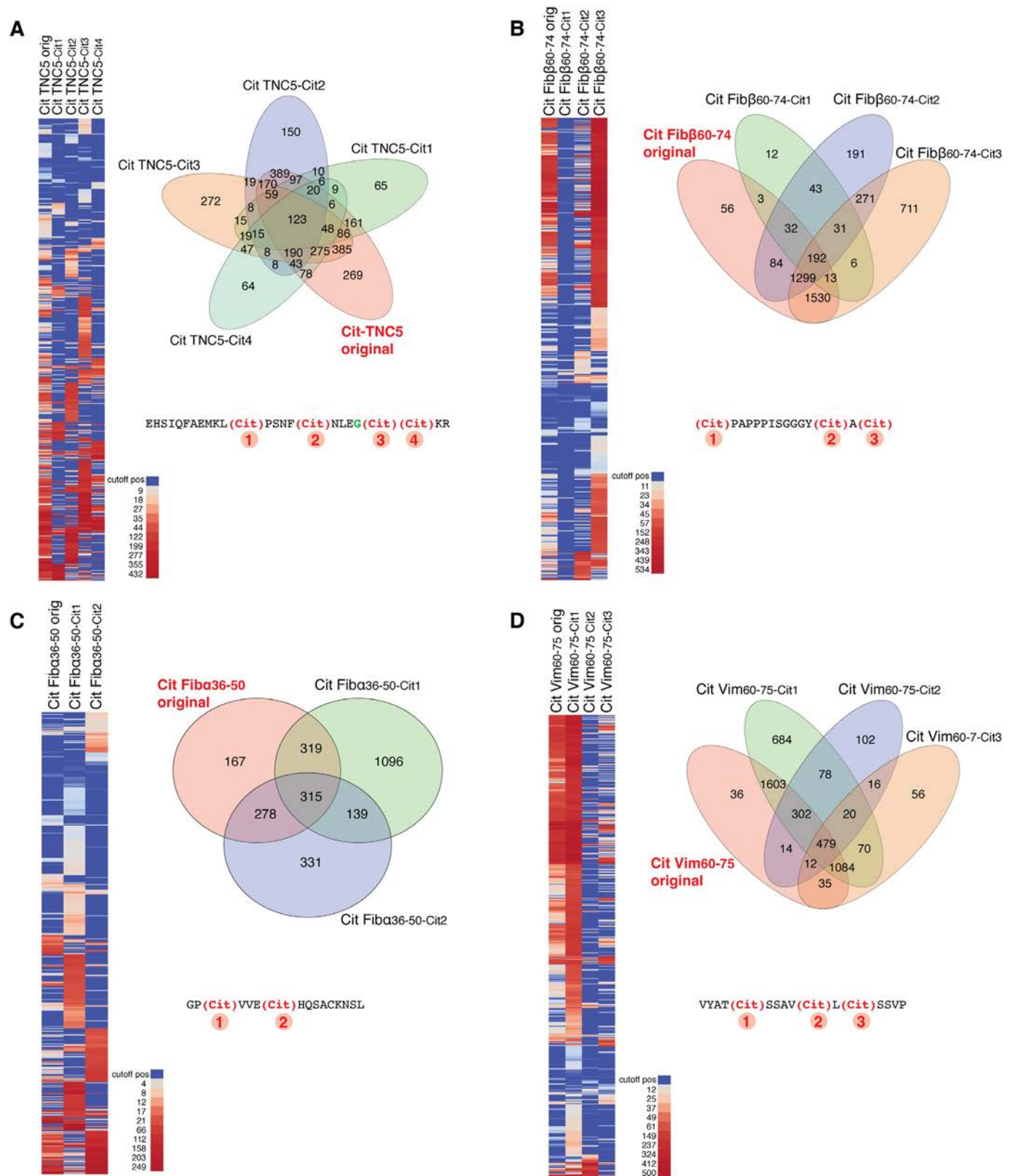


**Figure 1.** Serological anticitrullinated protein antibody patterns reveal the preferential binding of autoantibodies to citrulline motifs. A total of 6,907 patients with rheumatoid arthritis were screened for binding to citrulline-containing peptides. The figure visualizes binding to four peptide families with the original peptide with multiple citrulline and the equivalent single-citrulline peptides. Patients with any positive signal within the families are shown: (A) Cit TNC5 in 3,115 positive patients, (B) Cit Fib $\beta$ <sub>60-74</sub> in 4,474 positive patients, (C) Cit Fiba<sub>36-50</sub> in 2,645 positive patients, and (D) Cit Vim<sub>60-75</sub> in 4,591 positive patients. Heatmaps display positive signals in arbitrary units with the cut-off for positivity in the assays subtracted.

**Table 1.** Autoantibody frequencies in different RA cohorts\*

| Autoantibody assay              | Peptide sequence                                    | Single-cit | Group<br>A/B | Gly motif | Early RA <sup>a</sup><br>(n = 597),<br>n (%) | Cross-sectional 1 <sup>b</sup><br>(n = 3,510), n (%) | P value <sup>c</sup> | Cross-sectional 2 <sup>d</sup><br>(n = 2,800),<br>n (%) | P value <sup>c</sup> | All IgG<br>anti-CCP2-<br>(n = 2,183),<br>n (%) | All IgG anti-<br>CCP2+<br>(n = 4,724),<br>n (%) |
|---------------------------------|-----------------------------------------------------|------------|--------------|-----------|----------------------------------------------|------------------------------------------------------|----------------------|---------------------------------------------------------|----------------------|------------------------------------------------|-------------------------------------------------|
| <b>Cit-peptides</b>             |                                                     |            |              |           |                                              |                                                      |                      |                                                         |                      |                                                |                                                 |
| Cit Flib <sub>36-50</sub>       | GP(cit)WVF(cit)HQACKDSK                             | N          | N/A          | N/A       | 90 (15)                                      | 515 (15)                                             | NS (0.23)            | 474 (17)                                                | NS (0.30)            | 126 (6)                                        | 953 (20)                                        |
| Cit Flib <sub>36-50</sub> -Cit1 | GP(cit)WVF(cit)HQACKNSL                             | Y          | B            | N         | 143 (24)                                     | 931 (27)                                             | NS (0.19)            | 795 (28)                                                | <b>0.03</b>          | 202 (9)                                        | 1,667 (35)                                      |
| Cit Flib <sub>36-50</sub> -Cit2 | GPWV(cit)HQACKNSL                                   | Y          | B            | N         | 83 (14)                                      | 494 (14)                                             | NS (0.95)            | 486 (17)                                                | <b>0.04</b>          | 115 (5)                                        | 948 (20)                                        |
| Cit Flib <sub>36-52</sub>       | NEEGFSA(cit)GHRPLDKK                                | Y          | <b>A</b>     | Y         | 316 (53)                                     | 1,804 (51)                                           | NS (0.51)            | 1,419 (51)                                              | NS (0.32)            | 168 (8)                                        | 3,371 (71)                                      |
| Cit Flib <sub>60-74</sub>       | (cit)PAPPISGGV(cit)A(cit)                           | N          | N/A          | N/A       | 278 (47)                                     | 1,618 (46)                                           | NS (0.86)            | 1,313 (47)                                              | NS (0.89)            | 159 (7)                                        | 3,050 (64)                                      |
| Cit Flib <sub>60-74</sub> -Cit1 | (cit)PAPPISGGVYR                                    | Y          | B            | N         | 26 (4)                                       | 187 (5)                                              | NS (0.37)            | 119 (4)                                                 | NS (0.91)            | 90 (4)                                         | 242 (5)                                         |
| Cit Flib <sub>60-74</sub> -Cit2 | RPAPPISGGV(cit)AR                                   | Y          | B            | N         | 175 (29)                                     | 1,069 (30)                                           | NS (0.60)            | 899 (32)                                                | NS (0.19)            | 159 (7)                                        | 1,984 (42)                                      |
| Cit Flib <sub>60-74</sub> -Cit3 | RPAPPISGGVYR(cit)                                   | Y          | <b>A</b>     | N         | 318 (53)                                     | 2,001 (57)                                           | NS (0.09)            | 1,734 (62)                                              | <b>0.0001</b>        | 185 (8)                                        | 3,868 (82)                                      |
| Cit Flib <sub>307-324</sub>     | HQCHQEST(cit)GRSGRGRSGS                             | Y          | <b>A</b>     | Y         | 312 (52)                                     | 1,904 (54)                                           | NS (0.37)            | 1,533 (55)                                              | NS (0.28)            | 162 (7)                                        | 3,587 (76)                                      |
| Cit TNC1                        | VFLRRKVG(cit)ENFYQNW                                | Y          | B            | Y         | 137 (23)                                     | 723 (21)                                             | NS (0.19)            | 800 (29)                                                | <b>0.006</b>         | 93 (4)                                         | 1,567 (33)                                      |
| Cit TNC5                        | EHSIQFAEMKL(cit)PSNF(cit)NLEG                       | N          | N/A          | N/A       | 208 (35)                                     | 1,044 (30)                                           | <b>0.01</b>          | 1,147 (41)                                              | <b>0.006</b>         | 163 (8)                                        | 2,236 (47)                                      |
| Cit TNC5 <sub>Cit1</sub>        | EHSIQFAEMKL(cit)                                    | Y          | B            | N         | 47 (8)                                       | 380 (11)                                             | <b>0.03</b>          | 320 (12)                                                | <b>0.01</b>          | 95 (4)                                         | 652 (14)                                        |
| Cit TNC5 <sub>Cit2</sub>        | PSNFRNLEGRKR                                        | Y          | B            | N         | 112 (19)                                     | 628 (18)                                             | NS (0.61)            | 575 (21)                                                | NS (0.34)            | 75 (3)                                         | 1,240 (26)                                      |
| Cit TNC5 <sub>Cit3</sub>        | NLEGRKR                                             | Y          | B            | Y         | 171 (29)                                     | 771 (22)                                             | <b>0.0005</b>        | 797 (28)                                                | NS (0.92)            | 92 (4)                                         | 1,647 (35)                                      |
| Cit TNC5 <sub>Cit4</sub>        | EHSIQFAEMKLPSNFRNLEGR(cit)                          | Y          | B            | N         | 102 (17)                                     | 456 (13)                                             | <b>0.008</b>         | 401 (14)                                                | NS (0.09)            | 59 (3)                                         | 900 (19)                                        |
| Cit Vim <sub>60-75</sub>        | WVAT(cit)SSAV(cit)LC(cit)SSVP                       | N          | N/A          | N/A       | 297 (50)                                     | 1,670 (48)                                           | NS (0.33)            | 1,598 (57)                                              | <b>0.001</b>         | 124 (6)                                        | 3,441 (73)                                      |
| Cit Vim <sub>60-75</sub> -Cit1  | WVAT(cit)SSAVLRSSVP                                 | Y          | <b>A</b>     | N         | 342 (57)                                     | 2,186 (62)                                           | <b>0.03</b>          | 1,792 (64)                                              | <b>0.002</b>         | 246 (11)                                       | 4,074 (86)                                      |
| Cit Vim <sub>60-75</sub> -Cit2  | WVATSSAV(cit)LRSSVP                                 | Y          | B            | N         | 73 (12)                                      | 450 (13)                                             | NS (0.74)            | 500 (18)                                                | <b>0.0007</b>        | 91 (4)                                         | 932 (20)                                        |
| Cit Vim <sub>60-75</sub> -Cit3  | WVATSSAVLR(cit)SSVP                                 | Y          | B            | N         | 140 (23)                                     | 786 (22)                                             | NS (0.56)            | 846 (30)                                                | <b>0.0009</b>        | 77 (4)                                         | 1,695 (36)                                      |
| <b>Acet/Carb-peptides</b>       |                                                     |            |              |           |                                              |                                                      |                      |                                                         |                      |                                                |                                                 |
| Carb Flib <sub>307-324</sub>    | HQCHQEST(carb)GRSGRGRSGS                            | N/A        | N/A          | N/A       | 192 (32)                                     | 1,045 (30)                                           | NS (0.25)            | 1,084 (39)                                              | <b>0.003</b>         | 148 (7)                                        | 2,173 (46)                                      |
| Acet Flib <sub>307-324</sub>    | HQCHQEST(acet)GRSGRGRSGS                            | N/A        | N/A          | N/A       | 168 (28)                                     | 891 (25)                                             | NS (0.16)            | 903 (32)                                                | NS (0.05)            | 83 (4)                                         | 1,879 (40)                                      |
| Acet His <sub>41-18</sub> K5    | SGRG(acet)                                          | N/A        | N/A          | N/A       | 45 (7.5)                                     | 276 (7.9)                                            | NS (0.87)            | 295 (11)                                                | <b>0.03</b>          | 84 (4)                                         | 532 (11)                                        |
| Acet His <sub>41-18</sub> K16   | GGKGLGKGGAKRHRK/L<br>SGRGKGGKGLGKGG(acet)<br>RHRKVL | N/A        | N/A          | N/A       | 55 (9.2)                                     | 334 (9.5)                                            | NS (0.88)            | 344 (12)                                                | <b>0.04</b>          | 92 (4)                                         | 641 (14)                                        |
| <b>CCP2</b>                     |                                                     |            |              |           |                                              |                                                      |                      |                                                         |                      |                                                |                                                 |
| IgG anti-CCP2                   | -                                                   | -          |              |           | 385 (64)                                     | 2,437 (69)                                           | <b>0.02</b>          | 1,902 (68)                                              | NS (0.11)            | N/A                                            | N/A                                             |
| IgA anti-CCP2                   | -                                                   | -          |              |           | 174 (29)                                     | 1,065 (30)                                           | NS (0.59)            | 873 (31)                                                | NS (0.35)            | 24 (1)                                         | 2,088 (44)                                      |
| <b>RF</b>                       |                                                     |            |              |           |                                              |                                                      |                      |                                                         |                      |                                                |                                                 |
| IgM RF                          | -                                                   | -          |              |           | 371 (62)                                     | 2,211 (63)                                           | NS (0.71)            | 1,851 (66)                                              | NS (0.07)            | 497 (23)                                       | 3,936 (83)                                      |
| IgA RF                          | -                                                   | -          |              |           | 237 (39)                                     | 1,140 (32)                                           | <b>0.0007</b>        | 936 (33)                                                | <b>0.004</b>         | 208 (9.5)                                      | 2,105 (44)                                      |
| IgG RF                          | -                                                   | -          |              |           | 147 (25)                                     | 499 (14)                                             | <b>&lt;0.0001</b>    | 483 (17)                                                | <b>&lt;0.0001</b>    | 78 (3.6)                                       | 1,051 (22)                                      |

\* P values <0.05 are significant and are marked with bold font. CCP2, cyclic citrullinated peptide; N, no; N/A, not available; NS, not significant; RA, rheumatoid arthritis; RF, rheumatoid factor; Y, yes.

<sup>a</sup> Early disease-modifying antirheumatic drug-naïve patients with RA from the Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen cohort and Swedish Rheumatology Quality registry biobank with <90 days after diagnosis.

<sup>b</sup> Swedish Rheumatology Quality registry biobank (excluding patients included in the "early RA" dataset).

<sup>c</sup> Fisher's exact tests comparing frequencies with the disease-modifying antirheumatic drug-naïve early RA cohort IgG reactivity with Cit/Carb/Acet-peptides were measured with antigen microarray. CCP2 and RF were measured by fluorescent enzyme immunoassay.

<sup>d</sup> Danish and Norwegian patients (excluding Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen patients included in the "early RA" dataset).

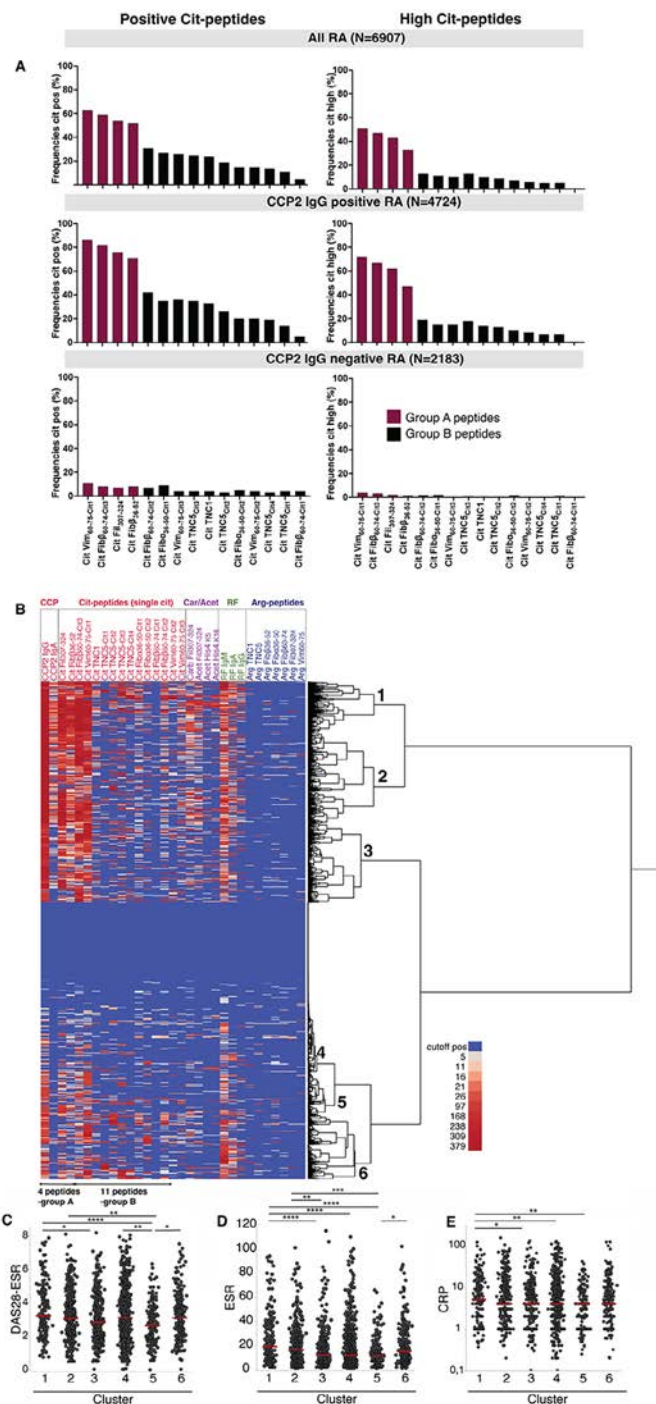


**Figure 2.** Autoantibody profiles in RA. (A) Frequencies of anticitrullinated protein antibody binding to different cit-peptides. Frequencies of positive cit-peptide signals (left) and high signals (right) in all patients with RA (top), IgG anti-CCP2-positive patients (middle), or IgG anti-CCP2-negative patients (low). “Group A peptides” with the highest frequency of positivity are highlighted in red. The cut-off for positivity was based on the 98th percentile of healthy controls. The cut-off for high signal was set to three times the positive cut-off. (B) Unsupervised hierarchical clustering illustrates patient subsets based on autoantibody reactivity patterns. Clustering by Ward method is shown for positive antibody signals in arbitrary units with the cut-off for positivity in the assays subtracted. The data include RF (IgM, IgA, and IgG), CCP2 (IgG and IgA), four peptides with carbamylated or acetylated sites (Carb/Acet), 15 peptides with single-citrulline sites, and seven arginine control peptides. Six distinct patient clusters could be identified as indicated. (C–E) Disease activity by DAS28, ESR (mm/h) and CRP levels (mg/L) by patient autoantibody profile cluster, including patients from the Swedish Rheumatology Quality registry biobank with available clinical data  $\pm 14$  days from sampling. Statistical analysis with Kruskal-Wallis test with Dunn correction of multiple comparison. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . CCP2, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28-ESR, Disease Activity Score 28 with erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; RF, rheumatoid factor.

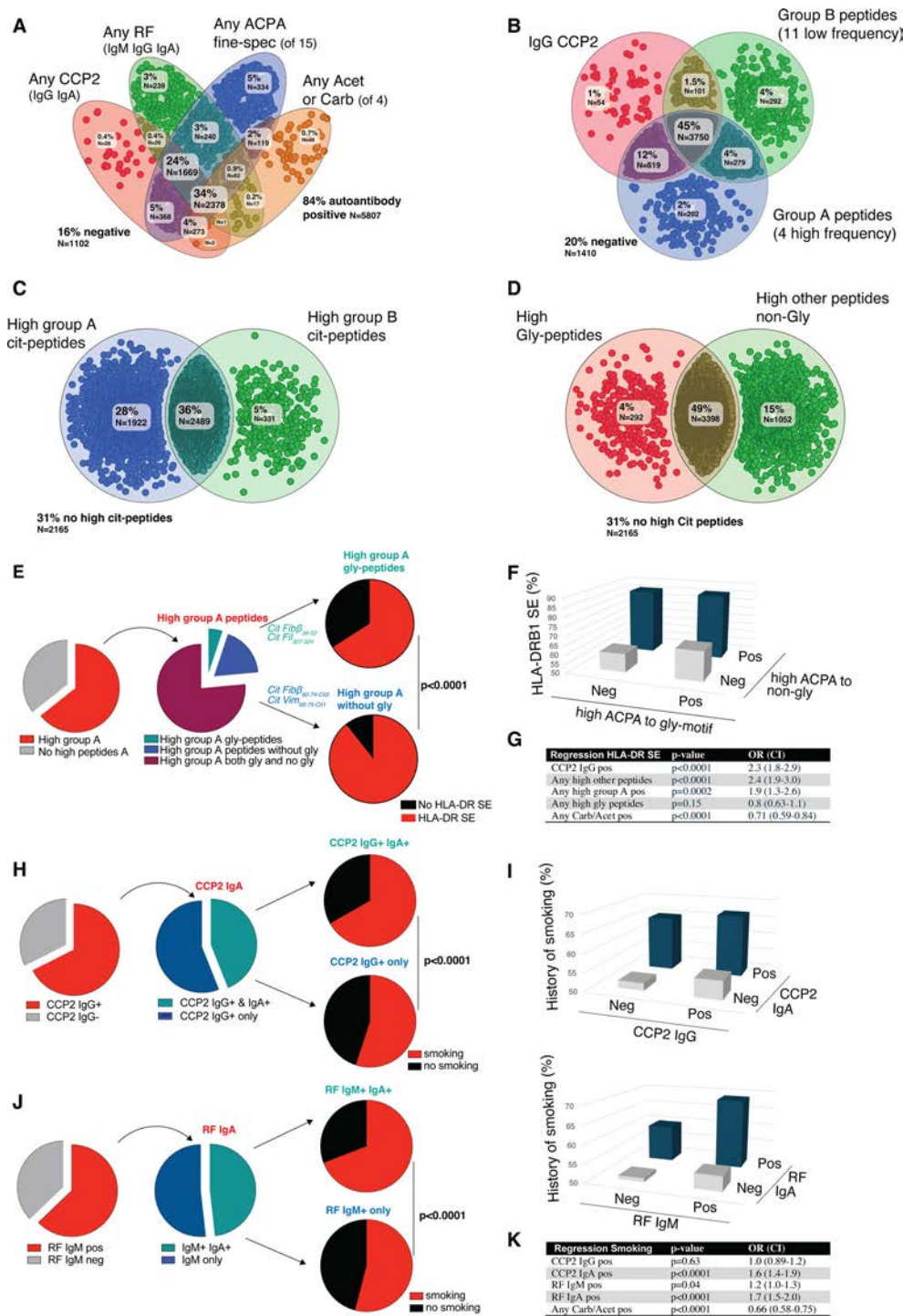


**Figure 3.** Autoantibody subset and association with rheumatoid arthritis risk factors. Data is shown for 6,907 patients with rheumatoid arthritis. (A) Overlap between anti-CCP2 (IgG and IgA), RF (IgM, IgA and IgG), 15 ACPA fine specificities using single-citrulline peptides, and four antihomocitrulline (Carb) and anti-acetyl-lysine (Acet) reactivities. (B) Overlap between IgG anti-CCP2, four high-frequency ACPAs (group A peptides), and 11 low-frequency ACPAs (group B). (C) Overlap between group A and group B ACPAs. (D) ACPA reactivity to peptides with glycine motifs (cit-gly and gly-cit) compared with 11 peptides without glycine. (E) Analysis of HLA-DRB1 SE allele frequency based on high group A reactivity and reactivity to only gly-motif peptides (Cit Fib $\beta_{36-52}$  and Cit Fil $_{307-324}$ ) versus other nonglycine peptides (Cit Fib $\beta_{60-74}$ -Cit $\alpha_{38}$  and Cit Vim $_{60-75}$ -Cit $\alpha_{11}$ ). (F) Total frequency of HLA-DRB1 SE in patients based on high cit-motif reactivity (n = 5,387 with HLA data). (H and J) History of smoking in CCP2 IgG+ IgA+ versus CCP2 IgG+ only and RF IgM+ IgA+ versus RF IgM+ only, respectively. (I) Smoking in patients based on CCP2 IgG/IgA positivity or RF IgM/IgA positivity (n = 3,112 with smoking data). (G and K) Logistic regression analysis for (G) HLA-DRB1 SE or (K) smoking with parameters as categorical values. ACPA, anticitrullinated protein antibody; CCP2, cyclic citrullinated peptide; CI, confidence interval; OR, odds ratio (yes/no); RF, rheumatoid factor; SE, shared epitope.

higher CCP2 levels (mean  $\pm$  SD:  $187 \pm 204$ ; median 98 vs  $102 \pm 135$ ; median 46 AU/mL) (Supplementary Figure 8), but the average peptide signals on the array was slightly lower for gly-motif peptides (Supplementary Figure 9).

**Patient clusters based on autoantibody profiles show differences in ACPA composition and disease activity.**

Unsupervised clustering with the Ward method including the 15 single-citrulline peptides, all isotypes of CCP2 and RF, and the homocitrulline and acetyl-lysine peptides illustrates the complex autoantibody profiles in the patients (Figure 2B; Table 2). Six main clusters of patients were identified, of which two captured patients with very high CCP2 IgG levels (clusters 1–2), one with moderate-to-high antibody levels (cluster 3), two with lower levels (clusters 5–6), and one cluster captured the seronegative group (cluster 4). The clusters also differed in CCP2 IgA positivity, Carb/Acet positivity, RF reactivity, and the proportion of different peptide subsets as well as HLA-DRB1 and smoking (Table 2; Supplementary Table 7). The cohort origin did not seem to affect the clustering (Supplementary Table 7). Notably, the patient subsets defined by the six clusters displayed significant differences in disease activity and inflammation by Disease Activity Score 28 (DAS28), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Tender Joint Count (TJC), and Swollen Joint Count (SJC) but not in the Patient Global Assessment (PGA) or in reported pain. Of the individual components, ESR and TJC28 remained statistically significant across the clusters when adjusting for sex, age, and early DMARD-naïve disease (Table 2). The subsets with moderate antibody levels had the lowest disease activity (cluster 5, average DAS28 = 2.8), and patients displayed higher RA disease activity in the high antibody cluster (cluster 1, average DAS28 = 3.6) as well as the seronegative cluster (cluster 4, average DAS28 = 3.3) (Figure 2C–E). No associations between comorbidities and antibody profiles were observed (Supplementary Table 8).

Importantly, the composition of ACPA fine specificities based on recognized cit-motifs was significantly different between the antibody-positive clusters. Regarding the ACPAs targeting different cit-motifs, we noted that even if clusters 1 and 2 had high multireactivity, the signals to the positive glycine-containing peptides were higher in cluster 2. The moderate antibody clusters 5 and 6 had a higher proportion of non-glycine cit-motif ACPA compared with others, but there were also differences in signal intensity for positive peptides between these two clusters so that glycine cit-motif signals were higher in cluster 5. In regression analysis, we found that having high reactivity to glycine cit-peptides or high reactivity to non-glycine cit-peptides independently correlated with patient clusters also when adjusting for CCP2 IgG levels, RF IgM levels, number of ACPA fine specificities, sex, and age. When instead dichotomizing the patients into four subgroups based only on ACPA fine specificity patterns of peptides with and without glycine cit-motifs (Figure 3), we found that

the double positive group (gly and non-gly ACPA) displayed the highest disease activity by DAS28 followed by the gly-motif only group (Supplementary Figure 10 and Supplementary Table 9).

**HLA-DRB1 SE alleles are more strongly associated with non-glycine cit-peptide ACPA specificities.**

The frequency of presence of SE allele positivity among patients with RA was 77% (Table 3). As expected, we observed a strong association between SE alleles and CCP2 IgG positivity, with 85% of CCP2 IgG+ patients carrying SE alleles (odds ratio [OR] = 3.9 95% confidence interval [CI] 3.4–4.5). There was a dose-dependent relationship in which patients with two copies of SE alleles had higher CCP2 IgG levels and a higher number of ACPA fine specificities (Supplementary Figure 11). RF and Carb/Acet reactivities were also associated with a higher frequency of SE alleles but had a lower OR (RF IgM OR = 2.2 95% CI 1.9–2.5; anti-Carb/Acet OR = 1.6 95% CI 1.4–1.8). However, if the patients were first stratified for CCP2 IgG positivity, so that CCP2 IgG+ patients with and without additional Carb or Acet reactivity were compared, there was no statistically significant association of Carb/Acet with SE alleles (Table 3), as most patients were concordant for SE and CCP2 IgG. Yet, patients that were CCP2 IgG negative but had high cit-peptide reactivity ( $n = 223$ ) had a modest but significant increase in HLA SE allele positivity (69% vs 57%;  $P = 0.002$ ; OR = 1.6 95% CI 1.2–2.2), which was diminished when excluding RF IgM positives (OR = 1.1 95% CI 0.75–1.7).

Importantly, there were striking differences in patient subsets based on ACPA fine specificities to glycine motif peptides or non-glycine ACPA in terms of association with SE alleles with the OR for non-gly motif ACPA positivity being twice as high as for ACPAs binding gly-motif peptides. To avoid bias by low-frequency ACPA fine specificities, we next compared patients with high group A peptide reactivity but with either only high non-gly motif ACPAs or only high gly-motif ACPA reactivity, and we found a significant difference in SE allele frequency in both the SRQb dataset (90% vs 62%; OR = 4.6, 95% CI 2.6–7.9) and the independent replication dataset from Danish and Norwegian patients (89% vs 67%; OR = 4.1, 95% CI 2.3–7.4). There was no difference in RF positivity between these groups. When the cohorts were combined, dichotomized for CCP2 IgG positivity, and all peptides (A and B) included in the analysis, the frequency of SE was 90% in CCP2 IgG+ patients with high non-gly motif ACPA versus 67% in CCP2 IgG+ patients with only high gly-motif ACPA (OR = 4.5, 95% CI 1.9–11.0;  $P = 0.0005$ ). Regression analysis confirmed that high ACPA reactivity to gly-motif peptides did not associate with SE alleles in contrast with high ACPA to non-gly motif peptides (Figure 3).

**Glycine motif ACPA are associated with a history of smoking.**

We also investigated ACPA fine specificity in relation to smoking. Patient-reported smoking status was available for 5,530 patients, showing a 58% overall history of cigarette

**Table 2.** Autoantibody distribution in different patient clusters\*

|                                                  | Cluster 1<br>(n = 692) | Cluster 2<br>(n = 1,265) | Cluster 3<br>(n = 1,100) | Cluster 4<br>(n = 2,361)      | Cluster 5 (n = 580)    | Cluster 6 (n = 905)    |
|--------------------------------------------------|------------------------|--------------------------|--------------------------|-------------------------------|------------------------|------------------------|
| <b>Autoantibodies</b>                            |                        |                          |                          |                               |                        |                        |
| CCP2 IgG levels, mean ± SD                       | 560 ± 104              | 575 ± 59                 | 245 ± 118                | 6 ± 12                        | 67 ± 45                | 82 ± 78                |
| CCP2 IgG, n (%)                                  | 692 (100)              | 1,265 (100)              | 1,090 (99)               | 272 (16)                      | 520 (90)               | 782 (86)               |
| CCP2 IgA levels, mean ± SD                       | 43 ± 28                | 34 ± 27                  | 12 ± 16                  | 2.2 ± 3.8                     | 5.2 ± 9.2              | 6.7 ± 11               |
| CCP2 IgA, n (%)                                  | 597 (86)               | 978 (77)                 | 332 (30)                 | 35 (1.5)                      | 53 (9)                 | 116 (13)               |
| RF IgM levels, mean ± SD                         | 164 ± 191              | 77 ± 100                 | 48 ± 69                  | 5.7 ± 11                      | 27 ± 38                | 93 ± 107               |
| RF IgM, n (%)                                    | 658 (95)               | 1,124 (89)               | 892 (81)                 | 559 (24)                      | 404 (70)               | 795 (88)               |
| RF IgG levels, mean ± SD                         | 65 ± 102               | 35 ± 42                  | 24 ± 34                  | 7.9 ± 15                      | 16 ± 21                | 40 ± 70                |
| RF IgG, n (%)                                    | 275 (40)               | 335 (26)                 | 157 (14)                 | 68 (2.9)                      | 55 (9.5)               | 239 (26)               |
| RF IgA levels, mean ± SD                         | 41 ± 28                | 30 ± 26                  | 24 ± 24                  | 7.9 ± 13                      | 18 ± 20                | 31 ± 27                |
| RF IgA, n (%)                                    | 452 (65)               | 641 (51)                 | 405 (37)                 | 191 (8.1)                     | 163 (28)               | 459 (51)               |
| Carb/Acet, n (%)                                 | 623 (90)               | 935 (74)                 | 573 (52)                 | 280 (12)                      | 184 (32)               | 303 (33)               |
| High group A only, n (%)                         | 165 (25)               | 572 (45)                 | 550 (50)                 | 161 (7)                       | 325 (56)               | 148 (16)               |
| High group B only, n (%)                         | 1 (0.1)                | 14 (1)                   | 14 (1)                   | 135 (6)                       | 46 (8)                 | 121 (13)               |
| High both A and B peptides, n (%)                | 527 (76)               | 674 (53)                 | 509 (46)                 | 71 (3)                        | 178 (31)               | 529 (58)               |
| High gly-peptides only, n (%)                    | 1 (0.1)                | 43 (3)                   | 80 (7)                   | 62 (3)                        | 79 (13)                | 26 (3)                 |
| High non-gly-peptides only, n (%)                | 3 (0.4)                | 56 (4)                   | 186 (17)                 | 247 (10)                      | 213 (37)               | 347 (38)               |
| High both gly and non-gly, n (%)                 | 688 (99)               | 1,161 (92)               | 807 (73)                 | 58 (3)                        | 257/580 (44)           | 425 (47)               |
| Ratio gly signal/non-gly, mean ± SD <sup>a</sup> | 1.1 ± 0.43             | 2.7 ± 0.32               | 2.4 ± 1.7                | 2.9 ± 0.60                    | 5.2 ± 0.53             | 1.1 ± 0.44             |
| Cit Flb <sub>36-52</sub> , n (%)                 | 607 (88)               | 932 (74)                 | 478 (43)                 | 76 (1.7)                      | 76 (13)                | 128 (14)               |
| Cit Fl <sub>307-324</sub> , n (%)                | 663 (96)               | 1,098 (87)               | 697 (63)                 | 51 (2.1)                      | 217 (37)               | 265 (29)               |
| Cit Flb <sub>60-74-Cit3</sub> , n (%)            | 665 (96)               | 1,097 (87)               | 830 (75)                 | 95 (4)                        | 245 (42)               | 317 (35)               |
| Cit Vim <sub>60-75-Cit1</sub> , n (%)            | 669 (97)               | 1,024 (81)               | 798 (73)                 | 102 (4.3)                     | 328 (57)               | 585 (65)               |
| Number cit-peptide+<br>(of 15), mean ± SD        | 8.7 ± 2.6              | 6.6 ± 2.4                | 5.7 ± 2.4                | 1.0 ± 1.8                     | 4.2 ± 2.2              | 5.3 ± 3.0              |
| <b>Summary autoantibodies</b>                    |                        |                          |                          |                               |                        |                        |
| High CCP2 IgG                                    | High CCP2 IgG          | High CCP2 IgG            | Moderate-high CCP2 IgG   | Low antibody/<br>seronegative | Low CCP2 IgG           | Moderate-low CCP2 IgG  |
| High CCP2 IgA                                    | High CCP2 IgA          | High CCP2 IgA            | Moderate-low CCP2 IgA    | –                             | Low CCP2 IgA           | Low CCP2 IgA           |
| High cit-pep                                     | Moderate-high cit-pep  | Moderate-high cit-pep    | Moderate cit-pep         | –                             | Moderate cit-pep       | Moderate cit-pep       |
| A and B; gly and non-gly                         | A>B; gly and non-gly   | A>B; gly and non-gly     | A>B; gly and non-gly     | –                             | A>B; gly < non-gly     | A and B; gly < non-gly |
| High RF                                          | High RF                | Moderate RF              | Moderate RF              | –                             | Moderate-low RF        | High RF                |
| High Carb/Acet                                   | Moderate Carb/Acet     | Moderate Carb/Acet       | Moderate Carb/Acet       | –                             | Moderate-low Acet/Carb | Moderate-low Acet/Carb |
| DAS28-ESR, mean ± SD [n]                         | 3.59 ± 1.6 [171]       | 3.26 ± 1.4 [371]         | 3.10 ± 1.5 [311]         | 3.30 ± 1.6 [660]              | 2.75 ± 1.3 [158]       | 3.2 ± 1.4 [227]        |
| PGA, mean ± SD [n]                               | 37 ± 27 [188]          | 34 ± 27 [410]            | 32 ± 26 [350]            | 36 ± 27 [735]                 | 34 ± 24 [34]           | 33 ± 26 [251]          |
| SJC28, mean ± SD [n]                             | 3.0 ± 4.0 [183]        | 2.4 ± 3.2 [406]          | 2.1 ± 3.6 [344]          | 2.6 ± 4.3 [730]               | 2.2 ± 3.6 [170]        | 2.2 ± 3.6 [253]        |
| TJC28, mean ± SD [n]                             | 3.4 ± 5.0 [182]        | 2.6 ± 4.2 [406]          | 2.9 ± 4.3 [344]          | 3.5 ± 5.0 [730]               | 1.7 ± 2.8 [170]        | 2.6 ± 3.7 [253]        |
| ESR, mean ± SD [n]                               | 25 ± 21 [181]          | 21 ± 17 [388]            | 17 ± 17 [334]            | 18 ± 18 [697]                 | 15 ± 13 [164]          | 20 ± 17 [238]          |
| CRP, mean ± SD [n]                               | 11 ± 16 [186]          | 9.7 ± 16 [409]           | 8.0 ± 14 [352]           | 9.1 ± 17 [740]                | 5.5 ± 6.6 [174]        | 8.4 ± 15 [250]         |
| Pain, mean ± SD [n]                              | 37 ± 27 [186]          | 34 ± 26 [407]            | 34 ± 28 [350]            | 37 ± 28 [730]                 | 32 ± 23 [174]          | 34 ± 27 [251]          |

\* CCP2, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28-ESR, Disease Activity Score 28 with erythrocyte sedimentation rate; PGA, Physician Global Assessment; RF, rheumatoid factor; SJC28, Swollen Joint Count 28; TJC28, Tender Joint Count 28.

<sup>a</sup> Ratio of the average array signal for positive gly-motif peptides/average signal for positive non-gly-peptides. Only positive signals above cut-off were included in the calculation.

<sup>b</sup> Clinical parameter from the time of blood sampling ±14 days available in the Swedish Rheumatology Quality registry biobank (SRQb).

**Table 3.** Association of autoantibody subsets with smoking and HLA-DRB1 SE alleles\*

| Subgroup                   | Smoking          |                      |               | HLA-DRB1 SE                                             |                      |               |                                              |                       |
|----------------------------|------------------|----------------------|---------------|---------------------------------------------------------|----------------------|---------------|----------------------------------------------|-----------------------|
|                            | Smoking, n (%)   | P value <sup>a</sup> | OR [95% CI]   | Swedish SE+<br>(77%, 2,055/2,652) <sup>b</sup><br>n (%) | P value <sup>a</sup> | OR [95% CI]   | SE+ (77%, 2,096/2,735) <sup>c</sup><br>n (%) | OR [95% CI]           |
| CCP2 IgG pos               | 2,247/3,737 (60) | <b>&lt;0.0001</b>    | 1.4 [1.2-1.5] | 1,569/1,832 (85)                                        | <b>&lt;0.0001</b>    | 3.6 [3.0-4.4] | 1,588/1,870 (85)                             | 3.9 [3.3-4.8]         |
| CCP2 IgG neg               | 939/1,793 (52)   | -                    | -             | 509/820 (62)                                            | -                    | -             | 508/865 (59)                                 | -                     |
| Any CCP2                   | 2,260/3,757 (60) | <b>&lt;0.0001</b>    | 1.4 [1.2-1.5] | 1,572/1,838 (85)                                        | <b>&lt;0.0001</b>    | 3.6 [2.9-4.4] | 1,596/1,881 (85)                             | 4.0 [3.3-4.8]         |
| CCP2 neg                   | 926/1,773 (52)   | -                    | -             | 506/814 (62)                                            | -                    | -             | 500/854 (59)                                 | -                     |
| Any RF                     | 2,225/3,632 (61) | <b>&lt;0.0001</b>    | 1.5 [1.4-1.7] | 1,458/1,762 (83)                                        | <b>&lt;0.0001</b>    | 2.1 [1.7-2.5] | 1,539/1,894 (81)                             | 2.2 [1.8-2.7]         |
| RF neg                     | 961/1,898 (51)   | -                    | -             | 620/890 (70)                                            | -                    | -             | 557/841 (66)                                 | -                     |
| CCP2 IgG+ IgA              | 1,096/1,643 (67) | <b>&lt;0.0001</b>    | 1.6 [1.4-1.9] | 704/829 (85)                                            | NS (0.74)            | -             | 726/853 (85)                                 | NS (0.85)             |
| CCP2 IgG+ only             | 1,151/2,094 (55) | -                    | -             | 858/1,003 (86)                                          | -                    | -             | 862/1,017 (85)                               | -                     |
| RF IgM+ IgA                | 1,159/1,677 (69) | <b>&lt;0.0001</b>    | 1.9 [1.6-2.2] | 679/832 (81)                                            | NS (0.37)            | -             | 715/906 (79)                                 | 0.03 0.69 [0.54-0.88] |
| RF IgM only                | 972/1,800 (54)   | -                    | -             | 697/836 (84)                                            | -                    | -             | 783/928 (84)                                 | -                     |
| Any Carb/Acet              | 1,326/2,326 (57) | NS (0.44)            | -             | 836/1,026 (82)                                          | <b>&lt;0.0001</b>    | 1.5 [1.2-1.8] | 1,029/1,258 (82)                             | -                     |
| No Carb/Acet               | 1,860/3,204 (58) | -                    | -             | 1,219/1,626 (75)                                        | -                    | -             | 1,067/1,476 (72)                             | -                     |
| CCP2 IgG, any Carb/Acet    | 1,224/2,115 (57) | <b>0.001</b>         | 0.8 [0.7-0.9] | 781/930 (84)                                            | NS (0.13)            | -             | 973/1,160 (84)                               | NS (0.11)             |
| CCP2 IgG, no Carb/Acet     | 1,023/1,622 (63) | -                    | -             | 781/902 (87)                                            | -                    | -             | 615/710 (87)                                 | -                     |
| Peptide A high             | 2,102/3,494 (60) | <b>&lt;0.0001</b>    | 1.3 [1.2-1.5] | 1,429/1,653 (86)                                        | <b>&lt;0.0001</b>    | 3.8 [3.1-4.6] | 1,543/1,812 (85)                             | 3.8 [3.1-4.6]         |
| No peptide A high          | 1,084/2,036 (53) | -                    | -             | 626/999 (63)                                            | -                    | -             | 553/923 (60)                                 | -                     |
| Gly-peptide high           | 1,776/2,898 (61) | <b>&lt;0.0001</b>    | 1.4 [1.2-1.5] | 1,149/1,353 (85)                                        | <b>&lt;0.0001</b>    | 2.4 [2.0-2.9] | 1,294/1,536 (84)                             | 2.6 [2.2-3.2]         |
| No gly-peptide high        | 1,410/2,632 (54) | -                    | -             | 906/1,299 (70)                                          | -                    | -             | 802/1,199 (67)                               | -                     |
| Non-gly-peptide high       | 2,116/3,541 (60) | <b>&lt;0.0001</b>    | 1.3 [1.1-1.4] | 1,462/1,687 (87)                                        | <b>&lt;0.0001</b>    | 4.1 [3.4-4.9] | 1,552/1,823 (85)                             | 3.9 [3.2-4.7]         |
| No non-gly high            | 1,070/1,989 (54) | -                    | -             | 593/965 (62)                                            | -                    | -             | 544/912 (62)                                 | -                     |
| Group A high, non-gly only | 371/667 (56)     | <b>0.03</b>          | 1.5 [1-2.1]   | 300/335 (90)                                            | <b>&lt;0.0001</b>    | 4.6 [2.6-7.9] | 272/305 (89)                                 | 4.1 [2.3-7.4]         |
| Group A high, Gly only     | 119/184 (65)     | -                    | -             | 62/95 (65)                                              | -                    | -             | 54/81 (67)                                   | -                     |

\* Smoking status was available in the Swedish Rheumatology Quality registry biobank (SRQb) and Danish Nationwide Clinical Rheumatology Registry/Danish Rheumatologic Biobank (DANBIO/DRB). P values <0.05 are significant and are marked with bold font. CCP2, cyclic citrullinated peptide; CI, confidence interval; NS, not significant; OR, odds ratio; RF, rheumatoid factor; SE, shared epitope.

<sup>a</sup> P value and OR are from Fisher exact tests.

<sup>b</sup> SEs by Illumina Global Screening Array (deCODE Genetics).

<sup>c</sup> For the Danish Nationwide Clinical Rheumatology Registry/Danish Rheumatologic Biobank (DANBIO/DRB), Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen (ARCTIC), Norwegian Antirheumatic Drug Register (NOR-DMARD), and Ultrasound in Rheumatoid Arthritis patients starting Biologic Treatment (ULRABIT), SE by Thermo Fisher Scientific customized genotyping.

smoking including both previous and current smokers. Although there was a significant association of CCP2 IgG positivity as well as group A cit-peptide positivity with smoking, it was rather modest. Intriguingly, patients with only high gly-motif ACPAs (including both A and B peptides) had a slightly higher frequency of a smoking than patients with only high non-gly motif ACPAs (65% vs 56%; OR = 1.4, 95% CI 1–2.0;  $P = 0.02$ ).

We also used IgA positivity as a comparison in the analysis to ascertain the robustness of the smoking data. Indeed, we could validate a significant association between a history of cigarette smoking and IgA positivity, in line with previous reports.<sup>7,30</sup> This was particularly striking when dichotomizing patients based on CCP2 IgA+ IgG+ double positivity compared with CCP2 IgG alone in which 67% versus 55% of the patients had a history of smoking, respectively (Table 3). A similar association was found for IgA RF. Here, we dichotomized RF IgM+ IgA+ double positivity and compared it with RF IgM+, for which 69% versus 54% had a history of smoking. Logistic regression analysis showed an independent association for positivity of CCP2 IgA (OR = 1.6, 95% CI 1.4–1.9) and RF IgA (OR = 1.7 95% CI 1.5–2.0) to smoking status (Figure 3).

## DISCUSSION

In this comprehensive serologic effort, we provide a granular profiling of autoantibodies in RA and demonstrate that ACPAs have preferential binding to small citrulline-containing epitopes, which is reflected in differential binding to single-citrulline peptides originating from the same peptide backbone. Our harmonized dataset from 6,900 patients enabled us to investigate the association of different ACPA subsets with RA risk factors. Strikingly, we found significant differences in HLA-DR SE allele association with fine specificities targeting nonglycine compared with glycine peptide epitopes. We could confirm an association of IgA autoantibody positivity with a history of cigarette smoking.<sup>7,30,31</sup> The observed associations may provide insights into how exposure to different classes of citrullinated antigen drives specific ACPA reactivity and the T cell dependence of such responses.

A complete lack of HLA association would indicate T cell-independent responses, and the low association for glycine-containing peptides, could implicate differences in T cell dependence in epitopes spreading between different ACPA responses. In line with our data is a report of difference in HLA SE allele association for ACPAs targeting a peptide derived from vimentin (no glycine peptide) compared with a peptide from fibrinogen (citrulline-glycine motif peptide),<sup>32</sup> but at the time this was not attributed to glycine motifs. Glycine, a small nonpolar amino acid, is often found in flexible or disordered protein regions. Cit-gly motifs are common in identified ACPA antigens,<sup>33</sup> including in the filaggrin cfc1 peptide. Furthermore, large unbiased peptide array screenings have confirmed a high prevalence of glycine adjacent to the citrulline in ACPA targets.<sup>6,28</sup> Interestingly, arg-gly motifs have

also been suggested to be preferential substrates for protein arginine deiminase 2 (PAD2) and PAD4 enzymes.<sup>34,35</sup> However, it remains to be elucidated if cit-gly citrullination is more dominating during physiologic conditions compared with during RA-associated hypercitrullination. Because T cell and B cell epitopes generally do not overlap, it is difficult to directly extrapolate the implications for antigen processing, presentation, and T cell tolerance. Moreover, further genetic association analysis is needed to determine if the gly-motif ACPAs may have an association with other major histocompatibility complex class II HLA alleles outside of the DRB1 or SE family. Similarly, we cannot currently deduce the biologic or temporal relation between the gly and non-gly cit-reactivities in the B cell response.

As expected based on previous studies, the inclusion of multiple antibody tests, including both RF and CCP2 isotype detection and different modified peptides, increased the seropositive group and identified autoantibody positive patients within the classical seronegative subgroup (CCP2 IgG–, and RF IgM–).<sup>7,17,36</sup> Yet, for clinical applications, multi-peptide tests need to be carefully redesigned and further evaluated to reduce the false discovery rate and increase the specificity for the combination of assays. Here we use the peptide array primarily to investigate ACPA patterns in patients with RA. Overall, the investigated ACPA fine specificities were relatively stable between cohorts despite potential differences in the inclusion criteria. Our novel panel of cit-peptides showed improved discrimination between different ACPA fine specificities. Moreover, different patient subsets could be identified in unsupervised clustering, which were also characterized by RF, CCP2 IgA, and Carb/Acet autoantibody levels. However, the clinical importance of these different autoantibody profiles remains to be investigated.

Owing to the multireactive nature of ACPAs, all investigated cit-peptides should be considered pseudoantigen capturing a certain ACPA reactivity pattern. They may not represent epitopes that are exposed in the citrullinated proteins, or the affinity of the ACPA may be higher to other cit-peptides/proteins expressing a similar motif that could be the primary targets. With that being said, the high frequency of binding to the peptide Cit Fibβ<sub>60–74</sub>-Cit3 with a C-terminal citrulline is particularly interesting given the suggested link between RA and the periodontal pathogen *Porphyromonas gingivalis*.<sup>37</sup> *P. gingivalis* expresses a PAD enzyme that preferentially citrullinate C-terminal arginine.<sup>38</sup>

Our data further support that ACPA specificity is determined by only a few amino acids adjacent to the citrulline. However, in several cases we could observe that one of the single-cit peptides had a higher frequency of positivity than did the original peptide with multiple citrullines. This could indicate that an arginine-to-citrulline conversion, resulting in a change from a positive to a neutral charge, outside of the dominating epitope, are reducing binding. This observation may suggest a more conformational component at play. The biochemical design of synthetic peptides (eg, biotin position and linker length in biotinylated peptides) have

also been shown to impact binding.<sup>39</sup> Nevertheless, available co-crystal structures of ACPA with cit-peptides show a hapten-like binding to the citrulline and more limited direct interactions with other side chains,<sup>40,41</sup> but certain amino acids or structures are hypothesized to sterically repulse binding.

Interestingly, the panel of 33 patient-derived monoclonal antibodies that were used for evaluations represented the serological profiles well. We observed high binding to the same preferential cit-peptides and similar binding patterns as in the patient antibody serology. Considering the multireactivity of ACPAs, it could be that patients with RA have a rather limited number of circulating ACPA clones that explains their ACPA fine specificity profiles. Indeed, a recent mass spectrometry study shows that the IgG1 ACPA response is unique to each patient and dominated by a small number of clones.<sup>42</sup>

Evidence links the risk of RA with smoking and other lung exposures, which implicates a central role for mucosal immunity.<sup>3</sup> We found that autoantibodies of the IgA isotype, both CCP2 IgA and RF IgA, were particularly strongly associated with a history of smoking, as previously reported.<sup>7,31</sup> However, serum IgA's direct link to mucosal responses is complex. Serum Igs are mostly expressed by long-lived cells in the bone marrow, but IgA-expressing cells can originate from prior mucosal responses.<sup>43</sup> IgA ACPA positivity is infrequent in IgG negative individuals and show a concordance with high IgG ACPA levels, IgG positivity for multiple cit-peptides, and IgG anti-Carb/Acet reactivity. Because human B cells class-switch from IgG to IgA, it can be hypothesized that smoking drives a strong chronic response resulting in epitope spreading and IgG to IgA class-switching.

Anti-Carb and anti-Acet autoantibodies were found in a subset of ACPA+ patients with RA and did not show any further association with SE or smoking when adjusting for CCP2 positivity. Nevertheless, this subset of patients is interesting because auto-reactivity to multiple modifications (Cit, Carb, or Acet) have previously been shown to be associated with more aggressive disease and higher risk of relapse after treatment.<sup>12,44,45</sup> In this study, we used modified filaggrin peptides to detect Carb and Acet binding in combination with acetylated histone 4. Although the histone 4 peptides represent natural lysine sites, which we have previous found to be acetylated and targets for multireactive ACPAs,<sup>14</sup> the filaggrin peptides are more artificial with the arginine/citrulline residue replaced with acetyl-lysine or homocitrulline. Hence, these peptides were similarly constructed as previously reported modified vimentin (mod-Vim58-69; Orgentec Diagnostika),<sup>7,12</sup> but because the peptide motif is different they may capture a slightly different subset than mod-Vim. Indeed, CCP2+ ACPA mAbs were found to be restricted in their cross-reactivity to these peptides depending on the peptide backbone (Supplementary Figure 2).

The use of multiple autoantibody tests distinguishes different ACPA profiles. We propose that different ACPA fine specificity combinations may be important for disease pathogenesis.

Indeed, patients with different autoantibody patterns either based on unsupervised clustering or ACPA fine specificity subtypes displayed statistically significant differences in disease activity components, in particular ESR and TJC. Patients with multi-peptide reactivities had higher disease activity than patients with fewer autoantibodies, but also a subset of seronegative patients had high activity. In model systems, ACPAs have been suggested to be directly involved in arthritis pathogenesis by interactions with different synovial cells (eg, neutrophils and osteoclasts) and by inducing pain, tenosynovitis, and bone loss.<sup>14,29,46–48</sup> Intriguingly, certain human ACPA clones have also been observed to have anti-inflammatory and antiatherogenic properties in experimental murine models.<sup>49–51</sup> Hence, different subsets of ACPAs are hypothesized have different functionality depending on their antigen-recognition.

In summary, we demonstrate that redefining ACPA fine specificity based on their recognition motif, rather than individual peptides, reveal distinct HLA-DR associations that provide essential indications about the B cell and T cells citrulline responses. Moreover, mapping the autoantibody response accelerates our understanding about seropositive disease and patient subsets.

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## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Grönwall confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

1. Aletaha D, Neogi T, Silman AJ, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European

- League Against Rheumatism collaborative initiative. *Ann Rheum Dis* 2010;69(9):1580–1588.
2. Mewar D, Coote A, Moore DJ, et al. Independent associations of anti-cyclic citrullinated peptide antibodies and rheumatoid factor with radiographic severity of rheumatoid arthritis. *Arthritis Res Ther* 2006; 8(4):R128.
  3. Klareskog L, Stolt P, Lundberg K, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum* 2006;54(1):38–46.
  4. Hensvold AH, Magnusson PK, Joshua V, et al. Environmental and genetic factors in the development of anticitrullinated protein antibodies (ACPAs) and ACPA-positive rheumatoid arthritis: an epidemiological investigation in twins. *Ann Rheum Dis* 2015;74(2):375–380.
  5. Raposo B, Klareskog L, Robinson WH, et al. The peculiar features, diversity and impact of citrulline-reactive autoantibodies. *Nat Rev Rheumatol* 2024;20(7):399–416.
  6. Zheng Z, Mergaert AM, Fahmy LM, et al. Disordered antigens and epitope overlap between anti-citrullinated protein antibodies and rheumatoid factor in rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(2): 262–272.
  7. Grönwall C, Liljefors L, Bang H, et al. A comprehensive evaluation of the relationship between different IgG and IgA anti-modified protein autoantibodies in rheumatoid arthritis. *Front Immunol* 2021;12: 627986.
  8. Hansson M, Mathsson L, Schleder T, et al. Validation of a multiplex chip-based assay for the detection of autoantibodies against citrullinated peptides. *Arthritis Res Ther* 2012;14(5):R201.
  9. Sokolove J, Bromberg R, Deane KD, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. *PLoS One* 2012;7(5):e35296.
  10. Willemze A, van der Woude D, Ghiddey W, et al. The interaction between HLA shared epitope alleles and smoking and its contribution to autoimmunity against several citrullinated antigens. *Arthritis Rheum* 2011;63(7):1823–1832.
  11. Sahlström P, Hansson M, Steen J, et al. Different hierarchies of anti-modified protein autoantibody reactivities in rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(10):1643–1657.
  12. Figueiredo CP, Bang H, Cobra JF, et al. Antimodified protein antibody response pattern influences the risk for disease relapse in patients with rheumatoid arthritis tapering disease modifying antirheumatic drugs. *Ann Rheum Dis* 2017;76(2):399–407.
  13. Jiang X, Trouw LA, van Wesemael TJ, et al. Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies. *Ann Rheum Dis* 2014;73(10):1761–1768.
  14. Lloyd KA, Wigerblad G, Sahlström P, et al. Differential ACPA binding to nuclear antigens reveals a PAD-independent pathway and a distinct subset of acetylation cross-reactive autoantibodies in rheumatoid arthritis. *Front Immunol* 2019;9:3033.
  15. Hecht C, Englbrecht M, Rech J, et al. Additive effect of anti-citrullinated protein antibodies and rheumatoid factor on bone erosions in patients with RA. *Ann Rheum Dis* 2015;74(12):2151–2156.
  16. Pertsinidou E, Saevardottir S, Manivel VA, et al. In early rheumatoid arthritis, anticitrullinated peptide antibodies associate with low number of affected joints and rheumatoid factor associates with systemic inflammation. *Ann Rheum Dis* 2024;83(3):277–287.
  17. Reed E, Hedström AK, Hansson M, et al. Presence of autoantibodies in “seronegative” rheumatoid arthritis associates with classical risk factors and high disease activity. *Arthritis Res Ther* 2020;22(1):170.
  18. Westerlind H, Grintborg B, Hammer HB, et al. Remission, response, retention and persistence to treatment with disease-modifying agents in patients with rheumatoid arthritis: a study of harmonised Swedish, Danish and Norwegian cohorts. *RMD Open* 2023;9(3):e003027.
  19. Saevardottir S, Stefansdottir L, Sulem P, et al; Members of the DBDS Genomic Consortium; Danish RA Genetics Working Group; Swedish Rheumatology Quality Register Biobank Study Group (SRQb). Multiomics analysis of rheumatoid arthritis yields sequence variants that have large effects on risk of the seropositive subset. *Ann Rheum Dis* 2022;81(8):1085–1095.
  20. Haavardsholm EA, Aga AB, Olsen IC, et al. Ultrasound in management of rheumatoid arthritis: ARCTIC randomised controlled strategy trial. *BMJ* 2016;354:i4205.
  21. Kringelbach TM, Grintborg B, Hogdall EV, et al; Biomarker Protocol Study Group. Identification of new biomarkers to promote personalised treatment of patients with inflammatory rheumatic disease: protocol for an open cohort study. *BMJ Open* 2018;8(2):e019325.
  22. Lie E, van der Heijde D, Uhlig T, et al. Treatment strategies in patients with rheumatoid arthritis for whom methotrexate monotherapy has failed: data from the NOR-DMARD register. *Ann Rheum Dis* 2011; 70(12):2103–2110.
  23. Hammer HB, Uhlig T, Kvien TK, et al. Pain catastrophizing, subjective outcomes, and inflammatory assessments including ultrasound: results from a longitudinal study of rheumatoid arthritis patients. *Arthritis Care Res (Hoboken)* 2018;70(5):703–712.
  24. Tidblad L, Westerlind H, Delcoigne B, et al. Comorbidities at diagnosis of rheumatoid arthritis: a population-based case-control study. *Rheumatology (Oxford)* 2021;60(8):3760–3769.
  25. Jia X, Han B, Onengut-Gumuscu S, et al. Imputing amino acid polymorphisms in human leukocyte antigens. *PLoS One* 2013;8(6): e64683.
  26. Hensvold A, Horuluoglu B, Sahlström P, et al. The human bone marrow plasma cell compartment in rheumatoid arthritis - clonal relationships and anti-citrulline autoantibody producing cells. *J Autoimmun* 2023;136:103022.
  27. Joshua V, Loberg Haarhaus M, Hensvold A, et al. Rheumatoid arthritis-specific autoimmunity in the lung before and at the onset of disease. *Arthritis Rheumatol* 2023;75(11):1910–1922.
  28. Steen J, Forsström B, Sahlström P, et al. Recognition of amino acid motifs, rather than specific proteins, by human plasma cell-derived monoclonal antibodies to posttranslationally modified proteins in rheumatoid arthritis. *Arthritis Rheumatol* 2019;71(2):196–209.
  29. Titcombe PJ, Wigerblad G, Sippl N, et al. Pathogenic citrulline-multispecific B cell receptor clades in rheumatoid arthritis. *Arthritis Rheumatol* 2018;70(12):1933–1945.
  30. Svård A, Skogh T, Alfredsson L, et al. Associations with smoking and shared epitope differ between IgA- and IgG-class antibodies to cyclic citrullinated peptides in early rheumatoid arthritis. *Arthritis Rheumatol* 2015;67(8):2032–2037.
  31. Masdottir B, Jónsson T, Manfredsdottir V, et al. Smoking, rheumatoid factor isotypes and severity of rheumatoid arthritis. *Rheumatology (Oxford)* 2000;39(11):1202–1205.
  32. Verpoort KN, Cheung K, Ioan-Facsinay A, et al. Fine specificity of the anti-citrullinated protein antibody response is influenced by the shared epitope alleles. *Arthritis Rheum* 2007;56(12):3949–3952.
  33. Trier NH, Dam CE, Olsen DT, et al. Contribution of peptide backbone to anti-citrullinated peptide antibody reactivity. *PLoS One* 2015; 10(12):e0144707.
  34. Assouhou-Luty C, Raijmakers R, Benckhuijsen WE, et al. The human peptidylarginine deiminases type 2 and type 4 have distinct substrate specificities. *Biochim Biophys Acta* 2014;1844(4):829–836.
  35. Rebak AS, Hendriks IA, Elsborg JD, et al. A quantitative and site-specific atlas of the citrullinome reveals widespread existence of citrullination and insights into PAD4 substrates. *Nat Struct Mol Biol* 2024; 31(6):977–995.

36. Rönnelid J, Hansson M, Mathsson-Alm L, et al. Anticitrullinated protein/peptide antibody multiplexing defines an extended group of ACPA-positive rheumatoid arthritis patients with distinct genetic and environmental determinants. *Ann Rheum Dis* 2018;77(2):203–211.
37. Bender P, Bürgin WB, Sculean A, et al. Serum antibody levels against *Porphyromonas gingivalis* in patients with and without rheumatoid arthritis - a systematic review and meta-analysis. *Clin Oral Investig* 2017;21(1):33–42.
38. Larsen DN, Mikkelsen CE, Kierkegaard M, et al. Citrullinome of *Porphyromonas gingivalis* outer membrane vesicles: confident identification of citrullinated peptides. *Mol Cell Proteomics* 2020;19(1):167–180.
39. Cornillet M, Babos F, Magyar A, et al. Seropositivity and antibody profiling of patients are dramatically impacted by the features of peptides used as immunosorbents: a lesson from anti-citrullinated protein/peptide antibody. *J Immunol* 2018;201(11):3211–3217.
40. Ge C, Xu B, Liang B, et al. Structural basis of cross-reactivity of anti-citrullinated protein antibodies. *Arthritis Rheumatol* 2019;71(2):210–221.
41. Kissel T, Ge C, Hafkenschied L, et al. Surface Ig variable domain glycosylation affects autoantigen binding and acts as threshold for human autoreactive B cell activation. *Sci Adv* 2022;8(6):eabm1759.
42. Stork EM, van Rijswijk DMH, van Schie KA, et al. Antigen-specific Fab profiling achieves molecular-resolution analysis of human autoantibody repertoires in rheumatoid arthritis. *Nat Commun* 2024;15(1):3114.
43. Mei HE, Yoshida T, Sime W, et al. Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood* 2009;113(11):2461–2469.
44. Ajeganova S, van Steenberg HW, Verheul MK, 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(1):112–118.
45. Truchetet ME, Dublanc S, Barnetche T, et al; Fédération Hospitalo-Universitaire ACRONIM. 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(12):2292–2302.
46. Jurczak A, Sandor K, Bersellini Farinotti A, et al. Insights into FcγR involvement in pain-like behavior induced by an RA-derived anti-modified protein autoantibody. *Brain Behav Immun* 2023;113:212–227.
47. Krishnamurthy A, Circiumaru A, Sun J, et al. Combination of two monoclonal ACPAs induced tenosynovitis, pain and bone loss in mice in a peptidyl arginine deiminase-4 dependent manner. *Arthritis Rheumatol* 2023;75(2):164–170.
48. Krishnamurthy A, Ytterberg AJ, Sun M, et al. Citrullination controls dendritic cell transdifferentiation into osteoclasts. *J Immunol* 2019;202(11):3143–3150.
49. Gomez AM, Brewer RC, Moon JS, et al. Anti-citrullinated protein antibodies with multiple specificities ameliorate collagen antibody-induced arthritis in a time-dependent manner. *Arthritis Rheumatol* 2024;76(2):181–191.
50. He Y, Ge C, Moreno-Giró À, et al. A subset of antibodies targeting citrullinated proteins confers protection from rheumatoid arthritis. *Nat Commun* 2023;14(1):691.
51. Raposo B, Afonso M, Israelsson L, et al. Divergent and dominant anti-inflammatory effects of patient-derived anticitrullinated protein antibodies (ACPA) in arthritis development. *Ann Rheum Dis* 2023;82(5):724–726.

# Incidence of Major Adverse Cardiovascular Events in Patients With Rheumatoid Arthritis Treated With JAK Inhibitors Compared With Biologic Disease-Modifying Antirheumatic Drugs: Data From an International Collaboration of Registries

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**Objective.** Our objective was to assess the incidence of major adverse cardiovascular events (MACEs) in patients with rheumatoid arthritis (RA) treated with JAK inhibitors (JAKi), tumor necrosis factor inhibitors (TNFi), or biologic disease-modifying antirheumatic drugs with other modes of action (bDMARD-OMA) in a multicountry, real-world population.

**Methods.** Patients with RA from 15 registries in the JAK-pot collaboration were included. MACE incidence was analyzed using two approaches: a within-registry analysis aggregating country-specific estimates from registers with >25 incident MACEs through meta-analysis and an individual-level data combined analysis. We used adjusted linear mixed Poisson regression to obtain incidence rate ratios (IRRs) of MACEs between treatment groups, accounting for multiple treatment courses.

**Results.** The study included 73,008 treatment courses (16,417 JAKi, 35,373 TNFi, and 21,218 bDMARD-OMA) and 828 incident MACEs among 51,233 patients. Median follow-up time was 1.3 years, with most of the follow-up concentrated in the first two years of treatment. Incidence rates were 7.0, 7.6, and 11.8 per 1,000 person-years for JAKi, TNFi, and bDMARD-OMA, respectively. Compared to TNFi, JAKi (within-registry adjusted IRR 0.89, 95% confidence interval [CI] 0.63–1.25) had similar incidence rates of MACEs and bDMARD-OMA had higher rates (within-registry adjusted IRR 1.35, 95% CI 1.10–1.66). Combined analysis showed similar results.

**Conclusion.** Observational data from the JAK-pot collaboration show no evidence of an increase in cardiovascular events during the first two years of use with JAKi compared to TNFi in the general RA population.

## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder that, when suboptimally controlled, may result in chronic joint

inflammation, progressive structural joint damage, and persistent pain. The disease is associated with an increased mortality rate, partly attributable to an increased risk of cardiovascular events, which occur at a higher rate in patients with RA compared to the

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general population.<sup>1,2</sup> Although the elevated cardiovascular risk in RA may be partly explained by traditional cardiovascular risk factors, available evidence suggests a direct role of systemic inflammation and altered lipoprotein profiles in the development of atherosclerosis and cardiovascular events.<sup>3,4</sup> Accordingly, treatment with disease-modifying antirheumatic drugs (DMARDs), such as tumor necrosis factor inhibitors (TNFi) or methotrexate, has been associated with a reduction in cardiovascular events in patients with RA.<sup>5,6</sup>

JAK inhibitors (JAKi) are the latest treatments developed for RA. They have shown substantial promise in the management of RA and have become an important option in the therapeutic arsenal against the disease. But concerns have been raised regarding their safety. ORAL Surveillance,<sup>7</sup> an open-label randomized controlled safety trial, demonstrated a numerically higher incidence of major adverse cardiovascular events (MACEs) with tofacitinib (one of the licensed JAKi), compared to TNFi, with a reported hazard ratio (HR) for tofacitinib versus TNFi of 1.33 (95% confidence interval [CI] 0.91–1.94). This did not meet the study's noninferiority criteria, although the difference was not statistically significant. These findings prompted the European Medicine Agency (EMA) and the US Food and Drug Administration to issue warnings for tofacitinib use.<sup>8,9</sup> Recent studies<sup>10–12</sup> based on observational data at the national level did not confirm these signals, although one study showed a higher risk of discontinuation for adverse events with tofacitinib compared to TNFi.<sup>13</sup> Current management guidelines recommend that JAKi may be used after consideration of pertinent risk factors for cardiovascular disease.<sup>14</sup> A comprehensive understanding of the cardiovascular safety profile of JAKi is of paramount importance for the safe use of this class of medication. The primary objective of this study was to compare the incidence of MACEs in patients with RA treated with JAKi to that of alternative treatment options, such as TNFi and biologic DMARDs with other modes of action (bDMARD-OMA) in a large, multicountry, real-world population.

## MATERIALS AND METHODS

**Patient sample.** The JAK-pot collaboration is an investigator-led, observational study that seeks to investigate the

clinical characteristics of patients treated with JAKi and bDMARDs for RA.<sup>13,15,16</sup> Local ethics committees (when required according to local legislation) approved the collection of data in each participating registry, and the Geneva Ethics Committee approved this specific study (CCER 2017-02278, amendment 2). This analysis included treatment courses from patients aged 18 or more, initiating either JAKi or bDMARDs from the date that JAKi were commercially available in each country (as early as 2013 for some countries, see Supplementary Table S1), to avoid confounding by underlying time-trends for MACEs. Patients could contribute multiple treatment courses, on the same class or on different drugs. Individual treatment course-level data were provided by the following 14 registers: ATTRA from the Czech Republic, BIOBADASER from Spain, BIOREG from Austria, Biorx.si from Slovenia, BSRBR-RA from the United Kingdom, GISEA from Italy, NOR-DMARD from Norway, RABBIT from Germany, REUMA.PT from Portugal, RHUMADATA from Canada, ROB-FIN from Finland, RRBR from Romania, SCQM from Switzerland and UCRCR from Greece. ARTIS, from Sweden, provided aggregated data and results from local analysis based on the same statistical analysis plan. We recognized that cross-registry variability could arise from differences in populations, data collection methods, coding systems, and exposure definitions. Detailed baseline characteristics by registry (including demographics, disease duration, and comorbidities), data collection methods and coding systems, and year of JAKi introduction can be found in Supplementary Tables S2, S3, and S1 of the Supplementary material, respectively.

**Exposure of interest and period.** The exposure of interest was the type of treatment course: JAKi (tofacitinib, baricitinib, filgotinib, or upadacitinib), TNFi (infliximab, etanercept, adalimumab, certolizumab, or golimumab), or bDMARD-OMA (abatacept, rituximab, sarilumab, or tocilizumab). The primary exposure period spanned from the initiation of treatment (baseline) until either the first recorded MACE, three months after discontinuation, start of a new treatment defined as the start of a new biologic or targeted synthetic DMARD (b/tsDMARD) molecule (biosimilar switching was not considered as a new treatment), end of participation in the register, date of death, or the end of the study period

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(November 2023), whichever came first. The end of the study period was set at an earlier date for some registries who could not provide more recent data (see Supplementary Material S4). The end of the exposure period for rituximab was set at one year after the last treatment administration because of its longer-lasting effects. To further assess the robustness of our findings, we performed sensitivity analyses using two alternative exposure periods: a shorter one, ending at the time of drug cessation, and a longer one, extending to six months after discontinuation, start of a new treatment, end of participation in the register, death, or the end of the study period, whichever came first.

**Study outcomes.** The primary outcome was the incidence of MACE, which was operationally defined as the occurrence of a stroke, myocardial infarction (MI), or transient ischemic attack (TIA). Patients with a history of the outcome were not excluded. Secondary end points included the incidence of the individual component events of MACEs (stroke, MI, or TIA). MACE was defined operationally based on specific *International Classification of Diseases* (ICD), *9th Revision*, ICD, *10th Revision*, and Medical Dictionary for Regulatory Activities (MedDRA) codes provided to each register (see Supplementary Tables S5 and S6). Details on the coding systems used by the registries and their data collection methods are provided in Supplementary Table S3.

**Covariates of interest.** Baseline covariates were chosen a priori based on clinical knowledge and the literature. They captured various patient, disease, and treatment characteristics, including sex, age, body mass index (BMI), disease activity (Clinical Disease Activity Index [CDAI] or Disease Activity Score in 28 joints if CDAI was not available in the register), disease duration (duration from symptoms onset until baseline or from the date of diagnosis until baseline if the date of symptoms onset was not available), seropositivity (rheumatoid factors and/or anticitrullinated protein antibody), number of previously used b/tsDMARDs (0, 1, 2, or  $\geq 3$ ), concomitant use of conventional synthetic DMARDs (csDMARDs) (none, methotrexate, other csDMARDs without methotrexate, methotrexate, and at least one other csDMARD), concomitant use of glucocorticoids (presence or absence), use of antiplatelet and anticoagulant medications, functional status (Health Assessment Questionnaire Disability Index), and C-reactive protein. All covariates were reassessed at each new treatment initiation to account for changes in patient characteristics over time.

Because the incidence of MACEs is heavily influenced by comorbidities, a modified Rheumatic Disease Comorbidity Index (RDCI)<sup>17</sup> was computed and included as a baseline covariate. This modified RDCI included the following comorbidities: lung disease, cardiovascular disease, hypertension, depression, diabetes, and cancer. Data on fracture, ulcer, and stomach problems were not collected and not used in this modified RDCI.

**Statistical methods.** The results are presented according to Strengthening the Reporting of Observational Studies in Epidemiology guidelines.<sup>18</sup> Baseline patient demographics and disease characteristics for each treatment group are described using counts and percentages for categorical variables, means and SDs for normally distributed continuous variables, and medians and interquartile ranges (IQRs) for nonnormally distributed data. The number of patients with valid (nonmissing) data are also provided. Missing covariate data were addressed using multiple imputations with chained equations (50 samples, predictive mean matching algorithm), with all covariates used for the propensity score included in the imputation model. The outcome variable was not imputed.

Because of constraints on cross-border data sharing, one registry could only provide aggregated data. Consequently, the analyses were conducted through two different approaches:

- Within-registry analysis: Analyses were performed independently within each registry data set. The results from these analyses were then combined using random-effects meta-analysis techniques, considering the potential heterogeneity across registries. Heterogeneity among the registries was quantified using the  $I^2$  statistic. Analyses were performed only if the number of events were  $\geq 25$  to avoid overadjustment. This analysis included data from five registries: Switzerland, Germany, Sweden, Spain, and the United Kingdom.
- Combined data analysis: Analyses were performed in a comprehensive manner, pooling data from all registries that provided individual treatment course-level data, to ensure sufficient statistical power for the analysis, particularly for more scarce outcomes. ARTIS was not included in this analysis.

The within-registry and combined analyses each involved different sets of registries, which can lead to variations in the observed results. Outcomes are reported as incidence rates, expressed as the number of events per 1,000 person-years (PYs) with 95% CIs. Unadjusted incidence rate ratios (IRRs) and 95% CIs were obtained using a Poisson regression model to compare crude incidence between treatment groups. Adjusted IRRs and 95% CIs were calculated using multivariable Poisson regression, adjusting for baseline confounders, as described previously. As rare events were analyzed, a propensity score approach with inverse probability weighting was employed to balance characteristics between treatment groups, within each register for within-registry analysis, and across combined registers for the combined data analyses. Absolute standardized mean differences before and after weighting for the combined analysis are presented in Supplementary Figures S7 and S8. We accounted for clustering resulting from patients contributing more than one treatment course and incorporated the logarithm of exposure time as an offset. The TNFi group served as the reference for

computing IRRs. All analyses were conducted using the statistics software<sup>19</sup> R 4.2.1 with the mice library for multiple imputation.<sup>20</sup>

**Secondary analyses.** *Randomized controlled trial duplicate cohort and history of cardiovascular disease.* A subgroup analysis was conducted focusing on patients with an increased risk of MACEs. This “randomized controlled trial (RCT)-duplicate cohort” mirrored the primary inclusion criteria of the ORAL Surveillance study, specifically targeting patients aged 50 years or older with at least one cardiovascular risk factor (hypertension, hyperlipidemia, diabetes, smoking, or a history of stroke or MI). A subgroup of patients with a history of cardiovascular disease (past stroke, past MI, or other past cardiovascular problems), drawn from the entire study cohort, was also analyzed separately.

*Supplementary sensitivity analyses.* Further sensitivity analyses (S9-S13), along with their methods, can be found in the Supplementary Materials, including stratification of the bDMARD-OMA group by mode of action, exclusion of rituximab, analysis by prior b/tsDMARD use (bionative vs nonbionative), and evaluation of MACE incidence before and after EMA safety warnings for JAKi. Additionally, an emulated trial analysis (S14-S16) using propensity score matching is detailed to assess robustness of the primary analysis.

**Data sharing statement.** Data are available on reasonable request. All the data belong to the registries. Anonymized data can be shared as long as agreements are made with all participating registries.

## RESULTS

This analysis included 73,008 treatment courses from 15 registers (16,417 on JAKi, 35,373 on TNFi, and 21,218 on bDMARD-OMA) and 828 incident MACEs among 51,233 patients. Table 1 summarizes the baseline characteristics of the 54,905 treatment courses from the 14 registers that provided treatment course-level data. ARTIS contributed with 18,103 treatment courses, for which baseline characteristics are presented in Supplementary Table S17. Patients were predominantly female (76%), seropositive (80%), and 58.4 years old, on average, with a median disease duration of 10.0 years. Most of the patients on JAKi were treated with baricitinib (44%), followed by tofacitinib (30%), upadacitinib (21%), and filgotinib (5%). Baseline characteristics were similar between the two largest JAKi groups (baricitinib and tofacitinib), whereas patients on upadacitinib and filgotinib were less often bionative, with many (62% and 64%, respectively) having previous exposure to two or more previous b/tsDMARDs (Supplementary Table S18). Patients on JAKi had similar disease activity at baseline than patients on TNFi and slightly higher than patients on bDMARD-OMA. Other baseline characteristics, including BMI and comorbidities, were comparable for the three

treatment groups. Patients starting a TNFi had shorter disease duration (8 years vs 11 years in JAKi and 12 years in bDMARD-OMA), were more often taking their first-line b/tsDMARD therapy (45% vs 20% in JAKi and 16% in bDMARD-OMA) and were less often on monotherapy (28% vs 37% in JAKi and 35% in bDMARD-OMA) compared to the other alternative treatments. A total of 78% of treatments were started in the prewarning period before the EMA safety warnings.

**Incidence of MACEs.** *Overall analysis.* The median follow-up from treatment start for JAKi, TNFi, and bDMARD-OMA were 1.5 (IQR 0.7–2.7), 1.5 (IQR 0.7–2.8), and 1.0 (IQR 1.0–2.0) years, respectively. Table 2 presents PYs at risk, number of MACEs, incidence rates per 1,000 PYs, and crude and adjusted IRRs for the within-registry analysis (data from 5 registries) and combined data analysis (data from 14 registries). Additionally, Supplementary Table S19 presents the PYs at risk, number of events, and crude incidence rates observed after the first and second years of treatment. When analyzing the incidence of MACEs by country (within-registry analysis) including only registries with at least 25 MACEs, there were a total of 743 MACEs recorded. The overall crude incidence was 7.0, 7.6, and 11.8 per 1,000 PYs for JAKi, TNFi, and bDMARD-OMA, respectively, and not significantly different between JAKi and TNFi (crude IRR 1.12, 95% CI 0.91–1.39), but higher for bDMARD-OMA compared to TNFi (crude IRR 1.70, 95% CI 1.45–1.99). When adjusted for baseline covariates, no difference in the incidence in MACEs was noted between JAKi and TNFi (adjusted IRR 0.96, 95% CI 0.60–1.54), but the adjusted incidence was significantly higher for bDMARD-OMA (adjusted IRR 1.35, 95% CI 1.10–1.66). The  $I^2$  statistic, measuring the heterogeneity of the adjusted IRR was 43.5% for JAKi versus TNFi and 21.0 % for bDMARD-OMA versus TNFi, indicating moderate heterogeneity for JAKi and low heterogeneity for bDMARD-OMA.

In the combined data analysis, including data from the 14 registers who contributed individual treatment course-level data, 289 MACEs were recorded, for a crude incidence rate of 2.8 of 1,000 PYs. The crude incidence was significantly higher for bDMARD-OMA versus TNFi (crude IRR 1.49, 95% CI 1.15–1.93) and similar between JAKi and TNFi (crude IRR 0.99, 95% CI 0.73–1.34). When adjusting for confounding factors, we could not identify statistically significant differences in the incidence of MACE for bDMARD-OMA compared to TNFi (adjusted IRR 1.19, 95% CI 0.90–1.58), and the incidence of MACE remained similar between JAKi and TNFi (adjusted IRR 0.89, 95% CI 0.63–1.25).

*Different exposure periods and mode of action.* The results from the analyses with shorter (Supplementary Table S20) and longer (Supplementary Table S21) exposure times were consistent with the overall findings. Within-registry analysis showed the highest crude incidence rates for CTLA4-Ig and anti-CD20, both significantly higher than TNFi (Supplementary Table S9). The combined data analysis confirmed these findings, with CTLA4-Ig

**Table 1.** Baseline characteristics by treatment group (14 registries, individual treatment course-level data)\*

| Characteristic                         | JAKi (BARI, 44%; TOFA, 30%; UPA, 21%; FILGO, 5%) |                    | TNFi (ETA, 40%; ADA, 30%; CZP, 9%; GOLi, 8%; INF, 6%; unspecified, 4%) |                    | bDMARD-OMA (RITUX, 33%; TCZ, 32%; ABA, 24%; SARI, 7%; unspecified, <sup>a</sup> 4%) |                    |
|----------------------------------------|--------------------------------------------------|--------------------|------------------------------------------------------------------------|--------------------|-------------------------------------------------------------------------------------|--------------------|
|                                        | Valid (%)                                        | Value (n = 13,374) | Valid (%)                                                              | Value (n = 25,049) | Valid (%)                                                                           | Value (n = 16,482) |
| Treatment duration, median (IQR), y    | 100.0                                            | 1.39 (0.58–2.66)   | 100.0                                                                  | 1.33 (0.54–2.77)   | 100.0                                                                               | 0.99 (0.79–1.88)   |
| Age, mean (SD), y                      | 100.0                                            | 58.29 (12.22)      | 100.0                                                                  | 57.12 (13.34)      | 100.0                                                                               | 60.48 (12.45)      |
| Female, n (%)                          | 100.0                                            | 10,468 (78.3)      | 100.0                                                                  | 18,906 (75.5)      | 100.0                                                                               | 12,488 (75.8)      |
| Disease duration, median (IQR), y      | 93.9                                             | 10.96 (5.36–18.43) | 93.6                                                                   | 8.35 (3.70–16.00)  | 93.8                                                                                | 11.88 (5.97–20.00) |
| Seropositivity (RF and/or ACPA), n (%) | 83.6                                             | 9,028 (80.7)       | 78.8                                                                   | 15,237 (77.2)      | 79.4                                                                                | 11,096 (84.8)      |
| Previous b/ts DMARD, n (%)             | 98.9                                             |                    | 98.7                                                                   |                    | 98.8                                                                                |                    |
| 0                                      |                                                  | 2,667 (20.2)       |                                                                        | 11,030 (44.6)      |                                                                                     | 2,617 (16.1)       |
| 1                                      |                                                  | 3,192 (24.1)       |                                                                        | 6,900 (27.9)       |                                                                                     | 4,219 (25.9)       |
| 2                                      |                                                  | 2,570 (19.4)       |                                                                        | 3,349 (13.5)       |                                                                                     | 3,640 (22.4)       |
| 3 or more                              |                                                  | 4,796 (36.3)       |                                                                        | 3,443 (13.9)       |                                                                                     | 5,809 (35.7)       |
| Concomitant csDMARD at baseline, n (%) | 78.5                                             |                    | 83.3                                                                   |                    | 79.6                                                                                |                    |
| MTX                                    |                                                  | 3,420 (32.6)       |                                                                        | 7,305 (35.0)       |                                                                                     | 4,054 (30.9)       |
| MTX + other                            |                                                  | 890 (8.5)          |                                                                        | 2,831 (13.6)       |                                                                                     | 1,326 (10.1)       |
| Other                                  |                                                  | 2,304 (21.9)       |                                                                        | 4,903 (23.5)       |                                                                                     | 3,173 (24.2)       |
| None                                   |                                                  | 3,886 (37.0)       |                                                                        | 5,818 (27.9)       |                                                                                     | 4,560 (34.8)       |
| Concomitant GC, n (%)                  | 93.9                                             | 5,542 (44.2)       | 92.8                                                                   | 7,995 (34.4)       | 92.0                                                                                | 6,362 (42.0)       |
| CRP, mean (SD), mg/L                   | 77.8                                             | 11.34 (21.18)      | 71.7                                                                   | 11.43 (21.51)      | 67.6                                                                                | 12.25 (27.06)      |
| CDAI, mean (SD)                        | 33.7                                             | 25.01 (13.74)      | 25.1                                                                   | 23.73 (13.85)      | 20.9                                                                                | 22.16 (13.97)      |
| DAS28, mean (SD)                       | 51.9                                             | 4.75 (1.50)        | 42.9                                                                   | 4.60 (1.58)        | 45.6                                                                                | 4.31 (1.71)        |
| HAQ, mean (SD)                         | 32.1                                             | 1.23 (0.72)        | 24.8                                                                   | 1.13 (0.73)        | 21.8                                                                                | 1.18 (0.75)        |
| BMI, mean (SD)                         | 100.0                                            | 27.07 (5.82)       | 100.0                                                                  | 27.29 (6.26)       | 100.0                                                                               | 27.25 (6.03)       |
| Ever smoking, n (%)                    | 74.8                                             | 3,604 (36.0)       | 76.5                                                                   | 7,388 (38.5)       | 75.6                                                                                | 4,820 (38.7)       |
| Past myocardial infarction, n (%)      | 60.4                                             | 159 (2.0)          | 64.2                                                                   | 281 (1.7)          | 70.3                                                                                | 263 (2.3)          |
| Past stroke, n (%)                     | 62.4                                             | 112 (1.3)          | 69.5                                                                   | 265 (1.5)          | 68.0                                                                                | 218 (1.9)          |
| Hypertension, n (%)                    | 82.8                                             | 3,363 (30.4)       | 85.5                                                                   | 5,862 (27.4)       | 84.9                                                                                | 4,223 (30.2)       |
| Hyperlipidemia, n (%)                  | 70.4                                             | 1,721 (18.3)       | 64.7                                                                   | 2,306 (14.2)       | 65.0                                                                                | 1,991 (18.6)       |
| Diabetes, n (%)                        | 81.4                                             | 990 (9.1)          | 84.6                                                                   | 1,873 (8.8)        | 83.7                                                                                | 1,335 (9.7)        |

\* ABA, abatacept; ACPA, anticitrullinated protein antibody; ADA, adalimumab; BARI, baricitinib; bDMARD, biologic disease-modifying antirheumatic drug; BMI, body mass index; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; csDMARD, classical synthetic disease-modifying antirheumatic drug; CZP, certolizumab; DAS28, Disease Activity Score in 28 joints; ETA, etanercept; FILGO, filgotinib; GC, glucocorticoid; GOLi, golimumab; HAQ, Health Assessment Questionnaire; INF, infliximab; IQR, interquartile range; JAKi, JAK inhibitor; MTX, methotrexate; OMA, other modes of action; RF, rheumatoid factor; RITUX, rituximab; SARI, sarilumab; TCZ, tocilizumab; TNFi, tumor necrosis factor inhibitor; TOFA, tofacitinib; tsDMARD, targeted synthetic disease-modifying antirheumatic drug; UPA, upadacitinib.

<sup>a</sup> Because of internal regulation, one of the registries could only provide the category of the bDMARD (bDMARD-OMA or TNFi) but not the specific type of bDMARD-OMA or TNFi.

**Table 2.** Crude and adjusted incidence and IRRs of MACEs for within-registry and combined data analysis\*

|                                       | PYs    | MACEs | IR per 1,000 PYs (95% CI) | Unadjusted IRR (95% CI)       | Adjusted IRR (95% CI)         |
|---------------------------------------|--------|-------|---------------------------|-------------------------------|-------------------------------|
| Within-registry analysis <sup>a</sup> |        |       |                           |                               |                               |
| TNFi                                  | 45,432 | 344   | 7.57 (6.79–8.42)          | 1 (ref)                       | 1 (ref)                       |
| JAKi                                  | 16,786 | 117   | 6.97 (5.76–8.35)          | 1.12 (0.91–1.39)              | 0.96 (0.60–1.54)              |
| bDMARD-OMA                            | 23,951 | 282   | 11.77 (10.44–13.23)       | 1.70 (1.45–1.99) <sup>b</sup> | 1.35 (1.10–1.66) <sup>b</sup> |
| Combined data analysis <sup>c</sup>   |        |       |                           |                               |                               |
| TNFi                                  | 50,042 | 125   | 2.50 (2.08–2.98)          | 1 (ref)                       | 1 (ref)                       |
| JAKi                                  | 25,113 | 62    | 2.47 (1.89–3.16)          | 0.99 (0.73–1.34)              | 0.89 (0.63–1.25)              |
| bDMARD-OMA                            | 27,428 | 102   | 3.72 (3.03–4.51)          | 1.49 (1.15–1.93) <sup>b</sup> | 1.19 (0.90–1.58)              |

\* Adjusted analyses adjusted for sex, age, body mass index, disease activity, disease duration, seropositivity, number of previously used bDMARDs or targeted synthetic DMARDs, concomitant use of conventional synthetic DMARDs, concomitant use of glucocorticoids, use of antiplatelet and anticoagulant medications, functional status, C-reactive protein, and Rheumatic Disease Comorbidity Index. bDMARD, biologic disease-modifying antirheumatic drug; CI, confidence interval; IR, incidence rate; IRR, incidence rate ratio; JAKi, JAK inhibitor; MACE, major adverse cardiovascular event; OMA, other modes of action; PY, person-year; ref, reference; TNFi: tumor necrosis factor inhibitor.

<sup>a</sup> Within-registry unadjusted and adjusted analysis included data from registries with at least 25 MACEs: Switzerland, Germany, Sweden, Spain, and United Kingdom.

<sup>b</sup> P value < 0.05.

<sup>c</sup> Combined data analysis included all 14 registries excluding Sweden.

**Table 3.** Crude and adjusted incidence and IRRs of secondary outcomes for the combined data analysis\*

|                                                     | Within-registry analysis <sup>a</sup> |           |                           |                               |                               | Combined data analysis <sup>b</sup> |           |                           |                               |                       |
|-----------------------------------------------------|---------------------------------------|-----------|---------------------------|-------------------------------|-------------------------------|-------------------------------------|-----------|---------------------------|-------------------------------|-----------------------|
|                                                     | PYs                                   | Events, n | IR per 1,000 PYs (95% CI) | Unadjusted IRR (95% CI)       | Adjusted IRR (95% CI)         | PYs                                 | Events, n | IR per 1,000 PYs (95% CI) | Unadjusted IRR (95% CI)       | Adjusted IRR (95% CI) |
| Stroke                                              |                                       |           |                           |                               |                               |                                     |           |                           |                               |                       |
| TNFI                                                | NA                                    | NA        | NA                        | NA                            | NA                            | 49,532                              | 38        | 0.77 (0.54–1.05)          | 1 (ref)                       | 1 (ref)               |
| JAKi                                                | NA                                    | NA        | NA                        | NA                            | NA                            | 24,790                              | 17        | 0.69 (0.40–1.09)          | 0.89 (0.50–1.58)              | 0.78 (0.42–1.46)      |
| OMA                                                 | NA                                    | NA        | NA                        | NA                            | NA                            | 27,068                              | 25        | 0.92 (0.60–1.36)          | 1.20 (0.74–1.96)              | 0.97 (0.57–1.65)      |
| Myocardial infarction (includes DE, SE, SP, and UK) |                                       |           |                           |                               |                               |                                     |           |                           |                               |                       |
| TNFI                                                | 29,409                                | 138       | 4.69 (3.94–5.54)          | 1 (ref)                       | 1 (ref)                       | 50,193                              | 75        | 1.49 (1.18–1.87)          | 1 (ref)                       | 1 (ref)               |
| JAKi                                                | 11,075                                | 57        | 5.15 (3.90–6.67)          | 1.23 (0.91–1.66)              | 0.90 (0.61–1.32)              | 25,190                              | 39        | 1.55 (1.10–2.12)          | 1.04 (0.70–1.52)              | 0.91 (0.60–1.39)      |
| OMA                                                 | 16,173                                | 125       | 7.73 (6.43–9.21)          | 1.76 (1.40–2.22) <sup>c</sup> | 1.72 (1.07–2.78) <sup>c</sup> | 27,533                              | 62        | 2.25 (1.73–2.89)          | 1.51 (1.08–2.11) <sup>c</sup> | 1.31 (0.91–1.89)      |
| Transient ischemic attack                           |                                       |           |                           |                               |                               |                                     |           |                           |                               |                       |
| TNFI                                                | NA                                    | NA        | NA                        | NA                            | NA                            | 34,376                              | 16        | 0.46 (0.27–0.76)          | 1 (ref)                       | 1 (ref)               |
| JAKi                                                | NA                                    | NA        | NA                        | NA                            | NA                            | 14,149                              | 8         | 0.56 (0.24–1.11)          | 1.21 (0.52–2.84)              | 1.36 (0.51–3.61)      |
| OMA                                                 | NA                                    | NA        | NA                        | NA                            | NA                            | 17,470                              | 17        | 0.97 (0.57–1.56)          | 2.09 (1.06–4.13) <sup>c</sup> | 1.57 (0.74–3.35)      |

\* Adjusted analyses adjusted for sex, age, body mass index, disease activity, disease duration, seropositivity, number of previously used biologic disease-modifying antirheumatic drugs (DMARDs) or targeted synthetic DMARDs, concomitant use of conventional synthetic DMARDs, concomitant use of glucocorticoids, use of antiplatelet and anticoagulant medications, functional status, C-reactive protein, and Rheumatic Disease Comorbidity Index. CI, confidence interval; DE, Germany; IR, incidence rate; IRR, incidence rate ratio; JAKi, JAK inhibitor; NA, number of events considered too low to perform the analysis; OMA, other mode of action; PY, person-year; ref, reference; SE, Sweden; SP, Spain; TNFi, tumor necrosis factor inhibitor; UK, United Kingdom.

<sup>a</sup> Within-registry unadjusted and adjusted analysis included data from registries with at least 25 major adverse cardiovascular events: CH, DE, SE, SP, and UK.

<sup>b</sup> Combined data analysis included all 14 registries excluding SE.

<sup>c</sup> *P* value < 0.05.

and anti-CD20 demonstrating significantly higher crude IRRs compared to TNFi.

**Previous b/tsDMARD use.** Adjusted IRR for patients using JAKi as first-line treatment were marginally lower than for patients having already been treated with a b/tsDMARD (Supplementary Table S11). However, the small number of events in the bionative group limits the statistical power to draw definitive conclusions from this analysis.

**Effect of the EMA warnings and emulated targeted trial.** Patients taking JAKi after the EMA warnings tended to be younger than before, but other characteristics were similar (Supplementary Table S12 and S13). Crude rates for the combined analysis of MACEs were lower after the EMA warnings for the three treatment groups, and adjusted IRRs for JAKi versus TNFi were higher after than before. The analysis of the emulated trial design (see Supplementary Tables S14-S16) provided similar results to the main analysis.

**Incidence of secondary end points.** When looking at the specific end points of stroke, MI, and TIA, the incidence of stroke across treatment groups did not differ (Table 3). Specifically, the combined data analysis adjusted IRRs suggest that, after accounting for confounding factors, the incidence of strokes associated with JAKi is similar to that of TNFi and bDMARD-OMA. The crude incidence rates for MI was not statistically different for JAKi, although the point estimate was higher, especially in the within-registry analysis (crude IRR 1.23, 95% CI 0.91–1.66), and significantly higher for bDMARD-OMA compared to TNFi (crude IRR 1.76, 95% CI 1.40–2.22); a significant difference that was still observed after adjusting for confounding factors (adjusted IRR 1.72, 95% CI 1.07–2.78). The unadjusted IRR for TIA was significantly higher

for bDMARD-OMA, but adjusted analyses mitigated these differences.

**Subgroup analyses. RCT-duplicate cohort.** The cohort referred to as the RCT-duplicate represented 45% of all treatment courses ( $n = 32,877$ ). Supplementary Table S22 presents the baseline characteristics of this cohort from the 14 registries that provided individual treatment-level data, accounting for 23,217 treatments. Although the cohort shares some selection criteria with the ORAL Surveillance (older age and cardiovascular risk factor), it is important to note that patients in this cohort generally had lower baseline disease activity compared to those included in the ORAL Surveillance study. Outside these selection criteria, patients in the RCT-duplicate cohort were similar to the general cohort. A total of 573 MACEs were recorded in the within-registry data analyses (five registries), for a crude incidence of 13.0 of 1,000 PYs, notably higher than in the overall cohort. Comparative analyses in this elevated-risk group revealed consistent results with the overall JAK-pot population (adjusted IRR JAKi compared to TNFi 0.57, 95% CI 0.12–2.66 for within-registry analyses; adjusted IRR bDMARD-OMA compared to TNFi 1.11, 95% CI 0.43–2.94 for within-registry analyses) (Table 4). However, the wide CI for the adjusted IRR suggests considerable uncertainty in the estimate, due to the relatively small number of events in this subgroup.

**History of cardiovascular disease.** In the subpopulation of patients with a history of cardiovascular disease (12% of the population), 86 MACEs were recorded in the combined data analyses, for a crude incidence of 6.9 of 1,000 PYs, higher than in both the overall cohort and the RCT-duplicate cohort. Only

**Table 4.** Crude and adjusted incidence and IRRs of MACEs for within-registry and combined data analysis in the randomized controlled trial duplicate cohort (aged  $\geq 50$  years and with at least one cardiovascular risk factor)

|                                       | PYs    | MACEs | IR per 1,000 PYs (95% CI) | Unadjusted IRR (95% CI)       | Adjusted IRR (95% CI)         |
|---------------------------------------|--------|-------|---------------------------|-------------------------------|-------------------------------|
| Within-registry analysis <sup>a</sup> |        |       |                           |                               |                               |
| TNFi                                  | 21,628 | 258   | 11.93 (10.52–13.48)       | 1 (ref)                       | 1 (ref)                       |
| JAKi                                  | 9,027  | 91    | 10.08 (8.12–12.38)        | 1.02 (0.80–1.32)              | 0.57 (0.12–2.66)              |
| bDMARD-OMA                            | 13,256 | 224   | 16.90 (14.76–19.26)       | 1.46 (1.22–1.75) <sup>b</sup> | 1.11 (0.43–2.94)              |
| Combined data analysis <sup>c</sup>   |        |       |                           |                               |                               |
| TNFi                                  | 21,136 | 94    | 4.45 (3.59–5.44)          | 1 (ref)                       | 1 (ref)                       |
| JAKi                                  | 11,440 | 41    | 3.58 (2.57–4.86)          | 0.81 (0.56–1.17)              | 1.11 (0.55–2.22)              |
| bDMARD-OMA                            | 12,933 | 70    | 5.41 (4.22–6.84)          | 1.22 (0.89–1.66)              | 1.36 (1.07–1.72) <sup>b</sup> |

\* Adjusted analyses adjusted for sex, age, body mass index, disease activity, disease duration, seropositivity, number of previously used bDMARDs or targeted synthetic DMARDs, concomitant use of conventional synthetic DMARDs, concomitant use of glucocorticoids, use of antiplatelet and anticoagulant medications, functional status, C-reactive protein, and Rheumatic Disease Comorbidity Index. bDMARD, biologic disease-modifying antirheumatic drug; CI, confidence interval; IR, incidence rate; IRR, incidence rate ratio; JAKi, JAK inhibitor; MACE, major adverse cardiovascular event; OMA, other mode of action; PY, person-year; ref, reference; TNFi, tumor necrosis factor inhibitor.

<sup>a</sup> Within-registry unadjusted and adjusted analysis included data from registries with at least 25 MACEs: Switzerland, Germany, Sweden, Spain, and United Kingdom.

<sup>b</sup>  $P$  value  $< 0.05$ .

<sup>c</sup> Combined data analysis included all 14 registries excluding Sweden.

**Table 5.** Crude and adjusted incidence and IRRs of MACEs for within-registry and combined data analysis in patients with a history of cardiovascular disease\*

|                                       | PYs   | MACEs | IR per 1,000 PYs (95% CI) | Unadjusted IRR (95% CI) | Adjusted IRR (95% CI) |
|---------------------------------------|-------|-------|---------------------------|-------------------------|-----------------------|
| Within-registry analysis <sup>a</sup> |       |       |                           |                         |                       |
| TNFi                                  | 2,841 | 52    | 18.30 (13.67–24.00)       | 1 (ref)                 | NA                    |
| JAKi                                  | 1,607 | 26    | 16.18 (10.57–23.71)       | 1.00 (0.62–1.60)        | NA                    |
| bDMARD-OMA                            | 2,623 | 67    | 25.54 (19.80–32.44)       | 1.49 (0.77–2.88)        | NA                    |
| Combined data analysis <sup>b</sup>   |       |       |                           |                         |                       |
| TNFi                                  | 5,517 | 32    | 5.80 (3.97–8.19)          | 1 (ref)                 | 1 (ref)               |
| JAKi                                  | 2,827 | 19    | 6.72 (4.05–10.50)         | 1.16 (0.67–2.00)        | 1.06 (0.56–2.02)      |
| bDMARD-OMA                            | 4,068 | 35    | 8.60 (5.99–11.97)         | 1.48 (0.91–2.42)        | 1.40 (0.80–2.43)      |

\* Adjusted analyses adjusted for sex, age, body mass index, disease activity, disease duration, seropositivity, number of previously used bDMARDs or targeted synthetic DMARDs, concomitant use of conventional synthetic DMARDs, concomitant use of glucocorticoids, use of antiplatelet and anticoagulant medications, functional status, C-reactive protein, and Rheumatic Disease Comorbidity Index. *P* value < 0.05. bDMARD, biologic disease-modifying antirheumatic drug; CI, confidence interval; IR, incidence rate; IRR, incidence rate ratio; JAKi, JAK inhibitor; MACE, major adverse cardiovascular event; NA, number of events considered too low to perform the analysis; OMA, other mode of action; PY, person-year; ref, reference; TNFi, tumor necrosis factor inhibitor.

<sup>a</sup> Within-registry unadjusted and adjusted analysis included data from registries with at least 25 MACEs: Switzerland, Germany, Sweden, Spain, and United Kingdom.

<sup>b</sup> Combined data analysis included all 14 registries excluding Sweden.

combined data analyses were performed because of the insufficient sample size in individual registers (Table 5).

## DISCUSSION

In this large, international, collaborative study, we did not observe an increased incidence of MACEs in patients treated with JAKi compared to patients treated with other bDMARDs, with a point estimate of approximately 1 between JAKi and TNFi (adjusted IRR 0.96, 95% CI 0.60–1.54). We further did not observe an increased rate of any individual cardiovascular outcome. Although the combined and within-registry analyses differed in design, both yielded point estimates that support similar risk profiles for JAKi and TNFi. When restricting our analysis to an “at risk” population for cardiovascular events, the overall incidence of MACEs was higher, but the relative risk of MACEs between the treatment groups remained similar, whether in patients with similar inclusion criteria to the ORAL Surveillance study<sup>7</sup> or in patients with a history of cardiovascular disease or using an emulated trial design.

Our study contributes important data to the ongoing debate regarding cardiovascular safety profile of JAKi, sparked by the publication of the ORAL Surveillance study results. This randomized, open-label, noninferiority, event-driven, safety trial enrolled more than 4,000 patients with RA aged 50 years or more and with at least one cardiovascular risk factor. Patients were randomly assigned to receive either tofacitinib at a dosage of 5 mg or 10 mg twice daily or a TNFi. Although the difference was not statistically significant, tofacitinib did not meet the noninferiority criteria for MACEs, as the 95% CIs of the HRs exceeded the prespecified upper boundary of 1.8 (HR 1.24, 95% CI 0.81–1.91 for the 5-mg dose). In our study, the upper range of uncertainty

is lower than this upper boundary of 1.8 when comparing JAKi to TNFi (adjusted IRR 0.96, 95% CI 0.60–1.54).

Our approach is characterized by the inclusion of a broad, real-world population of patients with RA across multiple registries, reflecting routine clinical practice. In contrast to single-country studies, our cross-registry collaboration encompasses diverse RA populations across several nations, broadening the generalizability of our findings and enabling more comprehensive subgroup analyses. The eligibility criteria differed from ORAL Surveillance. Although we performed analyses that mimicked the two of the inclusion criteria (aged more than 50 years and at least one cardiovascular risk factor), another main criterion in the ORAL Surveillance study was related to inadequate RA disease control, a factor we did not replicate in our analysis. In addition, other specific criteria, including cotreatment regimens, were not part of our study’s design. Our study focused on a broader comparison across JAKi rather than looking at individual JAKi. ORAL Surveillance only studied tofacitinib, whereas our study included all commercially available JAKi, with a nearly equal distribution between baricitinib and tofacitinib. Additionally, our study population is mostly European. Although ORAL Surveillance included European patients, almost all of them were from Poland, with only 3% included from non-Polish countries. Polish patients have a very low access to second-line therapy (3% of patients) and are treated mainly with non-anti-inflammatory drugs and methotrexate in contrast to the majority of other countries in Europe.<sup>21</sup> The difference in health care coverage should also be considered when comparing the studies, as most European countries have an inclusive health coverage, which has been shown to associate with fewer untreated cardiovascular comorbidities or risk factors.<sup>22,23</sup>

Finally, the incidence of MACEs in absolute terms was lower in our study’s combined analysis, likely reflecting patient

selection, with patients on average having less active disease compared to those enrolled in ORAL Surveillance and probably less at risk of cardiovascular. However, when focusing on the within-registry analysis, which included data from five countries with a higher number of recorded events, the incidence rate of MACEs for patients treated with TNFi (7.6 per 1,000 PYs) was comparable to that reported in ORAL Surveillance (7.3 per 1,000 PYs). The possibility of underreporting MACEs in observational settings, particularly in registries with less frequent or incomplete follow-up, remains a consideration. Nevertheless, the incidence rates reported herein align with the ranges reported in existing observational studies on patients with RA and similar to ORAL Surveillance in the within-registry analysis.<sup>3,24</sup>

The absence of an increased incidence of MACEs in patients with RA treated with JAKi corroborates the results of other observational studies on the topic. A population-based cohort study<sup>25</sup> of the French national health system compared a group of patients initiating JAKi and a group initiating adalimumab, and found similar risk of MACEs (weighted HR 1.0, 95% CI 0.7–1.5) between the two groups. In the United States, the STAR-RA study<sup>26</sup> pooled three claims databases to analyze the cardiovascular outcome of more than 100,000 patients with RA, 12% of whom had initiated tofacitinib. They found no evidence of increased risk of cardiovascular outcome with tofacitinib versus TNFi both in the overall RA population (pooled weighted HR 1.01, 95% CI 0.83–1.23) and in the RCT-duplicate cohort (pooled weighted HR 1.24, 95% CI 0.90–1.24). Another real-world retrospective observational study<sup>27</sup> used Korean health insurance data to investigate the risk of MACE, and found no significant differences between JAKi and TNFi in the rate of MACEs. In the Netherlands, a cohort study<sup>11</sup> using prescription database also found no difference between JAKi and bDMARDs (IRR 0.99, 95% CI 0.70–1.41) for cardiovascular events. Finally, data from the ARTIS register<sup>10</sup> in Sweden and the RABBIT register<sup>12</sup> in Germany, both of which are part of the present JAK-pot study, have independently published very similar conclusions, reporting no increased risk with JAKi treatment. Their inclusion in our current analysis builds on these previously reported findings, reinforcing the consistency of the observed results.

Although the results of these studies occasionally vary and not all JAKi are always considered, these studies provide reassurance regarding the cardiovascular risk for patients currently receiving JAKi therapy. However, these studies, such as ours, may also be limited by the duration of follow-up (less than one-third of PYs were observed beyond two years), preventing us from drawing conclusions on long-term risks. Despite this limitation, our study, which includes 13,000 JAKi treatments and all available b/tsDMARDs in clinical practice, also likely represents the most significant collaborative effort of this kind to date, aimed at investigating the real-world implications of JAKi.

Our study has several limitations. First, despite our best efforts to harmonize cardiovascular events based on exhaustive

lists of the different coding (MedDRA, ICD coding, or text) used by the registries, some differences in reporting between registries might persist. Indeed, some registries are linked to hospitalization data, whereas others have adverse events only documented by the patient's rheumatologist. The resulting differences in incidence between registries, similar to what has been observed elsewhere,<sup>28</sup> can be a topic of concern if events are reported selectively by treatment groups. If the report of MACEs between treatment arms within each registry was to be expected, it would likely increase the incidence for JAKi, given the novelty of the treatment and the concerns raised by the ORAL Surveillance study. Such a difference was not observed here, and if existing, this bias would tend to overestimate the true relative risk of JAKi. The dual approach we adopted for the analysis, comprising both within-registry and pooled analyses, was designed to account for the difference of MACE incidence between registries and address possible differences in reporting.

A second limitation is the overall limited number of events, especially for secondary outcomes and subgroups analyses. Although we considered analyzing each JAKi agent individually, the event count was insufficient for reliable subgroup analyses. Additionally, we did not adjust the combined analysis for countries, as including this factor would have increased the complexity of the model and led to overfitting. As a result, the low number of events also prevented us from comparing specific JAKi to bDMARDs. Furthermore, only approximately 30% of the follow-up time extends beyond two years, with 20% of MACEs occurring after the two-year mark. This limited long-term follow-up may have prevented us from observing the significant differences observed in ORAL Surveillance, that appeared mostly after two years, although disparities in nonfatal MI rates were already evident during the second year. Finally, despite our best effort to account for confounding factors through propensity score inverse probably weighting adjustment, and a sensitivity analysis using propensity score matching (emulated trial), we cannot exclude residual confounding.

Despite these limitations, our study includes a large sample size, with the use of multiple registries across Europe and Canada, allowing us to adjust for a wide range of confounders. The larger pooled cohort also enhances the statistical precision of MACE risk estimates, particularly for these rarer outcomes. Although RCTs are the gold standard for assessing efficacy and safety in controlled conditions, real-world evidence complements these data by reflecting broader, more diverse patient populations and practice patterns. Finally, the extended follow-up in our real-world setting provides important long-term insights that complement the relatively shorter duration of RCTs. The robustness of our statistical approach, together with the consistency of the results yielded by the sensitivity and subgroup analyses strengthen our results. Our results suggest that, overall, patients with RA treated with JAKi do not incur an increased risk of MACEs

compared to those treated with alternative bDMARDs, particularly within the first two years of therapy.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Aymon confirms that all authors have provided the final approval of the version to be published and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## ROLE OF THE STUDY SPONSORS

AbbVie, Galapagos, Pfizer, and Eli Lilly had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by AbbVie, Galapagos, Pfizer, and Eli Lilly.

## REFERENCES

- Hanslidaar R, Vedder D, Baniaamam M, et al. Cardiovascular risk in inflammatory arthritis: rheumatoid arthritis and gout. *Lancet Rheumatol* 2021;3(1):e58–e70.
- Aviña-Zubieta JA, Choi HK, Sadatsafavi M, et al. Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. *Arthritis Rheum* 2008;59(12):1690–1697.
- Lauper K, Gabay C. Cardiovascular risk in patients with rheumatoid arthritis. *Semin Immunopathol* 2017;39(4):447–459.
- England BR, Thiele GM, Anderson DR, et al. Increased cardiovascular risk in rheumatoid arthritis: mechanisms and implications. *BMJ* 2018; 361:k1036.
- Low ASL, Symmons DPM, Lunt M, et al; British Society for Rheumatology Biologics Register for Rheumatoid Arthritis (BSRBR-RA) and the BSRBR Control Centre Consortium. Relationship between exposure to tumour necrosis factor inhibitor therapy and incidence and severity of myocardial infarction in patients with rheumatoid arthritis. *Ann Rheum Dis* 2017;76(4):654–660.
- Micha R, Imamura F, Wyler von Ballmoos M, et al. Systematic review and meta-analysis of methotrexate use and risk of cardiovascular disease. *Am J Cardiol* 2011;108(9):1362–1370.
- Ytterberg SR, Bhatt DL, Mikuls TR, et al; ORAL Surveillance Investigators. Cardiovascular and cancer risk with tofacitinib in rheumatoid arthritis. *N Engl J Med* 2022;386(4):316–326.
- Xeljanz (tofacitinib): initial clinical trial results of increased risk of major adverse cardiovascular events and malignancies (excluding NMSC) with use of tofacitinib relative to TNF—alpha inhibitors. European Medicine Agency; 2021. Accessed June 17, 2024. [https://www.ema.europa.eu/en/documents/dhpc/direct-healthcare-professional-communication-dhpc-xeljanz-tofacitinib-initial-clinical-trial-results-increased-risk-major-adverse-cardiovascular-events-and-malignancies-excluding-nm-sc-use-tofacitinib\\_en.pdf](https://www.ema.europa.eu/en/documents/dhpc/direct-healthcare-professional-communication-dhpc-xeljanz-tofacitinib-initial-clinical-trial-results-increased-risk-major-adverse-cardiovascular-events-and-malignancies-excluding-nm-sc-use-tofacitinib_en.pdf)
- Initial safety trial results find increased risk of serious heart-related problems and cancer with arthritis and ulcerative colitis medicine Xeljanz, Xeljanz XR (tofacitinib). US Food and Drug Administration FDA; 2021. Accessed June 17, 2024. <https://www.fda.gov/drugs/drug-safety-and-availability/initial-safety-trial-results-find-increased-risk-serious-heart-related-problems-and-cancer-arthritis>
- Bower H, Frisell T, di Giuseppe D, et al. Comparative cardiovascular safety with janus kinase inhibitors and biological disease-modifying antirheumatic drugs as used in clinical practice: an observational cohort study from Sweden in patients with rheumatoid arthritis. *RMD Open* 2023;9(4):e003630.
- Popa CD, Opdam MAA, den Broeder N, et al. Therapy with JAK inhibitors or bDMARDs and the risk of cardiovascular events in the Dutch rheumatoid arthritis population. *Rheumatology* 2023;63(8):2142–2146.
- Meissner Y, Schäfer M, Albrecht K, et al. Risk of major adverse cardiovascular events in patients with rheumatoid arthritis treated with conventional synthetic, biologic and targeted synthetic disease-modifying antirheumatic drugs: observational data from the German RABBIT register. *RMD Open* 2023;9(4): e003489.
- Aymon R, Mongin D, Bergstra SA, et al. Evaluation of discontinuation for adverse events of JAK inhibitors and bDMARDs in an international collaboration of rheumatoid arthritis registers (the ‘JAK-pot’ study). *Ann Rheum Dis* 2024;83(4):421–428.
- Smolen JS, Landewé RBM, Bergstra SA, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2022 update. *Ann Rheum Dis* 2023;82(1):3–18.
- Lauper K, Iudici M, Mongin D, et al. Effectiveness of TNF-inhibitors, abatacept, IL6-inhibitors and JAK-inhibitors in 31 846 patients with rheumatoid arthritis in 19 registers from the ‘JAK-pot’ collaboration. *Ann Rheum Dis* 2022;81(10):1358–1366.
- Pombo-Suarez M, Sanchez-Piedra C, Gómez-Reino J, et al. After JAK inhibitor failure: to cycle or to switch, that is the question – data from the JAK-pot collaboration of registries. *Ann Rheum Dis* 2023; 82(2):175–181.
- England BR, Sayles H, Mikuls TR, et al. Validation of the Rheumatic Disease Comorbidity Index. *Arthritis Care Res (Hoboken)* 2015; 67(6):865–872.
- von Elm E, Altman DG, Egger M, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol* 2008; 61(4):344–349.
- R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; 2023. Accessed October 10, 2023. <https://www.R-project.org/>
- van Buuren S, Groothuis-Oudshoorn K. mice: multivariate imputation by chained equations in R. *J Stat Softw* 2011;45(3):1–67.
- Batko B, Stajszczyk M, Świerkot J, et al. Prevalence and clinical characteristics of rheumatoid arthritis in Poland: a nationwide study. *Arch Med Sci* 2019;15(1):134–140.
- Putrik P, Ramiro S, Keszei AP, et al. Lower education and living in countries with lower wealth are associated with higher disease activity in rheumatoid arthritis: results from the multinational COMORA study. *Ann Rheum Dis* 2016;75(3):540–546.
- Putrik P, Ramiro S, Moltó A, et al. Individual-level and country-level socioeconomic determinants of disease outcomes in SpA: multinational, cross-sectional study (ASAS-COMOSPA). *Ann Rheum Dis* 2019;78(4):486–493.
- Arts EEA, Popa CD, Den Broeder AA, et al. Prediction of cardiovascular risk in rheumatoid arthritis: performance of original and adapted SCORE algorithms. *Ann Rheum Dis* 2016;75(4):674–680.
- Hoisnard L, Vegas LP, Dray-Spira R, et al. Risk of major adverse cardiovascular and venous thromboembolism events in patients with rheumatoid arthritis exposed to JAK inhibitors versus adalimumab: a nationwide cohort study. *Ann Rheum Dis* 2023;82(2): 182–188.

26. Khosrow-Khavar F, Kim SC, Lee H, et al. Tofacitinib and risk of cardiovascular outcomes: results from the Safety of Tofacitinib in Routine care patients with Rheumatoid Arthritis (STAR-RA) study. *Ann Rheum Dis* 2022;81(6):798–804.
27. Min HK, Kim H, Jeong HJ, et al. Risk of cancer, cardiovascular disease, thromboembolism, and mortality in patients with rheumatoid arthritis receiving Janus kinase inhibitors: a real-world retrospective observational study using Korean health insurance data. *Epidemiol Health* 2023;45:e2023045.
28. Crowson CS, Rollefstad S, Kitas GD, et al; A Trans-Atlantic Cardiovascular Risk Consortium for Rheumatoid Arthritis (ATACC-RA). Correction: challenges of developing a cardiovascular risk calculator for patients with rheumatoid arthritis. *PLoS One* 2017;12(4):e0175605.

# Association of Thigh Intramuscular Fat Infiltration With Incident Knee and Hip Osteoarthritis: A Longitudinal Cohort Study

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**Objective.** Intramuscular fat infiltration (IMFI) in the thigh is linked to metabolic inflammation and muscle dysfunction, which could contribute to osteoarthritis development. However, no longitudinal studies have examined the associations between thigh IMFI and incident knee and hip osteoarthritis.

**Methods.** We conducted a cohort study using UK Biobank data, including participants with baseline IMFI in thigh muscle assessed by magnetic resonance imaging and no history of knee or hip osteoarthritis. A total of 24,224 and 24,221 participants were included in analyses of IMFI of anterior and posterior thigh muscle with knee osteoarthritis, respectively, whereas 24,767 and 24,764 were included in analyses of IMFI of anterior and posterior thigh muscle for hip osteoarthritis, respectively. Cox Proportional-hazard models were used to estimate hazard ratios (HRs) for incident clinically diagnosed knee or hip osteoarthritis, adjusting for potential confounders.

**Results.** During a mean follow-up of 4.9 years, 472 participants developed knee osteoarthritis, and 387 developed hip osteoarthritis. IMFI in the anterior thigh was significantly associated with incident knee osteoarthritis, with adjusted HRs of 1.78 (95% confidence interval [CI] 1.19–2.65), 2.00 (95% CI 1.34–2.99), and 2.34 (95% CI 1.53–3.59) across the second to fourth quartiles, respectively ( $P$  for trend <0.01). Both anterior and posterior thigh IMFI were significantly associated with hip osteoarthritis ( $P$  for both trends <0.01). Dose-response relationships were observed in restricted spline models. The interaction and subgroup analyses support that the effect of IMFI is independent of body mass index and sex.

**Conclusion.** Elevated thigh IMFI is significantly associated with increased incidence of both knee and hip osteoarthritis. Interventions targeting IMFI reduction may help prevent osteoarthritis and mitigate its impact.

## INTRODUCTION

Osteoarthritis (OA) is the leading cause of lower extremity disability, affecting more than 600 million people worldwide, with the knee and hip most often affected.<sup>1</sup> As the global population ages and the prevalence of adiposity (excessive fat accumulation in the body) increase, the prevalence of this debilitating condition is rising

among both older and younger adults.<sup>2</sup> Despite the significant morbidity, economic burden, and social impact of OA,<sup>3</sup> current treatments are limited to symptom management because there are no disease-modifying drugs available to halt, slow, or reverse OA progression effectively.<sup>4</sup> Therefore, identifying modifiable risk factors and adopting optimal management measures are central to preventing and treating OA to alleviate its burden.

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Intramuscular fat infiltration (IMFI), characterized by the accumulation of adipose tissue in skeletal muscle, is a common feature of myopathic changes, increasing by 6% to 8% annually in the older population.<sup>5–8</sup> IMFI plays a distinct role in metabolic inflammation and muscle dysfunction, independent of body mass index (BMI) or visceral and subcutaneous fat.<sup>5</sup> Specifically, intramuscular adipose tissue originates from unique cellular sources (eg, muscle satellite stem cells),<sup>5</sup> and can exacerbate mitochondrial dysfunction, oxidative stress, and insulin resistance in muscle.<sup>9,10</sup>

It is linked also to the up-regulation of inflammatory cytokines, chemokines, and adipokines,<sup>9,10</sup> contributing to muscle atrophy, weakness, and a cycle of local myosteatosis and systemic inflammation.<sup>5,9</sup> These changes can disrupt joint biochemical and biomechanical homeostasis, which are closely related to OA pathogenesis.<sup>3</sup> Fortunately, it is a factor that may be modified through exercise interventions (eg, resistance training or circuit training),<sup>11,12</sup> dietary interventions (eg, whey protein and vitamin D, or long-term calorie restriction),<sup>13,14</sup> and drug therapy (eg, liraglutide).<sup>15</sup> However, the temporal relationship between baseline IMFI and incident OA remains unexplored. This lack of longitudinal evidence limits our knowledge and confidence of the role of muscle fitness in OA prevention and management.

Notably, OA is a heterogeneous disease with several differences in pathogenesis and epidemiologic patterns between knee and hip OA.<sup>16</sup> For example, the contribution of biomechanical factors to pathogenesis differs between knee OA (a bicondylar hinge joint) and hip OA (a more stable ball-and-socket joint),<sup>16</sup> with quadriceps strength being associated with both knee and hip OA,<sup>17–19</sup> whereas hamstring strength associates with hip OA rather than knee OA.<sup>20,21</sup> In addition, a higher proportion of type II muscle fibers, which are more sensitive to lipotoxicity,<sup>22</sup> are distributed in the anterior thigh muscles compared with the posterior thigh muscles.<sup>23</sup> These differences suggest that the association between IMFI and OA may vary across anatomic location. To address the previously mentioned knowledge gaps, we examined the association between IMFI in the anterior or posterior thigh and the incidence of knee or hip OA using data from the UK Biobank.

## PATIENTS AND METHODS

**Data source and study population.** The UK Biobank is a prospective cohort study comprising approximately 500,000 participants aged 40 to 69 years,<sup>24</sup> recruited from the UK NHS between 2006 and 2010 (reference 11/NW/0382). All participants provided written informed consent. Initial data collection involved questionnaires and physical assessments, with ongoing follow-up through linkage to national health-related datasets for monitoring outcomes. In 2014, a subset of 100,000 individuals was invited to participate in a multimodal imaging study, which included magnetic resonance imaging (MRI) of the thigh muscles.<sup>25</sup> The exclusion criteria for the 100,000 participants invited to additional MRI scans included (1) recent surgery or implants,

(2) inability to tolerate the scan, and (3) medical conditions impairing scan feasibility (Supplementary Methods).<sup>25</sup> For the current analysis, we included participants who had completed an IMFI assessment of the anterior or posterior thigh in at least one leg, using the imaging time as the baseline. The index date was defined as the date of baseline IMFI data collection. For the analysis of incident knee OA, we excluded individuals with a pre-existing diagnosis of knee OA at baseline and those without IMFI data. Similarly, for the analysis of incident hip OA, we excluded individuals with a pre-existing diagnosis of hip OA and those without IMFI data at baseline.

**IMFI measurements.** Participants were scanned using a Siemens MAGNETOM Aera 1.5-T MRI scanner (Siemens Healthineers) with a six-minute dual-echo Dixon Vibe protocol, which provided water and fat-separated volumetric data from the neck to the knees.<sup>26</sup> Scans were conducted with six slabs in participants in the supine position, without localizer.<sup>27</sup> Thigh muscle composition analysis was performed using automatic segmentation via AMRA Researcher (AMRA Medical AB).<sup>26,27</sup> Imaging quality across all centers was ensured by a centralized team through consistent protocols, standardized quality control measures (eg, system testing, phantom measurements, maintenance), and ongoing image monitoring by radiographers and external experts. Further details of the MRI acquisition and processing are provided in the Supplementary Methods.<sup>25–28</sup> In our study, IMFI was defined at the individual level as the fraction of fat infiltration within the “viable muscle tissue” (fat-free muscle volume [FFMV]). FFMV was defined as the volume of all voxels in the thighs with a fat fraction of <50%. If IMFI data were available for both thighs, the average of the two values was used. If data were missing for one thigh, the IMFI data from the available thighs were used. Participants with no data for either thigh were excluded.<sup>29</sup> The IMFI of the anterior thigh was calculated as the summation of the region of interest, including the quadriceps femoris, sartorius, and tensor fascia latae, with subcutaneous fat excluded. Similarly, the posterior thigh included the gluteus, iliacus, adductor, and hamstring muscles. The MRI measurements used in this study are publicly available through the UK Biobank.

**Assessment of outcomes.** The primary outcome was clinically diagnosed knee OA, and the secondary outcome was clinically diagnosed hip OA.<sup>30</sup> Both outcomes were defined using the *International Classification of Diseases* (ICD), *Ninth Revision* or ICD, *Tenth Revision* codes. These diagnoses were considered as the “first occurrence of health outcomes,” based on data linked from primary care records, hospital admission records, or self-reported medical conditions in the UK Biobank database. Incident knee or hip OA was defined as a hospital diagnosis of knee or hip OA occurring any time after the index date.

**Assessment of covariates.** Covariates were selected based on biologic plausibility and evidence from previous studies, including age,<sup>3,31</sup> sex,<sup>3,31</sup> BMI,<sup>3,31</sup> smoking status,<sup>32</sup> alcohol consumption,<sup>33</sup> educational attainments,<sup>34</sup> physical activity levels,<sup>31</sup> FFMV<sub>adjusted</sub>,<sup>35,36</sup> and handgrip strength.<sup>36</sup> These factors are known to influence both the IMFI and incident OA in similar contexts. Age was calculated from the date of birth to the baseline assessment. Sex was self-reported at baseline. BMI was determined using the weight-to-height<sup>2</sup> ratio from the anthropometrics data collected at recruitment and was categorized as normal (<25) or overweight (≥25). Smoking and drinking statuses were self-reported at recruitment and categorized as never, former, current, or missing. Educational attainments were self-reported and classified as college, noncollege, or unknown. Physical activity levels were assessed using the short-form International Physical Activity Questionnaire and categorized into three groups: low (<600 metabolic equivalent [MET]–min/wk), moderate (600 to <3,000 MET–min/wk), and high (≥3,000 MET–min/wk).<sup>37</sup> The  $FFMV_{adjusted} = FFMV_{raw} \times (100 - \text{fraction of intramuscular fat infiltration})/100$  in the anterior or posterior thigh,<sup>28</sup> removing subcutaneous tissue, large streaks of pure fat infiltration, and diffuse muscle fat infiltration. A previous study confirmed that this lean muscle measurement in the UK Biobank, which excludes all fat fractions, demonstrates an excellent linear correlation with the gold standard, dual-energy x-ray absorptiometry, with a correlation coefficient<sup>38</sup> (*r*) of 0.97. Handgrip strength was defined as the maximum grip strength of the left and right hands.

**Statistical analysis.** Participant characteristics were described using the mean and SD for continuous variables and percentages for binary or categorical variables. For the end point of incident knee OA, participants were observed until the first recorded diagnosis of knee OA, death, loss to follow-up, or the end of the study (December 31, 2021), whichever occurred first. We calculated incidence rates of knee OA by dividing the number of cases of incident knee OA by the person-years of follow-up. We estimated the hazard ratios (HRs) and 95% confidence intervals (CIs) for incident knee OA in the second, third, and fourth quartiles of IMFI in the anterior or posterior thigh, compared with the lowest quartile (reference group), using Cox proportional hazards models. A linear trend in the effect of IMFI on incident knee OA was tested by including the value of IMFI in the regression model. In the multivariable-adjusted model, we adjusted for age, sex, BMI, smoking status, alcohol status, educational attainments, physical activity levels, FFMV<sub>adjusted</sub> (measured by MRI at the same anatomic sites as IMFI), and handgrip strength.

Several supplemental analyses were performed to further elucidate the relationship between IMFI in the anterior or posterior thigh and incident knee OA. First, we employed a restricted cubic spline regression model to examine the dose-response relationship between thigh IMFI and incident knee OA. Second, to assess the

impact of BMI or sex, we examined both the multiplicative interaction and additive interaction effects of thigh IMFI and BMI or sex on the risk of incident knee OA.<sup>39</sup> Then, we evaluated these associations across two BMI subgroups (<25 and ≥25) or sex subgroups.

For the secondary outcome, we took the approach described previously to investigate the relationship between baseline IMFI in the anterior or posterior thigh and incident hip OA. A *P* value of <0.05 (two-sided) was considered statistically significant. Statistical analyses were conducted using SAS V.9.4 (SAS Institute, Inc).

**Data availability.** The UK Biobank data are publicly available. The statistical code and data derived for this study are available from the corresponding authors, provided the permission from the UK Biobank.

## RESULTS

**Characteristics of the study population.** A flow chart depicting the participant selection process is shown in Figure 1. A total of 24,224 participants were included in analyses of the associations between baseline IMFI in the anterior thigh and incident knee OA (Table 1). The mean age was 62.95 years (SD 7.52 years), the mean BMI was 26.61 (SD 4.23), and 52.27% were female. Compared with those in the reference group (lowest quartile), participants in higher IMFI quartiles of the anterior thigh were generally older and had higher BMIs, higher female proportion, lower education attainment, higher prevalence of smoking, lower physical activity levels, and lower FFMV and handgrip strength. The baseline characteristics of participants for the incident hip OA analysis (*n* = 24,767) across quartiles of IMFI in the anterior thigh were similar to those observed for participants in the incident knee OA analysis (Table 1).

A total of 24,221 and 24,764 participants were included in analyses of the associations between baseline IMFI in the posterior thigh and incident knee or hip OA, respectively (Figure 1). In the incident knee OA analyses, the mean age was 62.95 years (SD 7.52 years), the mean BMI was 26.61 (SD 4.23), and 52.26% were female (Supplementary Table 1). Compared with those in the reference group (lowest quartile), participants in higher IMFI quartiles of the posterior thigh were generally older and had higher BMIs, higher female proportion, lower education attainment, higher prevalence of smoking, lower physical activity levels, and lower FFMV and handgrip strength. For the incident hip OA analysis, the basic characteristics were similar to those observed for participants in the incident knee OA analysis (Supplementary Table 1).

**Association of baseline thigh IMFI with risk of incident knee OA.** For anterior thigh muscle analyses, during a mean follow-up of 4.88 years (SD 1.31 years), 472 participants developed knee OA. As shown in Table 2, the incidence rates of

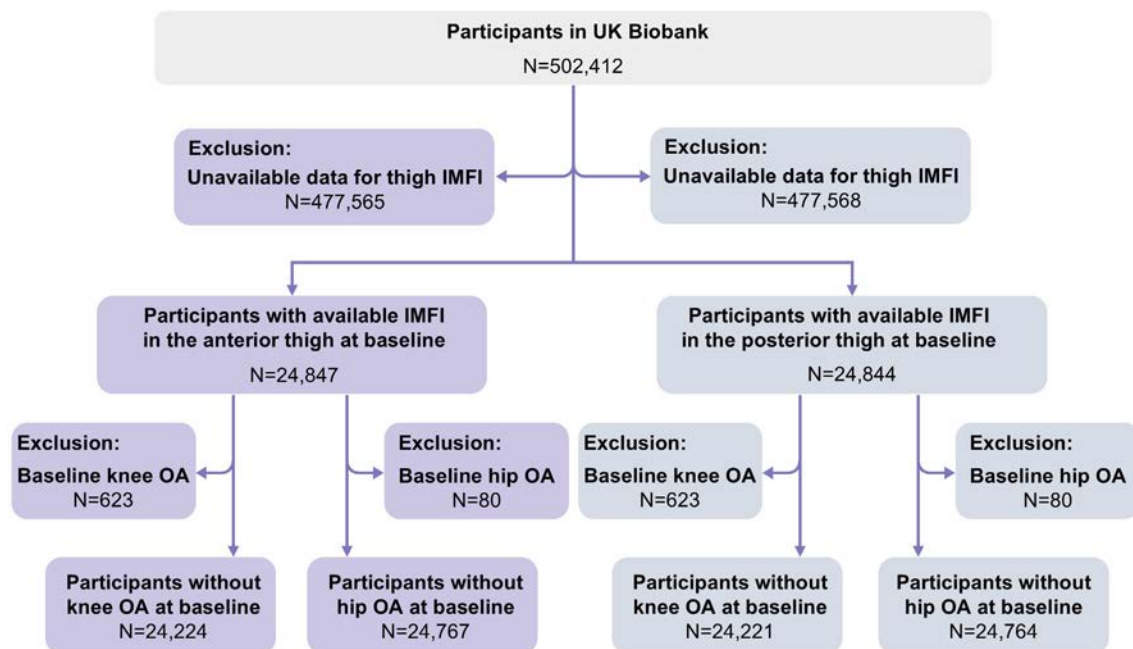


**Figure 1.** Flow chart of participants in the study. IMFI, intramuscular fat infiltration; OA, osteoarthritis.

knee OA were 1.56, 3.17, 4.13, and 7.12 per 1,000 person-years in the reference, second, third, and fourth quartiles of IMFI in the anterior thigh, respectively. Compared with the reference group, the adjusted HRs for incident knee OA were 1.78 (95% CI 1.19–2.65), 2.00 (95% CI 1.34–2.99), and 2.34 (95% CI 1.53–3.59) in the second, third, and fourth quartiles of IMFI in the anterior thigh, respectively ( $P$  for trend  $<0.01$ , Table 2). For posterior thigh muscle analyses, a total of 472 participants developed knee OA during a mean follow-up of 4.88 (SD 1.31) years. However, no significant association between IMFI in the posterior thigh muscle and incident knee OA was observed after adjusting for major confounders (Table 2).

Restricted spline regression models also demonstrated that higher baseline IMFI in the anterior thigh was associated with a higher risk of incident knee OA (Figure 2A). But no dose-response relationships were observed between baseline IMFI in the posterior thigh and the risk of incident hip OA (Figure 2C). We found no interactions between IMFI in the anterior thigh and BMI or sex in association with the risk of knee OA on either the additive ( $P > 0.05$ ) or multiplicative ( $P > 0.05$ ) scale. The subgroup analysis with different BMI or sex subgroups did not change the effect estimate materially (Figure 3).

**Association of baseline thigh IMFI with risk of incident hip OA.** For anterior thigh muscle analyses, during a mean follow-up of 4.89 years (SD 1.29 years), 387 participants developed hip OA. The incidence rates of hip OA were 1.93, 2.47, 3.14, and 5.24 per 1,000 person-years in the reference, second, third, and fourth quartiles of IMFI in the anterior thigh, respectively (Table 2). Compared with those in the reference

group, the adjusted HRs for incident hip OA in the second, third, and fourth quartiles were 0.94 (95% CI 0.63–1.41), 1.17 (95% CI 0.78–1.76), and 1.62 (95% CI 1.05–2.51), respectively ( $P$  for trend  $<0.01$ , Table 2). For posterior thigh muscle analyses, a total of 387 participants developed hip OA during a mean follow-up of 4.89 (SD 1.29) years. The incidence rates of hip OA were 1.80, 2.49, 3.33, and 5.22 per 1,000 person-years in the reference, second, third, and fourth quartiles of IMFI in the posterior thigh, respectively (Table 2). The adjusted HRs for incident hip OA in the second, third, and fourth quartiles were 1.01 (95% CI 0.68–1.50), 1.39 (95% CI 0.94–2.06), and 1.78 (95% CI 1.18–2.68), respectively ( $P$  for trend  $<0.01$ , Table 2), compared with the reference group.

Restricted spline regression models also demonstrated that higher baseline IMFI in both the anterior (Figure 2B) and posterior (Figure 2D) thigh was associated with a greater risk of incident hip OA. In addition, no interaction between IMFI in the anterior and posterior thigh muscle and BMI or sex was found to be associated with the risk of hip OA on either the additive ( $P > 0.05$ ) or multiplicative ( $P > 0.05$ ) scale. Similar patterns of associations were observed in subgroup analyses for each BMI or sex (Figure 4).

## DISCUSSION

To our knowledge, this is the first study to examine the relationship between thigh IMFI and the risk of incident OA. In this large population-based cohort study, we found that elevated IMFI in the anterior thigh was associated with a higher incidence of knee OA, and the elevated IMFI in both the anterior and posterior

**Table 1.** Baseline characteristics of participants\*

| Characteristic                                                      | Total          | IMFI of anterior thigh |               |               |               | Standardized mean difference |
|---------------------------------------------------------------------|----------------|------------------------|---------------|---------------|---------------|------------------------------|
|                                                                     |                | Q1 (lowest)            | Q2            | Q3            | Q4 (highest)  |                              |
| Knee OA                                                             |                |                        |               |               |               |                              |
| Participants, n                                                     | 24,224         | 6,056                  | 6,057         | 6,055         | 6,056         |                              |
| Age, mean ± SD, y                                                   | 62.95 ± 7.52   | 59.78 ± 7.33           | 62.40 ± 7.37  | 63.97 ± 7.19  | 65.66 ± 6.92  | 0.445                        |
| Female, n (%)                                                       | 12,661 (52.27) | 1,798 (29.69)          | 3,024 (49.93) | 3,655 (60.36) | 4,184 (69.09) | 0.453                        |
| BMI, mean ± SD                                                      | 26.61 ± 4.23   | 24.86 ± 3.22           | 25.71 ± 3.51  | 26.77 ± 3.87  | 29.09 ± 4.90  | 0.569                        |
| Education, n (%)                                                    |                |                        |               |               |               | 0.230                        |
| Noncollege                                                          | 7,597 (31.36)  | 1,679 (27.72)          | 1,810 (29.88) | 1,965 (32.45) | 2,143 (35.39) |                              |
| College                                                             | 14,584 (60.21) | 4,090 (67.54)          | 3,845 (63.48) | 3,524 (58.20) | 3,125 (54.60) |                              |
| Unknown                                                             | 2,043 (8.43)   | 287 (4.74)             | 402 (6.64)    | 566 (9.35)    | 788 (13.01)   |                              |
| Smoking, n (%)                                                      |                |                        |               |               |               | 0.151                        |
| Current                                                             | 1,542 (6.37)   | 352 (5.81)             | 354 (5.84)    | 394 (6.51)    | 442 (7.30)    |                              |
| Former                                                              | 7,898 (32.60)  | 1,647 (27.20)          | 1,855 (30.63) | 2,073 (34.24) | 2,323 (38.36) |                              |
| Never                                                               | 14,724 (60.78) | 4,048 (66.84)          | 3,839 (63.38) | 3,571 (58.97) | 3,266 (53.93) |                              |
| Unknown                                                             | 60 (0.25)      | 9 (0.15)               | 9 (0.15)      | 17 (0.28)     | 25 (0.41)     |                              |
| Alcohol, n (%)                                                      |                |                        |               |               |               | 0.039                        |
| Current                                                             | 23,019 (95.03) | 5,773 (95.33)          | 5,779 (95.41) | 5,742 (94.83) | 5,725 (94.54) |                              |
| Former                                                              | 539 (2.22)     | 128 (2.11)             | 127 (2.10)    | 146 (2.41)    | 138 (2.28)    |                              |
| Never                                                               | 651 (2.69)     | 153 (2.53)             | 147 (2.43)    | 160 (2.64)    | 191 (3.15)    |                              |
| Unknown                                                             | 15 (0.06)      | 2 (0.03)               | 4 (0.06)      | 7 (0.12)      | 2 (0.03)      |                              |
| Physical activity level, <sup>a</sup> n (%)                         |                |                        |               |               |               | 0.139                        |
| Low (%)                                                             | 3,847 (15.88)  | 780 (12.88)            | 893 (14.74)   | 979 (16.17)   | 1,195 (19.73) |                              |
| Moderate                                                            | 10,970 (45.28) | 2,921 (48.23)          | 2,877 (47.50) | 2,679 (44.24) | 2,493 (41.17) |                              |
| High                                                                | 5,658 (23.36)  | 1,616 (26.69)          | 1,420 (23.45) | 1,413 (23.34) | 1,209 (19.96) |                              |
| Unknown                                                             | 3,749 (15.48)  | 739 (12.20)            | 867 (14.31)   | 984 (16.25)   | 1,159 (19.14) |                              |
| White race, n (%)                                                   | 23,502 (97.02) | 5,834 (96.33)          | 5,869 (96.90) | 5,892 (97.31) | 5,907 (97.54) | 0.039                        |
| IMFI, mean anterior thigh ± SD                                      | 7.31 ± 1.86    | 5.29 ± 0.53            | 6.53 ± 0.29   | 7.62 ± 0.36   | 9.79 ± 1.54   | 3.371                        |
| Fat-free muscle volume of anterior thigh, <sup>b</sup> L            | 1.60 ± 0.46    | 1.88 ± 0.46            | 1.63 ± 0.43   | 1.50 ± 0.42   | 1.38 ± 0.37   | 0.641                        |
| Handgrip strength, kg                                               | 32.15 ± 10.48  | 37.60 ± 10.15          | 32.90 ± 10.00 | 30.27 ± 9.83  | 27.76 ± 9.29  | 0.545                        |
| Rates of death, n (%)                                               | 405 (1.67)     | 53 (0.88)              | 89 (1.47)     | 107 (1.77)    | 156 (2.58)    | 0.070                        |
| Rates of loss to follow-up, n (%)                                   | 5 (0.02)       | 2 (0.03)               | 1 (0.02)      | 1 (0.02)      | 1 (0.02)      | 0.005                        |
| Hip OA                                                              |                |                        |               |               |               |                              |
| Participants, n                                                     | 24,767         | 6,193                  | 6,191         | 6,192         | 6,191         |                              |
| Age, mean ± SD, y                                                   | 62.99 ± 7.51   | 59.80 ± 7.33           | 62.45 ± 7.37  | 64.01 ± 7.17  | 65.70 ± 6.91  | 0.446                        |
| Female, n (%)                                                       | 12,872 (51.97) | 1,823 (29.44)          | 3,066 (49.52) | 3,732 (60.27) | 4,251 (68.66) | 0.453                        |
| BMI, mean ± SD                                                      | 26.64 ± 4.25   | 24.88 ± 3.22           | 25.74 ± 3.51  | 26.80 ± 3.90  | 29.16 ± 4.91  | 0.574                        |
| Education, n (%)                                                    |                |                        |               |               |               | 0.231                        |
| Noncollege                                                          | 7,795 (31.47)  | 1,717 (27.72)          | 1,857 (30.00) | 2,013 (32.51) | 2,208 (35.66) |                              |
| College                                                             | 14,868 (60.03) | 4,185 (67.58)          | 3,914 (63.22) | 3,595 (58.06) | 3,174 (51.27) |                              |
| Unknown                                                             | 2,104 (8.50)   | 291 (4.70)             | 420 (6.78)    | 584 (9.43)    | 809 (13.07)   |                              |
| Smoking, n (%)                                                      |                |                        |               |               |               | 0.156                        |
| Current                                                             | 1,574 (6.36)   | 360 (5.81)             | 363 (5.86)    | 404 (6.53)    | 447 (7.22)    |                              |
| Former                                                              | 8,135 (32.85)  | 1,693 (27.34)          | 1,895 (30.61) | 2,133 (34.45) | 2,414 (38.99) |                              |
| Never                                                               | 14,998 (60.55) | 4,131 (66.70)          | 3,924 (63.38) | 3,638 (58.75) | 3,305 (53.39) |                              |
| Unknown                                                             | 60 (0.24)      | 9 (0.15)               | 9 (0.15)      | 17 (0.27)     | 25 (0.40)     |                              |
| Alcohol, n (%)                                                      |                |                        |               |               |               | 0.037                        |
| Current                                                             | 23,535 (95.03) | 5,904 (95.34)          | 5,906 (95.40) | 5,871 (94.82) | 5,854 (94.56) |                              |
| Former                                                              | 551 (2.22)     | 129 (2.08)             | 130 (2.10)    | 148 (2.39)    | 144 (2.33)    |                              |
| Never                                                               | 665 (2.69)     | 158 (2.55)             | 151 (2.44)    | 165 (2.66)    | 191 (3.08)    |                              |
| Unknown                                                             | 16 (0.06)      | 2 (0.03)               | 4 (0.06)      | 8 (0.13)      | 2 (0.03)      |                              |
| Physical activity level, <sup>a</sup> n (%)                         |                |                        |               |               |               | 0.137                        |
| Low (%)                                                             | 3,923 (15.84)  | 796 (12.85)            | 910 (14.70)   | 1,002 (16.18) | 1,215 (19.63) |                              |
| Moderate                                                            | 11,180 (45.14) | 2,978 (48.09)          | 2,922 (48.32) | 2,734 (44.15) | 2,546 (41.12) |                              |
| High                                                                | 5,830 (23.54)  | 1,665 (26.89)          | 1,472 (23.78) | 1,444 (23.32) | 1,249 (20.17) |                              |
| Unknown                                                             | 3,834 (15.48)  | 754 (12.17)            | 817 (13.20)   | 1,012 (16.35) | 1,181 (19.08) |                              |
| White race, n (%)                                                   | 24,028 (97.02) | 5,968 (96.37)          | 5,996 (96.85) | 6,024 (97.29) | 6,040 (97.56) | 0.039                        |
| IMFI, mean anterior thigh ± SD                                      | 7.32 ± 1.87    | 5.29 ± 0.53            | 6.54 ± 0.29   | 7.63 ± 0.36   | 9.82 ± 1.55   | 3.370                        |
| Fat-free muscle volume of anterior thigh, <sup>b</sup> mean ± SD, L | 1.60 ± 0.46    | 1.89 ± 0.46            | 1.63 ± 0.43   | 1.50 ± 0.42   | 1.38 ± 0.38   | 0.642                        |
| Handgrip strength, mean ± SD, kg                                    | 32.19 ± 10.50  | 37.64 ± 10.17          | 32.96 ± 10.02 | 30.30 ± 9.85  | 27.81 ± 9.31  | 0.544                        |

(Continued)

**Table 1.** (Cont'd)

| Characteristic                    | Total      | IMFI of anterior thigh |           |            |              | Standardized mean difference |
|-----------------------------------|------------|------------------------|-----------|------------|--------------|------------------------------|
|                                   |            | Q1 (lowest)            | Q2        | Q3         | Q4 (highest) |                              |
| Rates of death, n (%)             | 418 (1.69) | 54 (0.87)              | 92 (1.49) | 111 (1.79) | 161 (2.60)   | 0.071                        |
| Rates of loss to follow-up, n (%) | 6 (0.02)   | 2 (0.03)               | 1 (0.02)  | 1 (0.02)   | 2 (0.03)     | 0.007                        |

\* BMI, body mass index; FFMV, fat-free muscle volume; IMFI, intramuscular fat infiltration; MET, metabolic equivalent; OA, osteoarthritis; Q, quartile.

<sup>a</sup> We categorized participants into three mutually exclusive groups, specifically low (<600 MET-min/wk), moderate (600 to <3,000 MET-min/wk), and high (≥3,000 MET-min/wk) level of physical activity, based on a standard scoring criterion.

<sup>b</sup> The FFMV was adjusted as follows:  $FFMV_{adjusted} = FFMV_{raw} \times (100 - \text{fraction of intramuscular fat infiltration})/100$ .

thigh was related to an increased risk of hip OA. Statistically significant dose-response relationships were found for these associations. The results remained consistent and robust across subgroup and sensitivity analyses.

Previous cross-sectional or case-control studies have shown that thigh IMFI is positively associated with knee and hip OA.<sup>21,35,40,41</sup> Specifically, IMFI in the anterior thigh was significantly associated with worse Kellgren-Lawrence grades, cartilage and meniscus lesions, and worse pain or function.<sup>21,35,42</sup> A previous cohort study has reported that a reduction in IMFI in vastus medialis was associated with a reduced annual loss of cartilage volume in both medial tibial and patellar sites.<sup>43</sup> Consistent with

previous studies, our study, for the first time, demonstrated that thigh IMFI is strongly associated with incident OA. The IMFI in the anterior thigh was associated with both incident knee and hip OA, whereas IMFI in the posterior thigh was only related to incident hip OA.

Several explanations may account for the relationship between IMFI and the risk of incident OA at the knees and hips. During the aging process, ectopic lipid deposition (lipotoxicity) occurs in the thigh muscles, which are partially composed of type II muscle fibers that are sensitive to fat accumulation.<sup>22</sup> The accumulation of lipids and their derivatives within the muscle can induce mitochondrial dysfunction, disrupt fatty acid

**Table 2.** Association between IMFI in the anterior thigh muscle and incident knee or hip OA\*

|                                                  | Thigh IMFI at baseline |                  |                  |                  | <i>P</i> for trend |
|--------------------------------------------------|------------------------|------------------|------------------|------------------|--------------------|
|                                                  | Q1 (lowest)            | Q2               | Q3               | Q4 (highest)     |                    |
| IMFI in the anterior thigh and incident knee OA  |                        |                  |                  |                  |                    |
| Participants, n                                  | 6,056                  | 6,057            | 6,055            | 6,056            |                    |
| Incident cases, n                                | 46                     | 94               | 123              | 209              |                    |
| Mean follow-up time, mean ± SD, y                | 4.86 ± 1.22            | 4.90 ± 1.29      | 4.92 ± 1.33      | 4.85 ± 1.38      |                    |
| Incidence rate, per 1,000 person-years           | 1.56                   | 3.17             | 4.13             | 7.12             |                    |
| Crude HR (95% CI)                                | 1.0 (reference)        | 2.03 (1.43–2.89) | 2.64 (1.89–3.71) | 4.54 (3.30–6.24) | <0.01              |
| Adjusted HR <sup>a</sup> (95% CI)                | 1.0 (reference)        | 1.78 (1.19–2.65) | 2.00 (1.34–2.99) | 2.34 (1.53–3.59) | <0.01              |
| IMFI in the posterior thigh and incident knee OA |                        |                  |                  |                  |                    |
| Number of participants, n                        | 6,055                  | 6,057            | 6,053            | 6,056            |                    |
| Incident cases, n                                | 71                     | 90               | 136              | 175              |                    |
| Mean follow-up time, mean ± SD, y                | 4.93 ± 1.27            | 4.92 ± 1.29      | 4.88 ± 1.31      | 4.80 ± 1.34      |                    |
| Incidence rate, per 1,000 person-years           | 2.38                   | 3.02             | 4.61             | 6.03             |                    |
| Crude HR (95% CI)                                | 1.0 (reference)        | 1.27 (0.93–1.73) | 1.93 (1.45–2.57) | 2.51 (1.91–3.31) | <0.01              |
| Adjusted HR <sup>a</sup> (95% CI)                | 1.0 (reference)        | 1.11 (0.79–1.56) | 1.26 (0.90–1.78) | 1.26 (0.86–1.84) | 0.20               |
| IMFI in the anterior thigh and incident hip OA   |                        |                  |                  |                  |                    |
| Number of participants, n                        | 6,193                  | 6,191            | 6,192            | 6,191            |                    |
| Incident cases, n                                | 58                     | 75               | 96               | 158              |                    |
| Mean follow-up time, mean ± SD, y                | 4.86 ± 1.22            | 4.91 ± 1.28      | 4.93 ± 1.31      | 4.87 ± 1.35      |                    |
| Incidence rate, per 1,000 person-years           | 1.93                   | 2.47             | 3.14             | 5.24             |                    |
| Crude HR (95% CI)                                | 1.0 (reference)        | 1.28 (0.91–1.80) | 1.62 (1.17–2.24) | 2.69 (1.99–3.64) | <0.01              |
| Adjusted HR <sup>a</sup> (95% CI)                | 1.0 (reference)        | 0.94 (0.63–1.41) | 1.17 (0.78–1.76) | 1.62 (1.05–2.51) | <0.01              |
| IMFI in the posterior thigh and incident hip OA  |                        |                  |                  |                  |                    |
| Number of participants, n                        | 6,191                  | 6,191            | 6,191            | 6,191            |                    |
| Incident cases, n                                | 55                     | 76               | 101              | 155              |                    |
| Mean follow-up time, mean ± SD, y                | 4.94 ± 1.26            | 4.94 ± 1.27      | 4.90 ± 1.29      | 4.80 ± 1.33      |                    |
| Incidence rate, per 1,000 person-years           | 1.80                   | 2.49             | 3.33             | 5.22             |                    |
| Crude HR (95% CI)                                | 1.0 (reference)        | 1.38 (0.98–1.95) | 1.84 (1.33–2.56) | 2.88 (2.12–3.92) | <0.01              |
| Adjusted HR <sup>a</sup> (95% CI)                | 1.0 (reference)        | 1.01 (0.68–1.50) | 1.39 (0.94–2.06) | 1.78 (1.18–2.68) | <0.01              |

\* CI, confidence interval; HR, hazard ratio; IMFI, intramuscular fat infiltration; OA, osteoarthritis; Q, quartile.

<sup>a</sup> Adjusted for age, sex, body mass index, smoking, alcohol, educational attainments, physical activity levels, adjusted fat-free muscle volume, and handgrip strength.

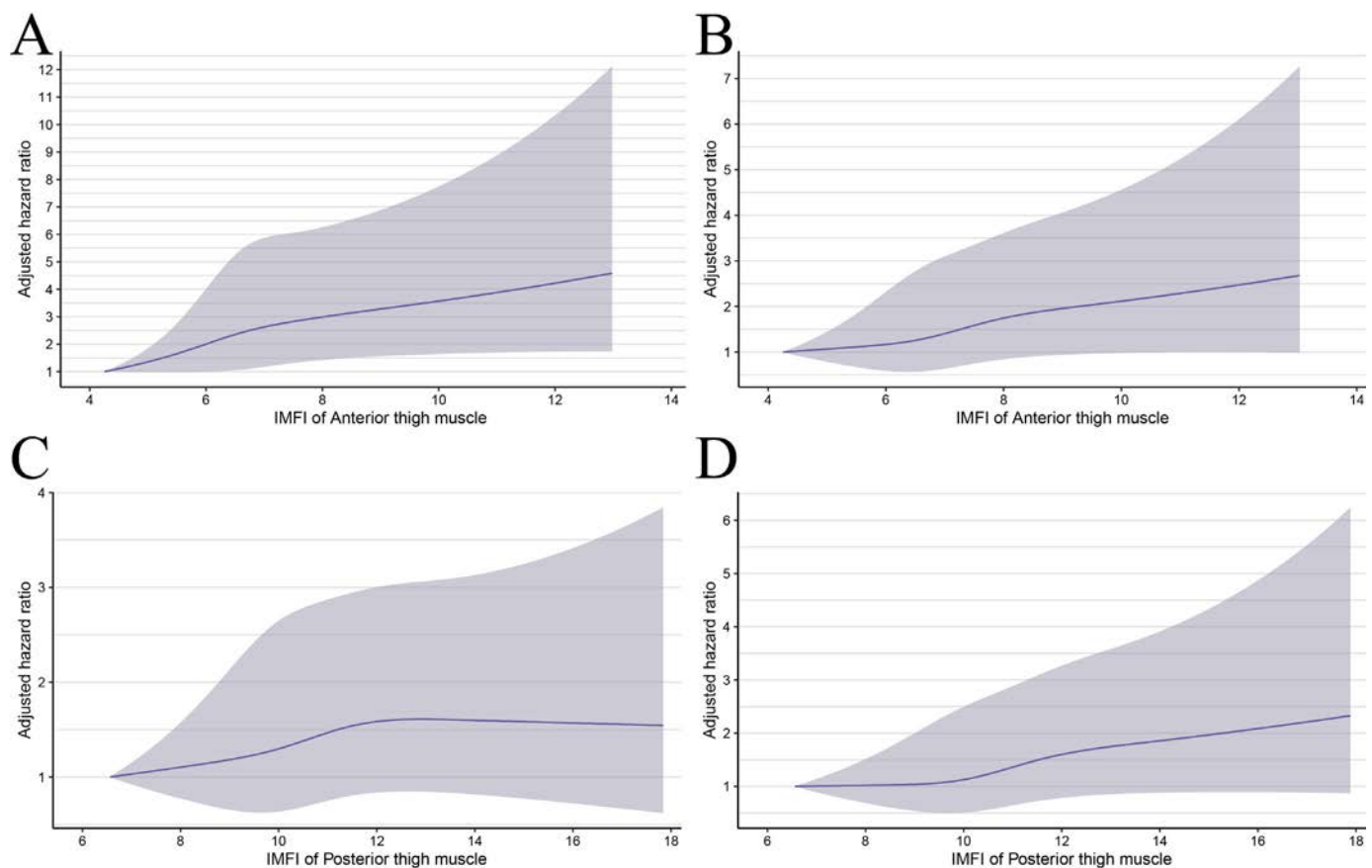


**Figure 2.** Restricted cubic spline regression models assessing the dose-response associations between thigh IMFI and incident knee and hip OA. (A) IMFI in anterior thigh and incident knee OA; (B) IMFI in anterior thigh and incident hip OA; (C) IMFI in posterior thigh and incident knee OA; (D) IMFI in posterior thigh and incident hip OA. IMFI, intramuscular fat infiltration; OA, osteoarthritis. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43159/abstract>.

$\beta$ -oxidation, increase reactive oxygen species production, and promote insulin resistance.<sup>5</sup> Additionally, intramuscular adipocytes can release free fatty acids and inflammatory mediators through a paracrine or endocrine-like mechanism, including inflammatory cytokines.<sup>5</sup> It has been demonstrated that inflammatory mediators are associated with OA, and cartilage degradation by destroying the extracellular matrix. This crosstalk between muscles and joints may contribute to the development of OA. In addition, IMFI can lead to muscle dysfunction and reduced muscle strength, which are closely related to the development of OA.<sup>17</sup> Notably, the anterior thigh muscles play a dominant role in knee OA but contribute less to the load on hip joint cartilage.<sup>44</sup> This may, in part, explain why our effect sizes were more significant for the risk of incident knee OA than hip OA. The posterior thigh muscles are the primary contributors to the mechanical stability of the hip joint,<sup>44</sup> with only the hamstrings among the posterior thigh muscles significantly contributing to knee flexion.<sup>45</sup> However, previous studies have found no significant association between reduced hamstring strength and knee OA.<sup>20,46</sup> Thus, despite increased IMFI in the posterior thigh muscles, their direct impact on knee OA development is likely limited.

IMFI is closely associated with obesity, in which excessive lipids are redistributed to other tissues.<sup>5</sup> However, the adipose tissue within muscles has at least three distinct cellular origins, including muscle satellite stem cells, fibroadipogenic progenitor cells resident in muscle, and adipose-derived stem cells that migrate from other adipose depots into the muscle tissue bed.<sup>5</sup> In addition, intramuscular adipose tissue plays unique roles in metabolic inflammation.<sup>5</sup> For example, intramuscular adipose tissue secretes significantly higher levels of inflammatory cytokines (eg, interferon- $\gamma$ , interleukin-2 [IL-2], and IL-13), chemokines (eg, CCL11 and IL-8), and adipokines (eg, resistin) than visceral and subcutaneous adipose tissue. Thus, we may not be able to simply equate IMFI with obesity completely. Our interaction and subgroup analyses indicated that the effect of thigh IMFI on the risk of incident OA could be independent of BMI. The previous study on cartilage volume also reported that the relationship between reduced vastus medialis fat and knee cartilage volume loss is independent of weight loss.<sup>43</sup>

In addition, our findings revealed that thigh IMFI was more strongly associated with knee OA in women, whereas it was associated with hip OA in men. This sex-specific pattern may be explained by the fact that women have larger knee valgus angle,

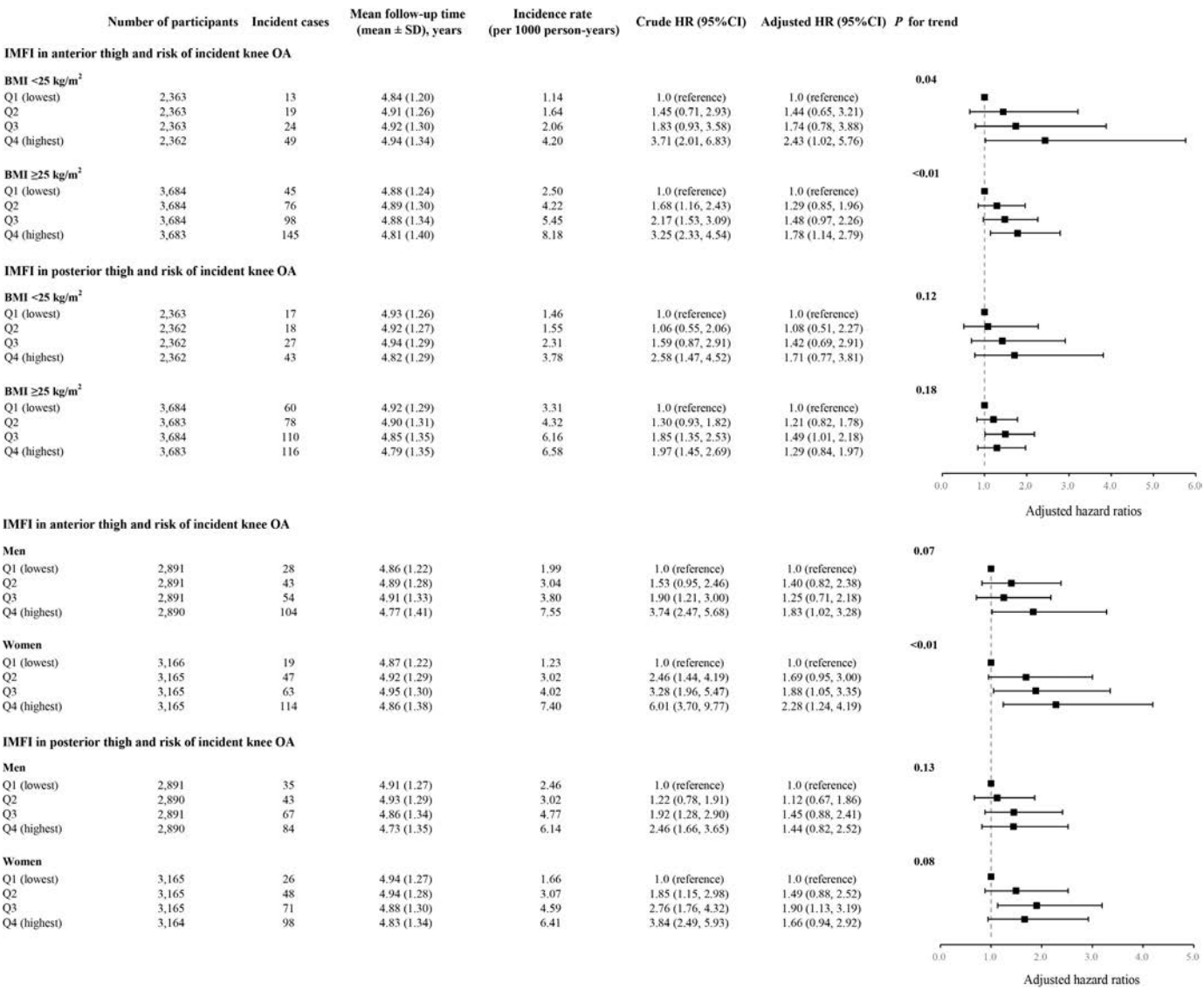


**Figure 3.** Associations between thigh IMFI and incident knee OA by BMI and sex. Data are shown as HRs with 95% CIs. Adjusted HRs are adjusted for age, sex, BMI, smoking, alcohol, educational attainments, physical activity levels, adjusted fat-free muscle volume, and handgrip strength. BMI, body mass index; CI, confidence interval; HR, hazard ratio; IMFI, intramuscular fat infiltration; OA, osteoarthritis; Q, quartile.

smaller knee cartilage volume, and greater fat accumulation in the anterior thigh, making them more susceptible to systemic inflammation and biomechanic effects induced by IMFI.<sup>47</sup> Previous research has also shown stronger associations between muscle weakness and knee OA in women than men.<sup>48</sup> Additionally, men have a larger femoral lesser trochanter, which may increase their susceptibility to hip OA.<sup>49</sup> Building on this, increased IMFI may further exacerbate stress imbalances in the hip joint, contributing to the development of hip OA. Notably, although further investigation is required to clarify the underlying mechanisms for these sex-specific associations, our study highlights the necessity of integrating sex-specific perspectives into clinical research and public health initiatives to develop tailored preventive and therapeutic strategies for knee and hip OA.

Our study has several strengths. Firstly, we used a standardized and precise assessment for thigh IMFI using volumetric MRI. The protocol and process for measuring thigh IMFI showed high repeatability and reproducibility.<sup>50</sup> Secondly, we performed interaction and subgroup analyses to assess the impact related to BMI and sex. Our findings further confirmed the robustness of the effect of thigh IMFI on incident knee and hip OA. In addition, we also adjusted for other important confounding variables in the current analysis, such as age, sex, physical activity level, FFMV of thigh, and handgrip strength, thus minimizing potential bias from major confounders.

However, there are several potential limitations of our study. Firstly, the UK Biobank does not have radiographic data for knee and hip OA. The clinical diagnosis of knee and hip OA was entirely

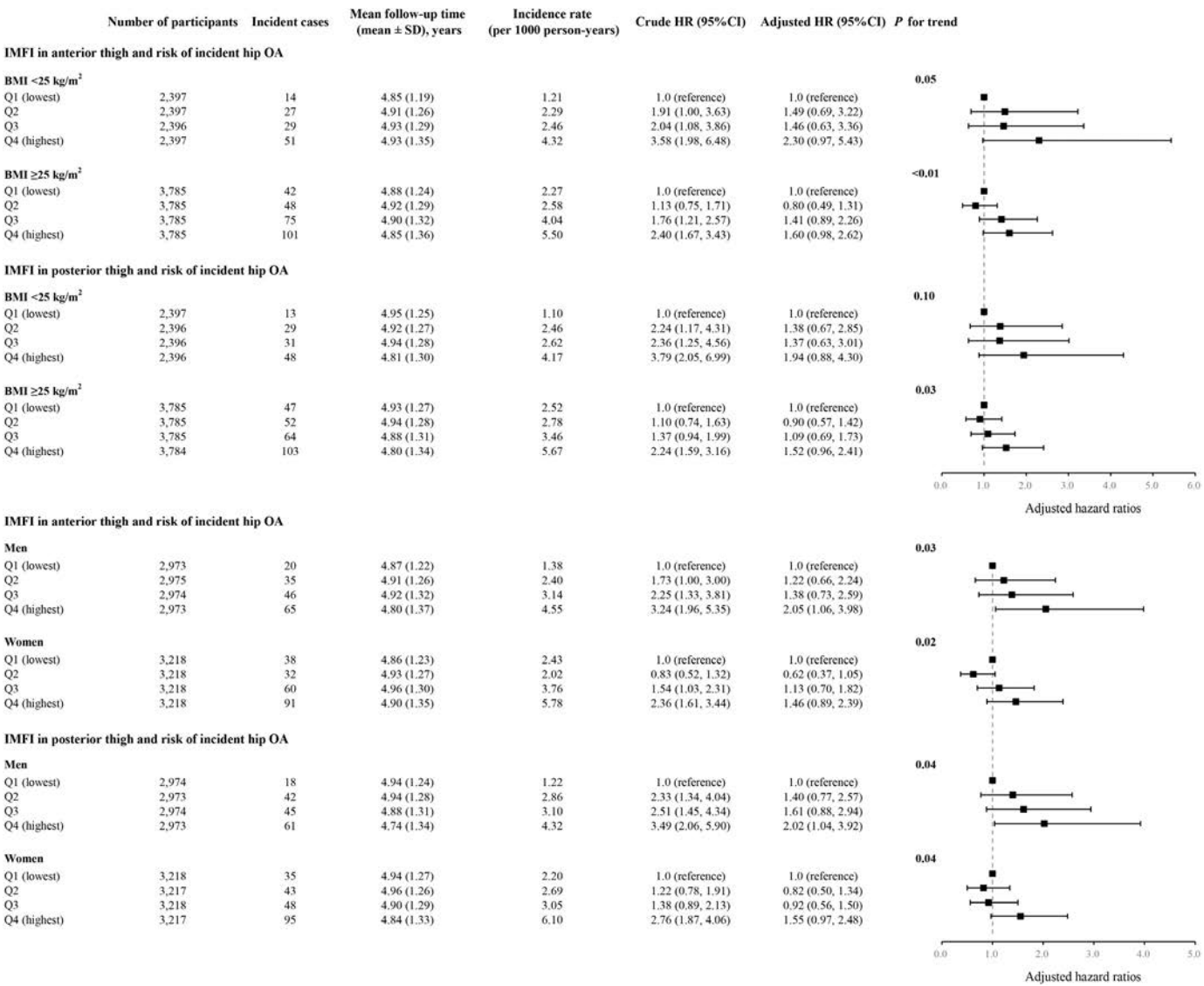


**Figure 4.** Associations between thigh IMFI and incident hip OA by BMI and sex. Data are shown as HRs with 95% CIs. Adjusted HRs are adjusted for age, BMI, smoking, alcohol, educational attainments, physical activity levels, adjusted fat-free muscle volume and handgrip strength. BMI, body mass index; CI, confidence interval; HR, hazard ratio; IMFI, intramuscular fat infiltration; OA, osteoarthritis; Q, quartile.

based on the ICD codes. Secondly, the UK Biobank is composed mostly of White individuals, and these findings may not be generalizable to other populations. Thirdly, due to data limitations in the database, we were unable to analyze the associations between individual muscle groups, such as the quadriceps or hamstrings, and incident knee or hip OA. Fourthly, although we adjusted for established clinical covariates, as with any observational study, our findings are subject to residual or unmeasured confounding. Lastly, although we explored the temporal relationship between IMFI and incident OA, further research is needed to investigate whether this association is causal.

Our study identified the association between IMFI in thigh muscles and incident OA, which was independent of BMI and sex, and provided robust and confirmatory evidence of the critical

role of IMFI as a risk factor for knee and hip OA. This may shed new light on potential OA pathogenesis, such as the synergistic interactions between muscle and joint degeneration. Furthermore, exercise training<sup>11,12</sup> and dietary interventions can reduce the thigh IMFI. Thus, our results further underscore the importance of implementing routine lower extremity stretching and strengthening exercise strategies and healthy diet styles to maintain muscle fitness, which should be beneficial for the prevention and management of OA and curbing its epidemic. In addition, our findings further highlight the potential to optimize muscle-targeted preventive strategies for OA by tailoring interventions based on anterior or posterior muscle groups and sex-specific differences.

In conclusion, this population-based study showed that thigh IMFI was positively associated with an increased risk of

incident knee and hip OA, with a strong dose-response relationship. Because IMFI is a modifiable risk factor, our findings may have important clinical and public health implications for the prevention and management of large joint OA.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Drs Wang and Lei confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

- GBD 2021 Diseases and Injuries Collaborators. Global incidence, prevalence, years lived with disability (YLDs), disability-adjusted life-years (DALYs), and healthy life expectancy (HALE) for 371 diseases and injuries in 204 countries and territories and 811 subnational locations, 1990–2021: a systematic analysis for the Global Burden of Disease Study 2021. *Lancet* 2024;403(10440):2133–2161.
- Weng Q, Chen Q, Jiang T, et al. Global burden of early-onset osteoarthritis, 1990–2019: results from the Global Burden of Disease Study 2019. *Ann Rheum Dis* 2024;83(7):915–925.
- Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *Lancet* 2019;393(10182):1745–1759.
- Duong V, Oo WM, Ding C, et al. Evaluation and treatment of knee pain: a review. *JAMA* 2023;330(16):1568–1580.
- Goodpaster BH, Bergman BC, Brennan AM, et al. Intermuscular adipose tissue in metabolic disease. *Nat Rev Endocrinol* 2023;19(5):285–298.
- Chen W, You W, Valencak TG, et al. Bidirectional roles of skeletal muscle fibro-adipogenic progenitors in homeostasis and disease. *Ageing Res Rev* 2022;80:101682.
- Xu Z, You W, Chen W, et al. Single-cell RNA sequencing and lipidomics reveal cell and lipid dynamics of fat infiltration in skeletal muscle. *J Cachexia Sarcopenia Muscle* 2021;12(1):109–129.
- Delmonico MJ, Harris TB, Visser M, et al; Health, Aging, and Body. Longitudinal study of muscle strength, quality, and adipose tissue infiltration. *Am J Clin Nutr* 2009;90(6):1579–1585.
- Li CW, Yu K, Shyh-Chang N, et al. Pathogenesis of sarcopenia and the relationship with fat mass: descriptive review. *J Cachexia Sarcopenia Muscle* 2022;13(2):781–794.
- Potes Y, Pérez-Martínez Z, Bermejo-Millo JC, et al. Overweight in the Elderly induces a switch in energy metabolism that undermines muscle integrity. *Ageing Dis* 2019;10(2):217–230.
- Holm PM, Blankholm AD, Nielsen JL, et al. Effects of neuromuscular control and strengthening exercises on MRI-measured thigh tissue composition and muscle properties in people with knee osteoarthritis - an exploratory secondary analysis from a randomized controlled trial. *Semin Arthritis Rheum* 2024;65:152390.
- de Almeida AC, Aily JB, Pedrosa MG, et al. A periodized training attenuates thigh intermuscular fat and improves muscle quality in patients with knee osteoarthritis: results from a randomized controlled trial. *Clin Rheumatol* 2020;39(4):1265–1275.
- Honaga K, Mori N, Akimoto T, et al. Investigation of the effect of nutritional supplementation with whey protein and vitamin D on muscle mass and muscle quality in subacute post-stroke rehabilitation patients: a randomized, single-blinded, placebo-controlled trial. *Nutrients* 2022;14(3):14.
- Shen W, Chen J, Zhou J, et al. Effect of 2-year caloric restriction on organ and tissue size in nonobese 21- to 50-year-old adults in a randomized clinical trial: the CALERIE study. *Am J Clin Nutr* 2021;114(4):1295–1303.
- Pandey A, Patel KV, Segar MW, et al. Effect of liraglutide on thigh muscle fat and muscle composition in adults with overweight or obesity: results from a randomized clinical trial. *J Cachexia Sarcopenia Muscle* 2024;15:1072–1083.
- Hall M, van der Esch M, Hinman RS, et al. How does hip osteoarthritis differ from knee osteoarthritis? *Osteoarthritis Cartilage* 2022;30(1):32–41.
- Øiestad BE, Juhl CB, Culvenor AG, et al. Knee extensor muscle weakness is a risk factor for the development of knee osteoarthritis: an updated systematic review and meta-analysis including 46 819 men and women. *Br J Sports Med* 2022;56(6):349–355.
- Loureiro A, Mills PM, Barrett RS. Muscle weakness in hip osteoarthritis: a systematic review. *Arthritis Care Res (Hoboken)* 2013;65(3):340–352.
- Judd DL, Thomas AC, Dayton MR, et al. Strength and functional deficits in individuals with hip osteoarthritis compared to healthy, older adults. *Disabil Rehabil* 2014;36(4):307–312.
- Knoop J, Dekker J, Klein JP, et al. Biomechanical factors and physical examination findings in osteoarthritis of the knee: associations with tissue abnormalities assessed by conventional radiography and high-resolution 3.0 Tesla magnetic resonance imaging. *Arthritis Res Ther* 2012;14(5):R212.
- Kumar D, Karampinos DC, MacLeod TD, et al. Quadriceps intramuscular fat fraction rather than muscle size is associated with knee osteoarthritis. *Osteoarthritis Cartilage* 2014;22(2):226–234.
- Tanner CJ, Barakat HA, Dohm GL, et al. Muscle fiber type is associated with obesity and weight loss. *Am J Physiol Endocrinol Metab* 2002;282(6):E1191–E1196.
- Johnson MA, Polgar J, Weightman D, et al. Data on the distribution of fibre types in thirty-six human muscles. An autopsy study. *J Neurol Sci* 1973;18(1):111–129.
- Sudlow C, Gallacher J, Allen N, et al. UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. *PLoS Med* 2015;12(3):e1001779.
- Littlejohns TJ, Holliday J, Gibson LM, et al. The UK Biobank imaging enhancement of 100,000 participants: rationale, data collection, management and future directions. *Nat Commun* 2020;11(1):2624.
- Linge J, Borga M, West J, et al. Body composition profiling in the UK Biobank Imaging study. *Obesity (Silver Spring)* 2018;26(11):1785–1795.
- West J, Dahlqvist Leinhard O, Romu T, et al. Feasibility of MR-based body composition analysis in large scale population studies. *PLoS One* 2016;11(9):e0163332.
- Karlsson A, Rosander J, Romu T, et al. Automatic and quantitative assessment of regional muscle volume by multi-atlas segmentation using whole-body water-fat MRI. *J Magn Reson Imaging* 2015;41(6):1558–1569.
- Linge J, Petersson M, Forsgren MF, et al. Adverse muscle composition predicts all-cause mortality in the UK Biobank imaging study. *J Cachexia Sarcopenia Muscle* 2021;12(6):1513–1526.
- Styrkarsdóttir U, Lund SH, Thorleifsson G, et al. Cartilage acidic protein 1 in plasma associates with prevalent osteoarthritis and predicts future risk as well as progression to joint replacements: results from the UK Biobank resource. *Arthritis Rheumatol* 2023;75(4):544–552.
- Roos EM, Arden NK. Strategies for the prevention of knee osteoarthritis. *Nat Rev Rheumatol* 2016;12(2):92–101.

32. Blagojevic M, Jinks C, Jeffery A, et al. Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. *Osteoarthritis Cartilage* 2010;18(1):24–33.
33. Liu T, Xu C, Driban JB, et al. Excessive alcohol consumption and the risk of knee osteoarthritis: a prospective study from the Osteoarthritis Initiative. *Osteoarthritis Cartilage* 2022;30(5):697–701.
34. Gill D, Karhunen V, Malik R, et al. Cardiometabolic traits mediating the effect of education on osteoarthritis risk: a Mendelian randomization study. *Osteoarthritis Cartilage* 2021;29(3):365–371.
35. Distefano G, Harrison S, Lynch J, et al. Skeletal muscle composition, power, and mitochondrial energetics in older men and women with knee osteoarthritis. *Arthritis Rheumatol* 2024;76(12):1764–1774.
36. Peng P, Wu J, Fang W, et al. Association between sarcopenia and osteoarthritis among the US adults: a cross-sectional study. *Sci Rep* 2024;14(1):296.
37. Chudasama YV, Khunti KK, Zaccardi F, et al. Physical activity, multimorbidity, and life expectancy: a UK Biobank longitudinal study. *BMC Med* 2019;17(1):108.
38. Borga M, West J, Bell JD, et al. Advanced body composition assessment: from body mass index to body composition profiling. *J Investig Med* 2018;66(5):1–9.
39. Schaubel DE, Wei G. Fitting semiparametric additive hazards models using standard statistical software. *Biom J* 2007;49(5):719–730.
40. Maly MR, Calder KM, Macintyre NJ, et al. Relationship of intermuscular fat volume in the thigh with knee extensor strength and physical performance in women at risk of or with knee osteoarthritis. *Arthritis Care Res (Hoboken)* 2013;65(1):44–52.
41. Zacharias A, Pizzari T, English DJ, et al. Hip abductor muscle volume in hip osteoarthritis and matched controls. *Osteoarthritis Cartilage* 2016;24(10):1727–1735.
42. Taniguchi M, Fukumoto Y, Yagi M, et al. A higher intramuscular fat in vastus medialis is associated with functional disabilities and symptoms in early stage of knee osteoarthritis: a case-control study. *Arthritis Res Ther* 2023;25(1):61.
43. Teichtahl AJ, Wluka AE, Wang Y, et al. Vastus medialis fat infiltration - a modifiable determinant of knee cartilage loss. *Osteoarthritis Cartilage* 2015;23(12):2150–2157.
44. Correa TA, Crossley KM, Kim HJ, et al. Contributions of individual muscles to hip joint contact force in normal walking. *J Biomech* 2010;43(8):1618–1622.
45. Mansfield PJ, Neumann DA. Structure and function of the knee. In: Mansfield PJ, Neumann DA, eds. *Essentials of Kinesiology for the Physical Therapist Assistant*. 3rd ed. Mosby; 2019. <https://www.sciencedirect.com/science/article/pii/B9780323544986000102>
46. Mohajer B, Dolatshahi M, Moradi K, et al. Role of thigh muscle changes in knee osteoarthritis outcomes: osteoarthritis initiative data. *Radiology* 2022;305(1):169–178.
47. Bartsch A, Anderson FL, Fredericson M, et al. Biomechanical and biological factors of sexual dimorphism in anterior knee pain: current concepts. *J ISAKOS* 2024;9(4):788–793.
48. Patterson BE, Girdwood MA, West TJ, et al. Muscle strength and osteoarthritis of the knee: a systematic review and meta-analysis of longitudinal studies. *Skeletal Radiol* 2023;52(11):2085–2097.
49. Faber BG, Macrae F, Jung M, et al. Sex differences in the radiographic and symptomatic prevalence of knee and hip osteoarthritis. *Front Endocrinol (Lausanne)* 2024;15:1445468.
50. Borga M, Ahlgren A, Romu T, et al. Reproducibility and repeatability of MRI-based body composition analysis. *Magn Reson Med* 2020;84(6):3146–3156.

# Regulation of Chondrocyte Metabolism and Osteoarthritis Development by Sirt5 Through Protein Lysine Malonylation

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**Objective.** Chondrocyte metabolic dysfunction plays an important role in osteoarthritis (OA) development during aging and obesity. Protein posttranslational modifications (PTMs) have recently emerged as an important regulator of cellular metabolism. We aim to study one type of PTM, lysine malonylation (MaK), and its regulator sirtuin 5 (Sirt5) in OA development.

**Methods.** Human and mouse cartilage tissues were used to measure SIRT5 and MaK levels. Both systemic and cartilage-specific conditional knockout mouse models were subject to high-fat diet treatment to induce obesity and OA. Proteomics analysis was performed in *Sirt5*<sup>−/−</sup> and wild-type chondrocytes. SIRT5 mutation was identified in the Utah Population Database.

**Results.** We found that SIRT5 decreases while MaK increases in the cartilage during aging. A combination of Sirt5 deficiency and obesity exacerbates joint degeneration in a sex-dependent manner in mice. We further delineate the malonylome in chondrocytes, pinpointing MaK's predominant impact on various metabolic pathways, such as carbon metabolism and glycolysis. Lastly, we identified a rare coding mutation in *SIRT5* that dominantly segregates in a family with OA. The mutation results in substitution of an evolutionally invariant phenylalanine to leucine (F101L) in the catalytic domain. The mutant protein results in a higher MaK level and decreased expression of cartilage extracellular matrix genes and upregulation of inflammation-associated genes.

**Conclusion.** We found that Sirt5-mediated MaK is an important regulator of chondrocyte cellular metabolism, and dysregulation of Sirt5-MaK could be an important mechanism underlying aging- and obesity-associated OA development.

## INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease that affects millions of people worldwide,<sup>1,2</sup> with no currently available disease-modifying drugs. One of the central features of OA is gradual loss of articular cartilage. It has been increasingly recognized that chondrocyte metabolic dysfunctions during various OA risk conditions such as aging, obesity, and injury are an

important mechanism for cartilage degeneration.<sup>3</sup> However, it is still unknown how dysregulated cellular metabolism happens and how to correct it for potential therapeutics development.

Protein posttranslational modifications (PTMs) are key regulators of cellular functions and metabolic pathways.<sup>4–6</sup> Abnormal accumulation of these PTMs in metabolically active organs, such as the liver, has been found to play an important role in the development of obesity- and aging-associated diseases.

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For example, increased acetylation of proteins in the liver that regulate fatty acid oxidation accelerates the development of metabolic syndrome.<sup>7</sup> We previously reported that there is an abnormal accumulation of protein posttranslational malonylation, succinylation, and acetylation at lysine residues in the cartilage of obese mice.<sup>8</sup> This presumably is due to accumulation of reactive acyl-group metabolic byproducts during obesity, such as malonyl-coenzyme A (CoA), succinyl-CoA, and acetyl-CoA, a phenomenon termed as “cellular carbon stress.”<sup>5</sup> Mammals possess seven sirtuins (Sirt1–7), several of which have the ability to remove those PTMs.<sup>4</sup> Sirt5 is a NAD<sup>+</sup>-dependent deacylase that can remove various acyl groups from lysine residues, such as malonyl,<sup>9</sup> succinyl,<sup>9</sup> and glutaryl<sup>10</sup> groups. Through its demalonylation activity, Sirt5 can modulate the levels of lysine malonylation (MaK), a PTM that has been demonstrated to mainly target glycolysis in the liver and is linked to metabolic disorders.<sup>11</sup> We reasoned that Sirt5–MaK signaling axis could potentially play an important role in chondrocyte functions and OA development because chondrocytes rely substantially on glycolysis to generate ATP.<sup>12–14</sup> Indeed, we previously discovered that Sirt5 expression declines in chondrocytes during aging, leading to increased MaK and altered glucose metabolism.<sup>15</sup> Moreover, obesity also increases MaK in chondrocytes, which may further impair their function and contribute to OA development.

In this study, we aimed to explore the age-related changes in Sirt5–MaK signaling and investigate the impact of SIRT5 deficiency in combination with obesity on chondrocyte function and elucidate the molecular mechanisms by which SIRT5 regulates cartilage homeostasis. Additionally, we identified a rare, dominantly inherited SIRT5 mutation (*SIRT5*<sup>F101L</sup>) in a familial OA cohort and examined its functional consequences in chondrocytes. Our findings provide new insights into the role of SIRT5 in cartilage metabolism and OA pathogenesis, offering potential avenues for therapeutic intervention in this debilitating disease.

## MATERIALS AND METHODS

**Animals and diet experiments.** Sirt5 heterozygous knockout mice (*B6;129-Sirt5*<sup>tm1FWa/J</sup>, stock 012757), *Aggrecan-Cre*<sup>ERT2</sup> mice (*B6.Cg-Acan*<sup>tm1(Cre/ERT2)Crm/J</sup>, stock 019148), and *Sirt5*<sup>flx/flx</sup> mice (*B6.129S(SJL)-Sirt5*<sup>tm1.1/cs/AuwJ</sup>, stock 033456) were purchased from The Jackson Laboratory. The mice were then bred to generate the genotype required for this study and treated with different diets. Detailed information can be found in the Supplementary Material. All animal studies were reviewed and approved by the American Association for Accreditation of Laboratory Animal Care International–accredited Institutional Animal Care and Use Committee at the Ohio University. Additional detailed methods are provided in the Supplementary Material.

**Sample size considerations.** Sample size was determined based on a combination of several factors: availability, 3Rs for animal studies, statistical powers, and previous research experience. For example, three young and old human cartilage samples were used as it is challenging to obtain human cartilage tissue with the required specifications, as the availability is often limited. As for the number of mice used for Figure 1D, we based our sample size on prior studies 29,30 investigating protein expression changes in mouse across aging, where similar sample sizes (*n*=3–5 per group) have been used to detect biologically relevant differences. Given the complexity and resource-intensive nature of aging studies in mice, we selected a sample size that balanced statistical power with ethical considerations, following the 3Rs (Replacement, Reduction, and Refinement) principles. Despite the relatively small sample size, our data showed a clear trend consistent with findings on human cartilage. The observed upregulation of MaK in aged mice (Figure 1C & 1D) supports the relevance of our results. As for the number of mice used in our animal studies, we determined the number of animals used in our study (*n*=6–10 per group) based on prior published studies 1,31,32 in the field, including our own, which have used similar sample sizes to detect biologically meaningful differences in the joint. This range has been sufficient in previous work to observe consistent and reproducible effects. Additionally, our findings demonstrate clear trends with statistical significance.

## RESULTS

**Sirt5 decreases while MaK increases in the cartilage during aging.** To investigate the impact of aging on MaK and Sirt5 in joint tissues, we conducted immunohistochemical staining of MaK and SIRT5 in human knee articular cartilage samples obtained from donors spanning different age groups. In the young group, a majority of chondrocytes exhibited robust SIRT5 expression, whereas MaK staining was faint. Conversely, in the elderly group, MaK expression was markedly enhanced, whereas SIRT5 staining was notably reduced (Figure 1A). Further quantitative analysis assessed the proportions of chondrocytes strongly stained, weakly stained, or unstained for SIRT5 and MaK relative to total chondrocyte counts. The results indicated a nearly 70% decline in the ratio of strongly SIRT5-stained chondrocytes in older donors compared with younger ones, whereas MaK expression showed an opposite trend (Figure 1B). To corroborate these findings in humans, we examined cartilage tissue from mice of different ages: young (25 weeks), middle-aged (48 weeks), and old (90 weeks). Consistently, we observed a significant age-associated increase in MaK levels (Figure 1C and 1D).

In summary, our findings suggest that aging is associated with altered expression patterns of MaK and SIRT5 in both human and mouse joint tissues, with MaK levels increasing significantly with age. These results highlight potential age-related changes in metabolic pathways within chondrocytes that merit further investigation.

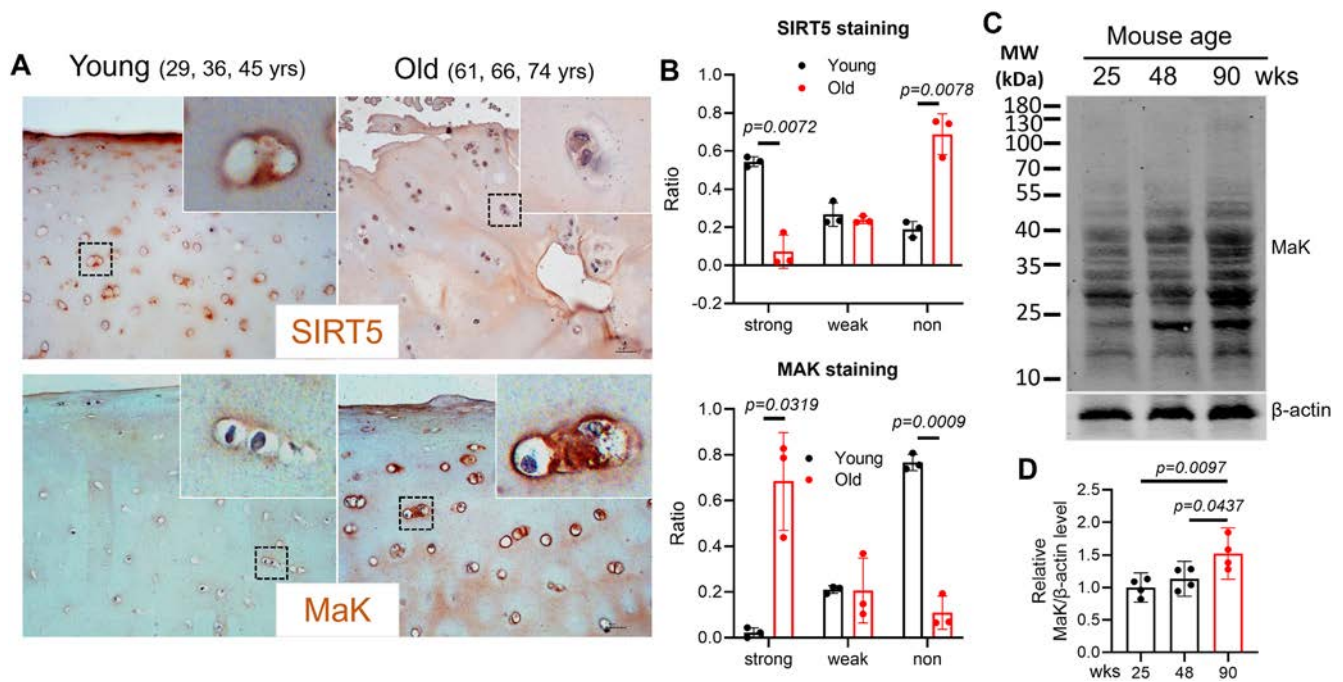


**Figure 1.** Sirt5 decreases, whereas MaK increases in cartilage during aging. (A) Immunohistochemical staining for SIRT5 and MaK in the human articular cartilage tissues from young and old donors ( $n = 3$ ). Insert boxes show the magnified images of the dashed squared area. (B) Quantification of the ratio of strongly, weakly, or nonstained chondrocytes to total chondrocytes. (C) Immunoblotting for MaK and  $\beta$ -actin in the articular cartilage tissues from mice of different ages (25, 48, and 90 weeks). (D) Quantification of the MaK relative to  $\beta$ -actin level ( $n = 4$ ). Data are expressed as the means  $\pm$  SD. MaK, lysine malonylation; MW, molecular weight; SIRT5, sirtuin 5.

### Sirt5 systemic knockout (*Sirt5*<sup>-/-</sup>) exacerbates high-fat diet-induced metabolic dysfunctions and OA development in mice in a sex-dependent manner.

In our previous work, we highlighted Sirt5 as a critical regulator of chondrocyte cellular metabolism,<sup>15</sup> noting that *Sirt5*<sup>-/-</sup> mice exhibit mild spontaneous cartilage degeneration by 10 months of age.<sup>8</sup> We also demonstrated that another major OA risk factor, obesity, also increases MaK in the cartilage tissue.<sup>15</sup> To explore the role of Sirt5 in high-fat diet (HFD)-induced OA development, we subjected both wild-type (WT) and *Sirt5*<sup>-/-</sup> mice to 20 weeks of either HFD or low-fat diet (LFD) to induce obesity. Before dietary intervention, female *Sirt5*<sup>-/-</sup> mice exhibited lower body weight compared with female WT mice, a distinction maintained under LFD conditions but gradually diminishing under HFD (Supplementary Figure 1A, left panel). Similar weight disparities were observed in male mice prediet and with LFD, although HFD induced less weight gain in *Sirt5*<sup>-/-</sup> mice compared with WT (Supplementary Figure 1B). Notably, despite lesser weight gain, *Sirt5*<sup>-/-</sup> mice displayed greater glucose intolerance than WT counterparts irrespective of sex (Supplementary Figure 1C and 1D), indicative of systemic metabolic dysfunctions associated with Sirt5 deficiency. Furthermore, analysis of body composition revealed that HFD resulted in reduced lean mass percentage in both WT and *Sirt5*<sup>-/-</sup> mice across sexes. Interestingly, HFD led to increased fat mass specifically in female *Sirt5*<sup>-/-</sup> mice, whereas both male WT and *Sirt5*<sup>-/-</sup> mice showed elevated fat mass under HFD conditions (Supplementary Figure 1E and 1F).

These findings underscore the complex interplay between Sirt5 and metabolic homeostasis, highlighting Sirt5 as a potential modulator of diet-induced metabolic changes.

Next, we evaluated the knee joints of these mice. Under LFD conditions, *Sirt5*<sup>-/-</sup> mice exhibited early signs of proteoglycan loss compared with WT mice (Figure 2A, upper panel). With HFD treatment, WT mice showed mild-to-moderate cartilage degeneration with superficial lesions across the joint surface, whereas *Sirt5*<sup>-/-</sup> mice displayed deeper and larger cartilage defects (Figure 2A, lower panel). Although no statistically significant differences were observed in OA Research Society International (OARSI) scores among the groups (Figure 2B and 2D), male *Sirt5*<sup>-/-</sup> mice notably exhibited higher Mankin scores compared with their WT counterparts (Figure 2C and 2E). HFD exacerbated these differences more prominently in male mice than in female mice (Figure 2C and 2E). Conversely, no significant differences were detected in osteophyte scores (Supplementary Figure 2A and 2F) or Tidemark duplication scores (Supplementary Figure 2C and 2H) between genotypes or sexes. *Sirt5*<sup>-/-</sup> mice also exhibited significantly higher cartilage damage scores, particularly exacerbated by HFD in male mice (Supplementary Figure 2B and 2G). Regarding Safranin-O staining, female *Sirt5*<sup>-/-</sup> mice showed more than twice the loss of staining intensity of WT mice in both LFD and HFD groups (Supplementary Figure 2D). In male mice, significant differences were observed only under HFD conditions (Supplementary Figure 2I). Hypertrophic chondrocytes

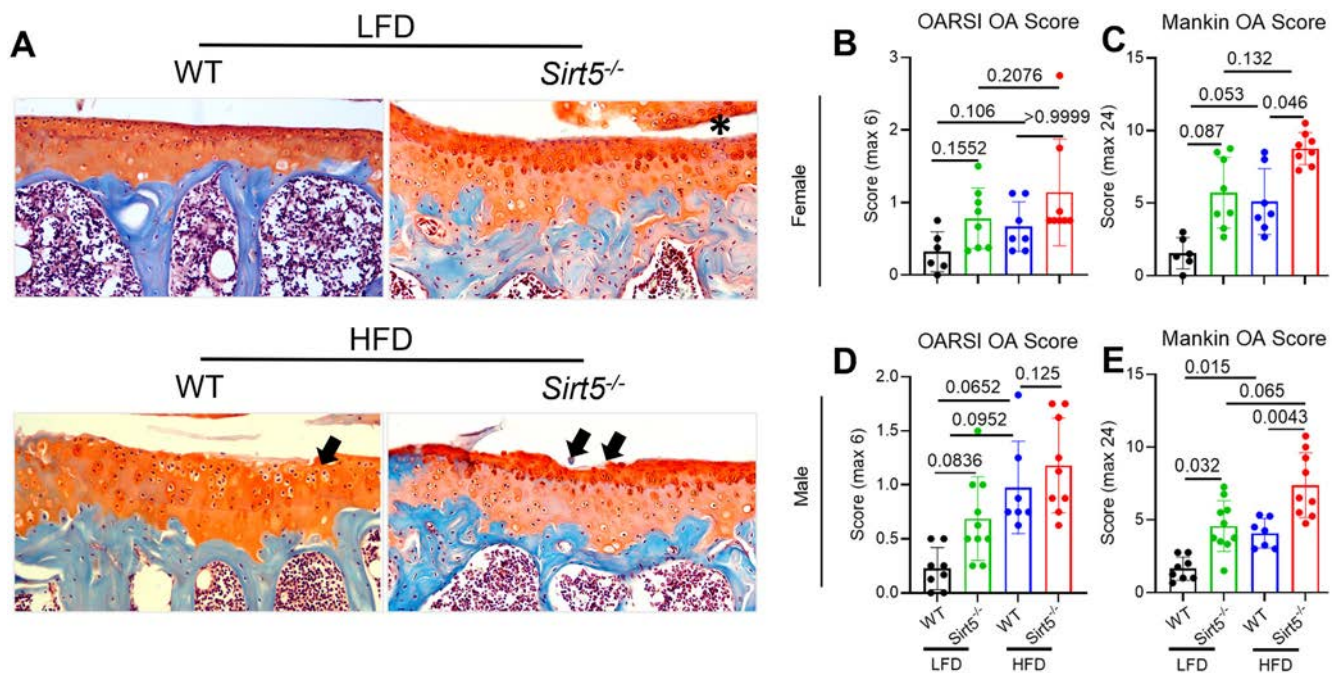


**Figure 2.** Sirtuin 5 systemic knockout mice develop more severe HFD-induced OA. (A) Fast Green/Safranin Orange staining of *Sirt5*<sup>-/-</sup> and WT mouse knee joints (28 weeks old). Pictures show the medial tibias. Asterisk indicates loss of proteoglycan staining and superficial degeneration. Arrows indicate cartilage defects. (B–E) Quantifications of OA severity in male and female *Sirt5*<sup>-/-</sup> and WT mouse knee joints. Two different scoring systems were used: OARS (B and D) and Mankin (C and E). Data are expressed as the means  $\pm$  SD. HFD, high-fat diet; LFD, low-fat diet; OA, osteoarthritis; OARS, Osteoarthritis Research Society International; WT, wild-type. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43164/abstract>.

were more prevalent in *Sirt5*<sup>-/-</sup> mice under both LFD and HFD conditions but were particularly pronounced in male mice (Supplementary Figure 2E and 2J). These findings underscore the differential impact of Sirt5 deficiency on cartilage integrity and OA progression, influenced by diet and gender-specific factors.

### **Sirt5 cartilage-specific knockout (*Sirt5*-CKO) does not affect systemic metabolism but promotes HFD-induced OA development in mice in a sex-dependent manner.**

To elucidate the specific role of Sirt5 in joint-specific OA development, we used *Aggrecan-Cre*<sup>ERT2</sup> mice crossed with *Sirt5*<sup>flox/flox</sup> mice to achieve cartilage-specific knockout of Sirt5. Similarly, both WT (*Sirt5*<sup>flox/flox</sup>) and *Sirt5* conditional knockout (*Sirt5*-CKO; *Aggrecan-Cre*<sup>ERT2</sup>; *Sirt5*<sup>flox/flox</sup>) mice were administered tamoxifen to induce *Sirt5* deletion, followed by exposure to either a HFD or LFD to induce obesity. Unlike systemic knockout models, WT and *Sirt5*-CKO mice showed similar weight gain under HFD conditions in both male and female mice (Supplementary Figure 3A and 3B). Additionally, both groups exhibited comparable glucose intolerance following HFD treatment, as evidenced by nearly two-fold increases in the area under the curve during the intraperitoneal glucose tolerance test compared with LFD groups, irrespective of sex (Supplementary Figure 3C and 3D). Furthermore, HFD led to a reduction in lean mass in both WT and *Sirt5*-CKO mice regardless of sex

(Supplementary Figure 3E). Interestingly, only WT female mice, but not *Sirt5*-CKO female mice, showed a significantly higher percentage of fat mass under HFD conditions, possibly due to greater variability in fat accumulation among *Sirt5*-CKO mice. Conversely, in male mice, both WT and *Sirt5*-CKO mice exhibited significantly increased fat mass percentages after HFD exposure (Supplementary Figure 3F). These findings highlight the joint-specific implications of Sirt5 in metabolic responses to diet-induced obesity, independent of its effects on systemic metabolism.

We then investigated the role of cartilage-specific Sirt5 knockout in OA development. Initially, we confirmed efficient deletion of Sirt5 specifically in cartilage, with no impact on other tissues such as muscle (Supplementary Figure 4A). Subsequent joint analysis revealed that HFD induced typical moderate superficial cartilage lesions, proteoglycan loss, and osteophyte formation in WT mice (Figure 3A). In contrast, *Sirt5*-CKO mice subjected to HFD exhibited more extensive cartilage defects covering approximately half to two-thirds of the articular surface, along with pronounced osteophyte development at the medial tibial side (Figure 3A). Similar to findings from systemic knockout studies, OARS scores did not show statistically significant differences among groups (Figure 3B and 3D). However, under HFD conditions, *Sirt5*-CKO male mice displayed significantly higher Mankin scores compared with WT mice, indicative of more severe OA in *Sirt5*-CKO mice (Figure 3C and 3E), consistent with histologic observations (Figure 3A). Although no statistical significance was observed for osteophyte

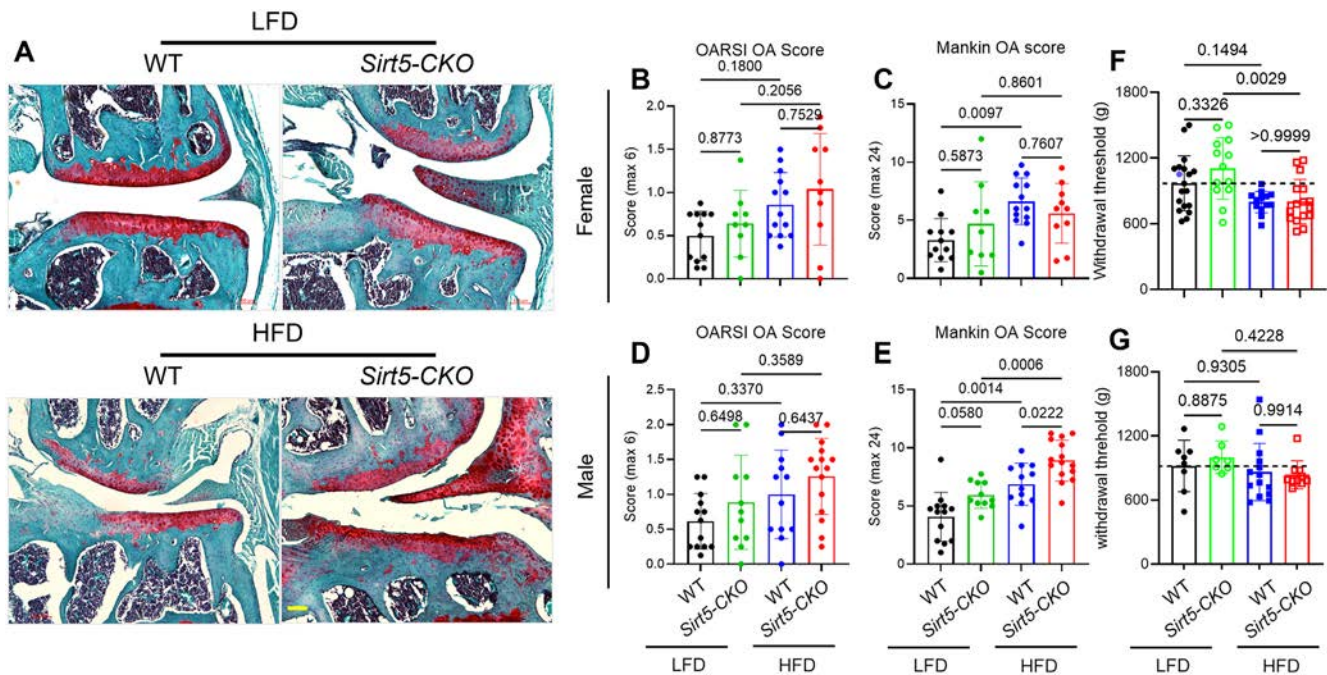


**Figure 3.** *Sirt5* cartilage-specific knockout mice (*Sirt5-CKO*) develop more severe HFD-induced OA. (A) Fast Green/Safranin Orange staining of *Sirt5-CKO* and WT mouse knee joints (32 weeks old). Pictures show the medial compartments, including both femur and tibia. (B–G) Quantifications of OA severity in male and female *Sirt5-CKO* and WT mouse knee joints. Two different scoring systems were used: OARSI (B and D) and Mankin (C and E). (F–G) Knee hyperalgesia as an indicator for OA pain assessed in the same mice. Data are expressed as the means  $\pm$  SD. HFD, high-fat diet; LFD, low-fat diet; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; WT, wild-type. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43164/abstract>.

scores (Supplementary Figure 4B and 4G), *Sirt5-CKO* mice exhibited higher scores, suggesting a trend toward increased osteophyte formation compared with WT. Additionally, *Sirt5-CKO* male mice showed higher cartilage damage scores under HFD conditions (Supplementary Figure 4C and 4H), with no differences in Tidemark duplication or hypertrophic chondrocyte scores observed in either sex (Supplementary Figure 4D, 4I, 4F, and 4K). In terms of proteoglycan staining, *Sirt5-CKO* male mice exhibited reduced staining compared with WT male mice (Supplementary Figure 4E and 4J).

Furthermore, we conducted a knee hyperalgesia assay using pressure application measurement (PAM) to assess joint pain. We observed no significant changes in knee withdrawal thresholds among female mice across different groups (Figure 3F). In contrast, *Sirt5-CKO* male mice under HFD conditions showed a significant reduction in knee withdrawal threshold compared with LFD, suggesting increased susceptibility to knee hyperalgesia in *Sirt5-CKO* male mice under obesity conditions compared with WT (Figure 3G). These results highlight the specific impact of cartilage-specific *Sirt5* deficiency on OA progression and pain sensitivity in a sex-dependent manner.

### ***Sirt5* deficiency in chondrocytes results in decreased expression of extracellular matrix proteins and increased expression of proinflammatory proteins.**

To investigate the role of *Sirt5* deficiency in chondrocyte

functions, we used proteomics to profile global protein expressions in WT and *Sirt5*<sup>−/−</sup> chondrocytes. Principal component analysis showed a nonoverlapping distribution of WT and *Sirt5*<sup>−/−</sup> samples with 70.7% of the variation explained by the principal component 1 (genotype) (Figure 4A), suggesting distinct proteomic profiles of WT and *Sirt5*<sup>−/−</sup> chondrocytes. Furthermore, differential abundance analysis further highlighted significant alterations, revealing 520 up-regulated and 566 down-regulated proteins in *Sirt5*<sup>−/−</sup> chondrocytes compared with WT (Figure 4B). Notably, key extracellular matrix (ECM) proteins like COL2a1 and COL11a2 were markedly down-regulated, whereas inflammation-associated mediators such as prostaglandin endoperoxide synthase 1 and 15-hydroxyprostaglandin dehydrogenase were prominently up-regulated in *Sirt5*<sup>−/−</sup> chondrocytes (Figure 4B). To delve deeper into these changes, we performed cluster analyses using the Online Search Tool for Retrieval of Interacting Genes/Proteins. The up-regulated proteins indicated involvement in crucial signaling pathways such as the MAPK cascade, integrin-mediated signaling, and actin cytoskeleton organization. Conversely, the down-regulated proteins were associated with metabolic pathways and collagen formation and/or cartilage development processes (Figure 4C). These findings align with the established roles of *Sirt5* in cellular metabolism and strongly support our hypothesis that *Sirt5* plays a pivotal role in cartilage tissue homeostasis and potentially in the development of OA.

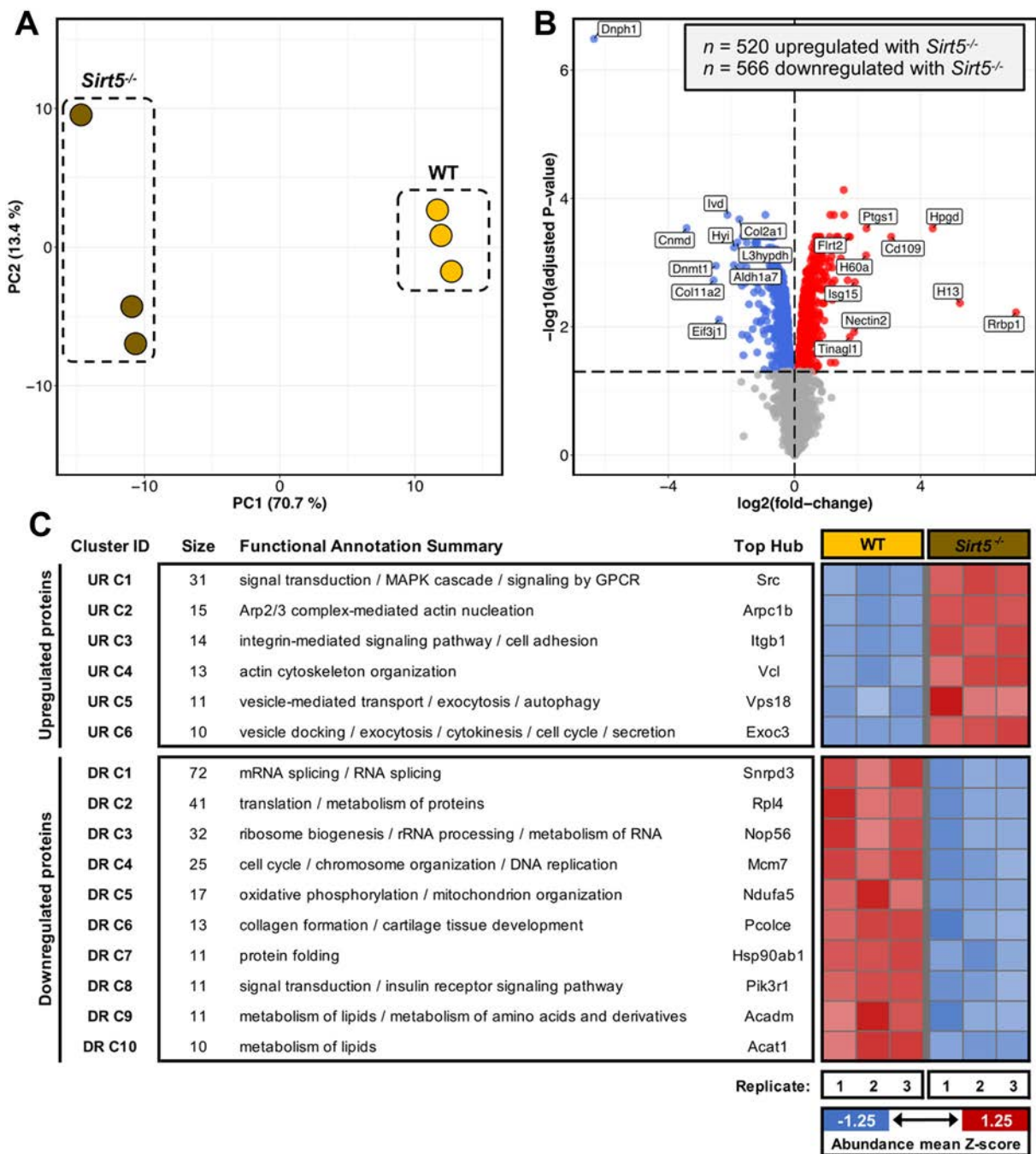


**Figure 4.** Sirt5 deficiency in chondrocytes results in decreased expression of extracellular proteins and increased expression of proinflammatory proteins. (A) Principal component analysis plot of *Sirt5*<sup>-/-</sup> and WT mouse chondrocyte proteomic samples (*n* = 3). (B) Volcano plot depicting protein differential regulation between *Sirt5*<sup>-/-</sup> vs WT samples. Red and blue denote significant up-regulation and significant down-regulation, respectively. The top 10 up-regulated and down-regulated proteins based on magnitude of log2 fold-change are labeled in plot. (C) Overview of protein interaction analysis. Given are all clusters of significantly up-regulated and down-regulated proteins that contain at least 10 proteins. Functional annotation summaries are representative based on enriched Gene Ontology biologic process and Reactome pathway terms. Top hubs have been defined based on eigenvector centrality. Accompanying heatmap depicts mean abundance z score of each cluster in each sample. GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; PC, principal component; rRNA, ribosomal RNA; WT, wild-type. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43164/abstract>.

**Sirt5-regulated MaK is mainly targeting metabolic enzymes in chondrocytes.** Our previous studies have demonstrated that Sirt5 plays a crucial role in regulating chondrocyte

cellular metabolism and the posttranslational modification of lysine residues through malonylation.<sup>15</sup> To investigate the extent of malonylation in primary chondrocytes from WT and *Sirt5*<sup>-/-</sup>

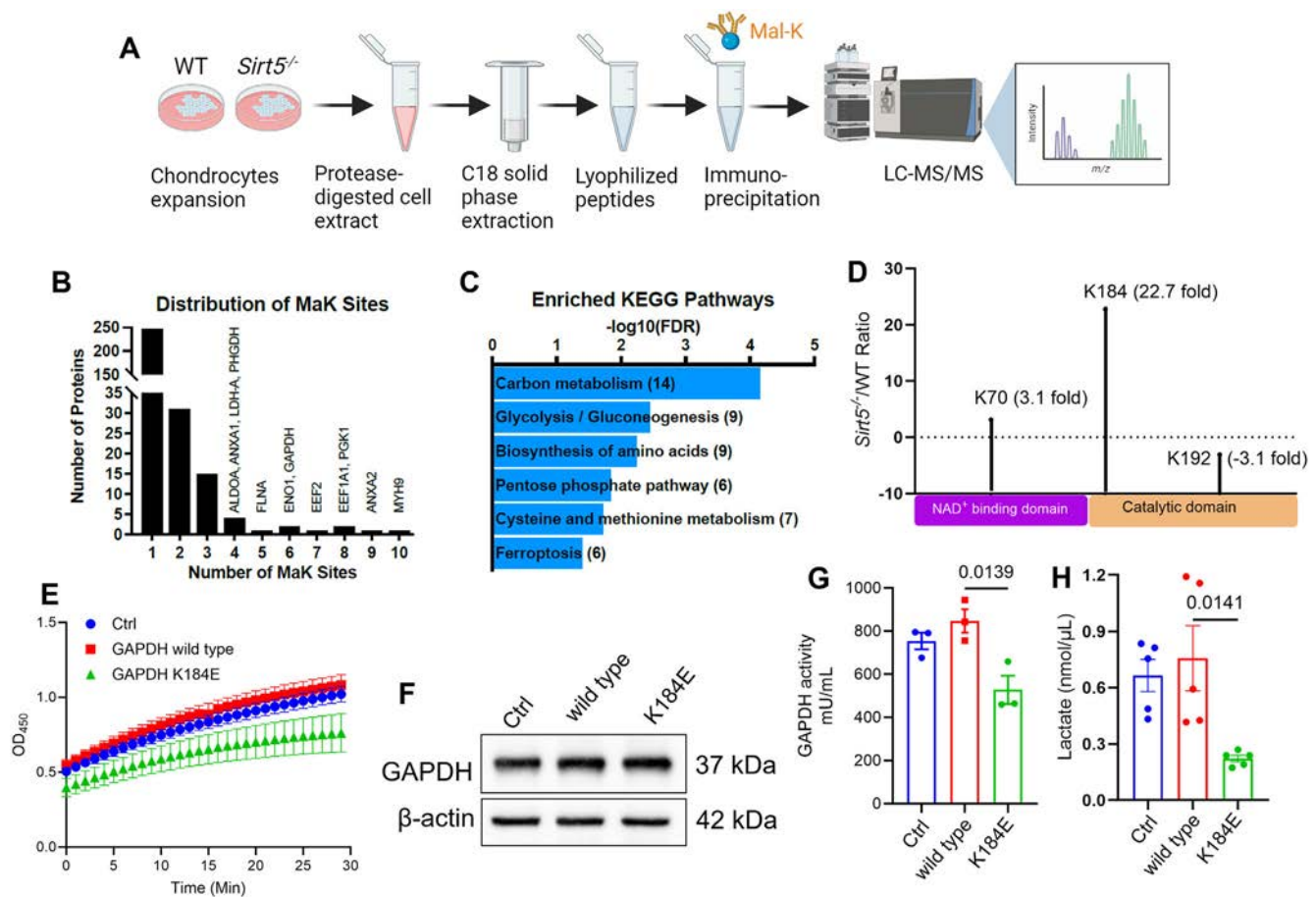


**Figure 5.** Enrichment and identification of lysine malonylome in mouse primary chondrocytes by label-free quantification. (A) Schematic description of experimental procedures of the label-free quantification of lysine malonylome. (B) Distribution of the number of MaK sites identified per protein in chondrocytes. Most malonylated proteins contain a single site, but some contain multiple sites. (C) KEGG pathway enrichment analysis of malonylated proteins in chondrocytes. Numbers in the brackets show the number of malonylated proteins in the pathways. (D) Sites of malonylation in the GAPDH protein with respect to its two functional domains, NAD binding, and catalytic domains. The ratio of malonylation between WT and *Sirt5*<sup>-/-</sup> samples are shown above. (E) GAPDH enzymatic activity kinetics assay. The assay includes on-transfected Ctrl, wild-type GAPDH, and K184E. (F) Western blotting for GAPDH to confirm successful transfection of WT and mutant GAPDH in chondrocytes. (G) Quantifications of GAPDH activity in mouse primary chondrocytes transfected with WT or mutant GAPDH. (H) Quantification of lactate production in mouse primary chondrocytes transfected with WT or mutant GAPDH. Data are expressed as the means ± SD. Ctrl, control; FDR, false discovery rate; K184E, mutant GAPDH; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, liquid chromatography mass spectrometry/mass spectrometry; MaK, lysine malonylation; WT, wild-type. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43164/abstract>.

mice, we employed a previously established workflow for antibody-based enrichment and identification of peptides from protein digests containing malonylation modifications (MaK) (Figure 5A). In total, we quantified over 1,000 malonylated peptides covering 469 protein sites across all samples. Among these sites, 152 exhibited a measurable difference between WT and *Sirt5*<sup>-/-</sup> chondrocytes. Notably, 52.3% of the identified proteins had a single malonylation site, whereas 47.7% had two or more MaK sites (Figure 5B).

We performed Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis on the malonylated proteins and found significant enrichment in pathways related to carbon metabolism and glycolysis and/or gluconeogenesis (Figure 5C). Glycolysis is a fundamental metabolic pathway in

chondrocytes, providing ATP, and our findings suggest that *Sirt5*-regulated malonylation targets key metabolic proteins in this context.

Given the enrichment of MaK modifications in glycolytic enzymes and the critical role of glycolysis in chondrocyte energy metabolism, we compared the relative abundance of MaK in all detected glycolytic enzymes between *Sirt5*<sup>-/-</sup> and WT chondrocytes. Among them, GAPDH, which catalyzes the conversion of glyceraldehyde 3-phosphate to glycerate 1,3-bisphosphate, showed significant dynamic regulation (with *Sirt5*<sup>-/-</sup>:WT ratios of 3.1, 22.7, and -3.1 for three lysine sites) (Figure 5D).

To explore the functional implications of malonylation on these glycolytic enzymes and their regulation by *Sirt5*, we investigated whether modification of the most malonylated lysine

184 affects GAPDH enzymatic activity. We generated a mutated mouse GAPDH expression vector in which lysine 184 was substituted with glutamate (K184E), mimicking MaK modification by introducing a negatively charged residue. Mouse primary chondrocytes were transfected with either WT or mutant GAPDH. A kinetic enzymatic assay revealed that the GAPDH K184E mutant significantly reduced enzymatic reaction rates compared with both WT GAPDH and nontransfected controls (Figure 5E, 5F, and 5G). Furthermore, we measured the final glycolysis product lactate and found that the GAPDH K184E mutant also significantly decreased lactate production compared with WT GAPDH and control groups (Figure 5G). These results underscore the functional impact of Sirt5-regulated malonylation on glycolytic enzymes in chondrocytes, highlighting a novel regulatory mechanism in cellular metabolism.

**Identification and functional analysis of a human OA-associated *SIRT5*<sup>F101L</sup> mutation.** We further explored the clinical relevance of our study. Using the Utah Population Database, we identified families in which OA segregates as a dominant trait, with multiple members diagnosed with early-onset or severe OA in various joints, including the first metatarsophalangeal joint, erosive hand OA, finger interphalangeal joint OA, thumb OA, or shoulder OA.<sup>16–18</sup> We completed genomic analyses for 151 families and identified a rare *SIRT5*-coding mutation (rs201979175, c. C303G:p.F101L, minor allele frequency 0.00004303) in one family that segregates dominantly with finger interphalangeal joint OA (hand OA). Members of this family also have OA in other joints, including the spine, wrist, and shoulder (Figure 6A). The *SIRT5*<sup>F101L</sup> mutation occurs in an evolutionarily conserved amino acid; the WT allele, phenylalanine, is invariant in the vertebrate lineage

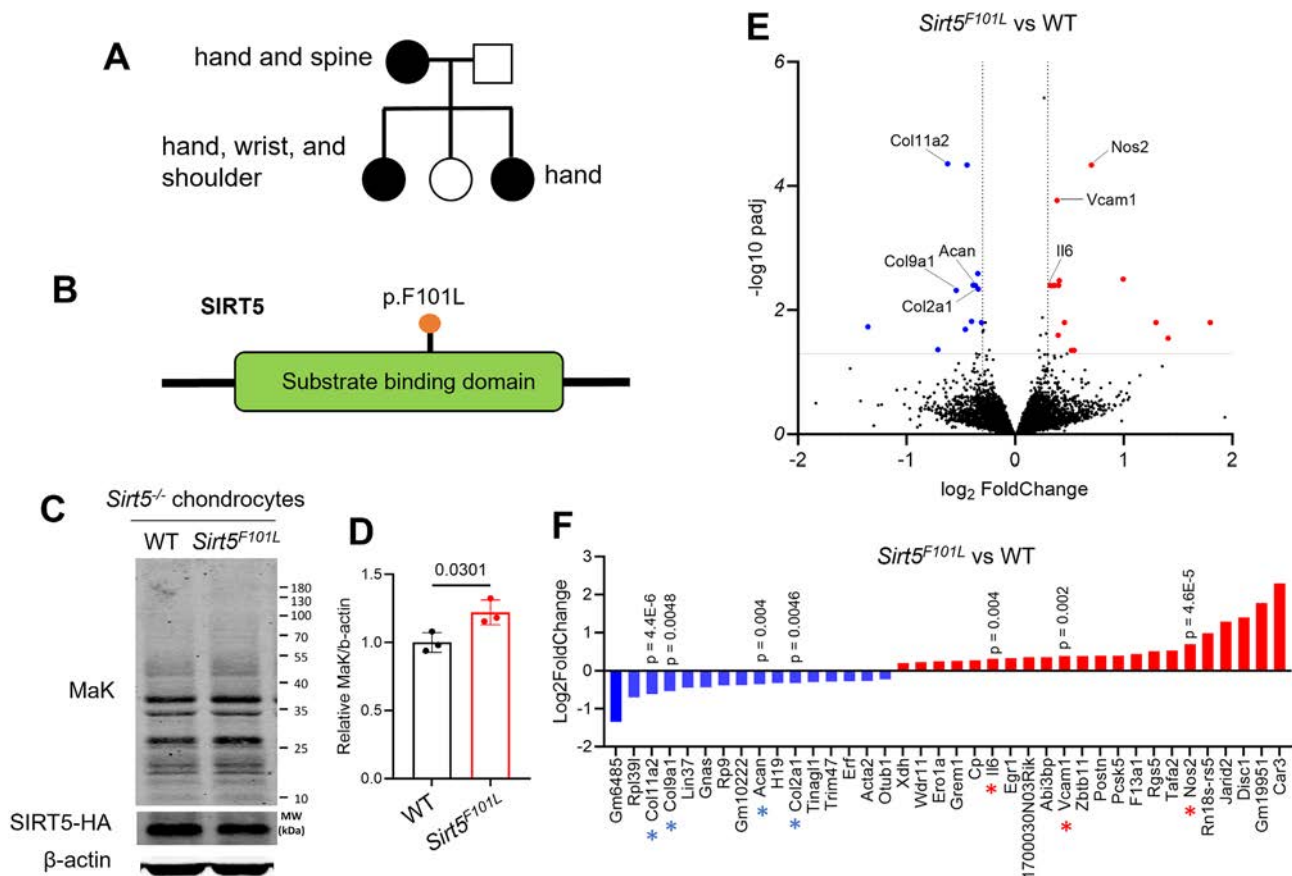


**Figure 6.** Identification of the human osteoarthritis (OA)-associated *SIRT5*<sup>F101L</sup> mutation. (A) A rare *SIRT5* coding mutation dominantly segregates in a family with hand OA. Circles, female mice; square, male mice; black circles and squares, individuals affected with hand OA and individuals that also had other joints affected. (B) Whole exome sequencing identified a mutation in a highly conserved amino acid (F101L) (F is invariant in the vertebrate lineage) in the substrate-binding domain of *SIRT5*. (C) Western blot analysis indicates chondrocytes transfected with *SIRT5*<sup>F101L</sup> mutant have a higher level of MaK compared with WT. (D) Quantification of the band intensity between WT and *SIRT5*<sup>F101L</sup> mutant in the blot in panel (C) ( $n = 3$ ). (E) Bulk RNA-sequencing analysis of primary mouse chondrocytes transfected with WT or *SIRT5*<sup>F101L</sup> mutant constructs. Expression of *Sirt5*<sup>F101L</sup> causes a reduction of multiple cartilage extracellular matrix genes (*Col11a2*, *Col9a1*, *Acan*, and *Col2a1*) and an increase of inflammation-associated genes (*Nos2*, *Il6*, and *Vcam1*) ( $n = 3$ ). (F) List of up-regulated or down-regulated genes as colored in the panel (E). Asterisk indicates statistically significantly changed genes;  $P$  values are shown above each gene. Data are expressed as the means  $\pm$  SD. MaK, lysine malonylation; *SIRT5*, sirtuin 5; WT, wild-type. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43164/abstract>.

(Figure 6B). The F101L mutation is located in the substrate-binding domain (Figure 6B) and is predicted to be damaging or disease-causing by multiple in silico pathogenicity prediction programs (eg, SIFT and MutationTaster). These data indicate that a rare, dominant coding mutation in *SIRT5* is associated with familial OA.

To determine if the *Sirt5*<sup>F101L</sup> mutation alters Sirt5 protein function, we tested if overexpression of *Sirt5*<sup>F101L</sup> in primary mouse chondrocytes affected protein MaK and gene expression. Primary mouse *Sirt5*<sup>-/-</sup> chondrocytes were transfected with WT or *Sirt5*<sup>F101L</sup>, and protein extracts were collected for MaK analysis. Immunoblot analysis indicates chondrocytes transfected with *Sirt5*<sup>F101L</sup> have a higher level of MaK compared with WT (Figure 6C and 6D). WT and *Sirt5*<sup>F101L</sup> transfected primary chondrocytes were also used for bulk RNA-sequencing analysis. Expression of *Sirt5*<sup>F101L</sup> caused a reduction of multiple cartilage ECM genes (*Col11a2*, *Col9a1*, *Acan*, and *Col2a1*) and an increase of inflammation-associated genes (*Nos2*, *Il6*, and *Vcam1*) (Figure 6F). These data are consistent with changes we observe in MaK levels and protein expression in *Sirt5*<sup>-/-</sup> chondrocytes, suggesting that *Sirt5*<sup>F101L</sup> is hypomorphic. In sum, our molecular, cellular, and genetic data support a central role for SIRT5 in maintaining chondrocytes homeostasis.

## DISCUSSION

Our study delves into the role of SIRT5 and its associated posttranslational MaK in chondrocyte metabolism and OA pathogenesis. We observed that aging and obesity lead to significant alterations in the expression and activity of SIRT5, contributing to cartilage degeneration and OA development. Our findings underscore the potential of targeting SIRT5 and its regulatory pathways as a therapeutic strategy for OA.

The sirtuin family of histone deacetylases has seven highly conserved NAD-dependent deacetylases, which were discovered in 1999 to 2000.<sup>19,20</sup> Sirtuins regulate key biologic processes in mammals, including many aspects of metabolism, stress response, and signaling.<sup>21</sup> In addition to their well-studied roles as lysine deacetylases, certain sirtuins can also remove other types of acyl modifications from lysine residues, including propionyl, butyryl, malonyl, succinyl, and the long-chain fatty acid–derived myristoyl and palmitoyl groups.<sup>9,10,22–24</sup> Sirtuins regulate numerous processes in vivo, including metabolism, DNA repair, metastasis, apoptosis, and translation while promoting longevity and protecting against cancer.<sup>21</sup> Sirt5 was initially reported as a lysine deacetylase found in both mitochondria and cytoplasm.<sup>25</sup> However, recent studies have shown it has low deacetylase activity and strong demalonylase and desuccinylase activity.<sup>9</sup> Sirt5 deficiency in liver causes hypermalonylation of proteins that are enriched in glycolytic pathway, as identified by mass spectrometry of immunoprecipitated malonyl-lysine peptides.<sup>11</sup> Consistently, *Sirt5*<sup>-/-</sup> hepatocytes show decreased production

of lactic acid and reduced glycolytic flux.<sup>11</sup> We previously reported that *Sirt5*<sup>-/-</sup> primary chondrocytes isolated in vitro have up-regulated MaK and impaired cellular metabolism.<sup>15</sup> In this study, we further discovered that SIRT5 decreases in accordance with an increase of MaK during aging in both human and mouse cartilage. MaK has also been strongly implicated in the pathogenesis of metabolic diseases. For example, a large-scale analysis of protein malonylation in livers of *db/db* mice showed up-regulated MaK of metabolic enzymes involved in glucose catabolism and fatty acid oxidation.<sup>26</sup> We also previously discovered that the MaK level is increased in the cartilage of obese mice compared with lean mice.<sup>8</sup> These early findings prompted us to further investigate the combined role of Sirt5 deficiency and obesity in OA development. Using both systemic and conditional knockout mouse models, our results demonstrated that obesity and Sirt5 deficiency synergistically promote chondrocyte metabolic dysfunction and cartilage degeneration. This finding potentially reveals a common signaling pathway through which aging and obesity—the two major risk factors for OA—converge to promote chondrocyte metabolic dysfunction and OA development.

Emerging evidence has suggested that acyl-based PTMs at lysine residues are crucial regulators of cellular metabolism. Proteomic studies identifying the malonylome, succinylome, and acetylome have found that metabolic proteins are enriched in these datasets.<sup>10,11,27–30</sup> Gene ontology analyses on acetylated proteins consistently reveal that metabolic pathways, including fatty acid metabolism, glycolysis, and amino acid metabolism, are among the top targets for these PTMs. However, it remains largely unknown how sites of malonylation, succinylation, and glutarylation identified in proteomic studies affect the activity of their targets and the pathophysiology at the whole-organismal level. Malonylation is a relatively unexplored PTM compared with succinylation and acetylation. Previous research has discovered that malonylation suppresses voltage-dependent anion channel 2,<sup>31</sup> mammalian target of rapamycin,<sup>32</sup> and GAPDH<sup>11</sup> while activating acetyl-CoA carboxylase 1.<sup>33</sup> Malonylation has been implicated in several pathophysiologic processes, including sepsis-induced myocardial dysfunction,<sup>31</sup> angiogenesis,<sup>32</sup> and hepatic steatosis.<sup>33</sup> In this study, we are the first to use affinity enrichment-based proteomics to identify the malonylome in chondrocytes. We demonstrated a similar suppressive effect of GAPDH malonylation on its activity and glycolysis in chondrocytes. Combined with our data from both systemic and conditional Sirt5 knockout mouse models, our findings provide strong evidence that MaK in cartilage tissue during aging and obesity conditions could be an important underlying molecular mechanism for OA development.

Our study provides significant insights into the sex-dependent effects of Sirt5 deficiency and HFD treatment on obesity and OA development in mice. We found that systemic Sirt5 knockout (*Sirt5*<sup>-/-</sup>) female mice maintained a lower body weight than WT under LFD, a difference that diminished with HFD. In male mice, *Sirt5*<sup>-/-</sup> mice gained less weight than WT under HFD,

indicating that Sirt5 deficiency may mitigate some HFD-induced weight gain, especially in male mice. Despite lesser weight gain, both male and female *Sirt5*<sup>-/-</sup> mice showed greater glucose intolerance compared with WT controls, highlighting metabolic dysfunctions linked to Sirt5 deficiency. HFD increased fat mass specifically in female *Sirt5*<sup>-/-</sup> mice, whereas both male WT and *Sirt5*<sup>-/-</sup> mice had elevated fat mass. This suggests that female *Sirt5*<sup>-/-</sup> mice may be more susceptible to HFD-induced adiposity despite their lower body weight. These findings differ from a previous study showing no significant metabolic abnormalities in *Sirt5*<sup>-/-</sup> mice under either diet,<sup>34</sup> possibly due to different HFD treatment durations (10 vs 20 weeks). The systemic metabolic differences between WT and *Sirt5*<sup>-/-</sup> were not seen in the *Sirt5*-CKO study. These findings underscore the importance of considering sex as a biologic variable in metabolic studies and suggest potential sex-specific mechanisms by which Sirt5 influences adiposity and glucose metabolism.

Our results showed that male *Sirt5*<sup>-/-</sup> mice had higher Mankin scores and more cartilage damage under HFD conditions than female mice. Similarly, *Sirt5*-CKO mice under HFD had more cartilage defects and higher Mankin scores compared with WT mice, with male mice showing more severe OA. The PAM assay demonstrated sex-dependent differences in OA pain. Whereas knee withdrawal thresholds did not change significantly in female mice across groups, male *Sirt5*-CKO mice under HFD had a significant reduction in knee withdrawal threshold compared with LFD. These findings indicate a greater susceptibility to diet-induced OA progression in male mice and a potential sex-specific response to Sirt5 deficiency in joint tissues. Our results are consistent with well-documented sex-dependent differences of obesity and OA pathogenesis in both clinical<sup>35,36</sup> and preclinical studies. For example, it was found that male mice seem to be developing more consistent OA pathologies in response to different risk factors such as joint injury<sup>37,38</sup> and aging.<sup>39</sup> Because C57BL/6J female mice are typically more resistant to the obesogenic effects of a HFD treatment,<sup>40,41</sup> they are often excluded from obesity-associated OA studies.<sup>42</sup> Our results, to the best of our knowledge, are the first to report that, similar to joint injury-induced OA, obesity-associated OA appears to be more severe in male mice compared with female mice.

The identification of the human *SIRT5*<sup>F101L</sup> mutation in a family with dominantly inherited OA provides valuable insights into the genetic underpinnings of OA and underscores the critical role of SIRT5 in joint health. Our findings suggest that this rare mutation may reduce SIRT5 functions and contribute to the pathogenesis of OA through alterations in chondrocyte metabolism. The *SIRT5*<sup>F101L</sup> mutation was identified in a multigenerational family with a high incidence of OA, affecting various joints, including the spine, wrist, and shoulder. The evolutionary conservation of the phenylalanine residue at position 101 and its predicted pathogenicity by multiple in silico tools strongly indicate the functional importance of this amino acid. The mutation's

location in the substrate-binding domain suggests a potential impact on SIRT5's enzymatic activity, further supported by our functional assays demonstrating that *Sirt5*<sup>F101L</sup> has decreased demalonylase activity and disrupts gene expression in chondrocytes. Many (if not all) of the previously identified OA-associated mutations are non-null, even those identified from family studies.<sup>43–49</sup> By studying a hypomorphic human OA-associated allele of *Sirt5*<sup>F101L</sup>, we could potentially uncover novel metabolic and posttranslational effects that we could not detect using a null allele of *Sirt5*. Our ongoing effort includes creating a novel mouse model harboring the *Sirt5*<sup>F101L</sup> mutation, examine its susceptibility to different OA risk factors, and identify potential chemical modulators to increase its enzymatic activity.

Our study also is not without limitations. Firstly, the mild OA phenotype observed in mice during aging and obesity may not fully capture the complexity of the disease's progression in patients with more severe pain. Secondly, the use of proteomics in in vitro cell culture may not accurately reflect the in vivo environment, potentially affecting the translatability of the results. Thirdly, more human cartilage tissues are needed to confirm the changes of SIRT5 and MaK during aging. Additionally, because chondrocytes were pooled for identifying the malonylome, the ability to perform statistical analysis for this experiment is limited. Lastly, although the data we generated using cells transfected with *Sirt5*<sup>F101L</sup> mutant provides valuable insights, they may not entirely replicate the physiologic conditions in vivo. Therefore, more research directly in the *Sirt5*<sup>F101L</sup> mutant mice is needed to understand the mutation's role in OA development.

In conclusion, our study has elucidated the critical role of Sirt5 and MaK in chondrocyte metabolism and the pathogenesis of OA during aging and obesity. We have demonstrated that Sirt5 deficiency, combined with obesity, significantly exacerbates joint degeneration in mice and that MaK predominantly impacts metabolic pathways such as carbon metabolism and glycolysis. Furthermore, the discovery of a rare coding mutation in SIRT5 that segregates in a family with hand OA highlights the potential genetic contributions to OA development. These findings suggest that dysregulation of the Sirt5-MaK axis could be a pivotal mechanism underlying OA development associated with aging and obesity, offering new avenues for therapeutic interventions.

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## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Zhu confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements. We thank Dr. Martin Lotz from Scripps Research for providing us with human cartilage samples.

## REFERENCES

- Wallace IJ, Worthington S, Felson DT, et al. Knee osteoarthritis has doubled in prevalence since the mid-20th century. *Proc Natl Acad Sci USA* 2017;114(35):9332–9336.
- Yelin E, Weinstein S, King T. The burden of musculoskeletal diseases in the United States. *Semin Arthritis Rheum* 2016;46(3):259–260.
- Batushansky A, Zhu S, Komaravolu RK, et al. Fundamentals of OA. An initiative of Osteoarthritis and Cartilage. Obesity and metabolic factors in OA. *Osteoarthritis Cartilage* 2022;30(4):501–515.
- Carrico C, Meyer JG, He W, et al. The mitochondrial acylome emerges: proteomics, regulation by sirtuins, and metabolic and disease implications. *Cell Metab* 2018;27(3):497–512.
- Trub AG, Hirschey MD. Reactive acyl-CoA species modify proteins and induce carbon stress. *Trends Biochem Sci* 2018;43(5):369–379.
- Wagner GR, Hirschey MD. Nonenzymatic protein acylation as a carbon stress regulated by sirtuin deacylases. *Mol Cell* 2014;54(1):5–16.
- Hirschey MD, Shimazu T, Jing E, et al. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. *Mol Cell* 2011;44(2):177–190.
- Liu H, Rosol TJ, Sathaseelan R, et al. Cellular carbon stress is a mediator of obesity-associated osteoarthritis development. *Osteoarthritis Cartilage* 2021;29(9):1346–1350.
- Du J, Zhou Y, Su X, et al. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. *Science* 2011;334(6057):806–809.
- Tan M, Peng C, Anderson KA, et al. Lysine glutarylation is a protein posttranslational modification regulated by SIRT5. *Cell Metab* 2014;19(4):605–617.
- Nishida Y, Rardin MJ, Carrico C, et al. SIRT5 regulates both cytosolic and mitochondrial protein malonylation with glycolysis as a major target. *Mol Cell* 2015;59(2):321–332.
- Hollander JM, Zeng L. The emerging role of glucose metabolism in cartilage development. *Curr Osteoporos Rep* 2019;17(2):59–69.
- Rajpurohit R, Mansfield K, Ohyama K, et al. Chondrocyte death is linked to development of a mitochondrial membrane permeability transition in the growth plate. *J Cell Physiol* 1999;179(3):287–296.
- Tchetina EV, Markova GA. Regulation of energy metabolism in the growth plate and osteoarthritic chondrocytes. *Rheumatol Int* 2018;38(11):1963–1974.
- Zhu S, Batushansky A, Jopkiewicz A, et al. Sirt5 deficiency causes posttranslational protein malonylation and dysregulated cellular metabolism in chondrocytes under obesity conditions. *Cartilage* 2021;13(2\_suppl):1185S–1199S.
- Gavile CM, Kazmers NH, Novak KA, et al. Familial clustering and genetic analysis of severe thumb carpometacarpal joint osteoarthritis in a large statewide cohort. *J Hand Surg Am* 2022;47(10):923–933.
- Juryneć MJ, Gavile CM, Honeggar M, et al. NOD/RIPK2 signalling pathway contributes to osteoarthritis susceptibility. *Ann Rheum Dis* 2022;81(10):1465–1473.
- Kazmers NH, Meeks HD, Novak KA, et al. Familial clustering of erosive hand osteoarthritis in a large statewide cohort. *Arthritis Rheumatol* 2021;73(3):440–447.
- Frye RA. Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochem Biophys Res Commun* 1999;260(1):273–279.
- Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* 2000;273(2):793–798.
- Hall JA, Dominy JE, Lee Y, et al. The sirtuin family's role in aging and age-associated pathologies. *J Clin Invest* 2013;123(3):973–979.
- Imai S, Armstrong CM, Kaerberlein M, et al. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 2000;403(6771):795–800.
- Jiang H, Khan S, Wang Y, et al. SIRT6 regulates TNF- $\alpha$  secretion through hydrolysis of long-chain fatty acyl lysine. *Nature* 2013;496(7443):110–113.
- Peng C, Lu Z, Xie Z, et al. The first identification of lysine malonylation substrates and its regulatory enzyme. *Mol Cell Proteomics* 2011;10(12):M111.012658.
- Mahlknecht U, Ho AD, Letzel S, et al. Assignment of the NAD-dependent deacetylase sirtuin 5 gene (SIRT5) to human chromosome band 6p23 by in situ hybridization. *Cytogenet Genome Res* 2006;112(3–4):208–212.
- Du Y, Cai T, Li T, et al. Lysine malonylation is elevated in type 2 diabetic mouse models and enriched in metabolic associated proteins. *Mol Cell Proteomics* 2015;14(1):227–236.
- Park J, Chen Y, Tishkoff DX, et al. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. *Mol Cell* 2013;50(6):919–930.
- Rardin MJ, He W, Nishida Y, et al. SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks. *Cell Metab* 2013;18(6):920–933.
- Weinert BT, Schölz C, Wagner SA, et al. Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep* 2013;4(4):842–851.
- Rardin MJ, Newman JC, Held JM, et al. Label-free quantitative proteomics of the lysine acetylome in mitochondria identifies substrates of SIRT3 in metabolic pathways. *Proc Natl Acad Sci USA* 2013;110(16):6601–6606.
- She H, Tan L, Du Y, et al. VDAC2 malonylation participates in sepsis-induced myocardial dysfunction via mitochondrial-related ferroptosis. *Int J Biol Sci* 2023;19(10):3143–3158.
- Bruning U, Morales-Rodríguez F, Kalucka J, et al. Impairment of angiogenesis by fatty acid synthase inhibition involves mTOR malonylation. *Cell Metab* 2018;28(6):866–880.e15.
- Cao H, Cai Q, Guo W, et al. Malonylation of acetyl-CoA carboxylase 1 promotes hepatic steatosis and is attenuated by ketogenic diet in NAFLD. *Cell Rep* 2023;42(4):112319.
- Yu J, Sadhukhan S, Noriega LG, et al. Metabolic characterization of a Sirt5 deficient mouse model. *Sci Rep* 2013;3(1):2806.
- Segal NA, Nilges JM, Oo WM. Sex differences in osteoarthritis prevalence, pain perception, physical function and therapeutics. *Osteoarthritis Cartilage* 2024;32(9):1045–1053.
- Tschon M, Contartese D, Pagani S, et al. Gender and sex are key determinants in osteoarthritis not only confounding variables. a systematic review of clinical data. *J Clin Med* 2021;10(14):10.

37. Hwang HS, Park IY, Hong JI, et al. Comparison of joint degeneration and pain in male and female mice in DMM model of osteoarthritis. *Osteoarthritis Cartilage* 2021;29(5):728–738.
38. Komaravolu RK, Mehta-D'souza P, Conner T, et al. Sex-specific effects of injury and beta-adrenergic activation on metabolic and inflammatory mediators in a murine model of post-traumatic osteoarthritis. *Osteoarthritis Cartilage* 2024;32(9):1097–1112.
39. Geraghty T, Obeidat AM, Ishihara S, et al. Age-associated changes in knee osteoarthritis, pain-related behaviors, and dorsal root ganglia immunophenotyping of male and female mice. *Arthritis Rheumatol* 2023;75(10):1770–1780.
40. Casimiro I, Stull ND, Tersey SA, et al. Phenotypic sexual dimorphism in response to dietary fat manipulation in C57BL/6J mice. *J Diabetes Complications* 2021;35(2):107795.
41. Gelineau RR, Arruda NL, Hicks JA, et al. The behavioral and physiological effects of high-fat diet and alcohol consumption: sex differences in C57BL6/J mice. *Brain Behav* 2017;7(6):e00708.
42. Zhu S, Donovan EL, Makosa D, et al. Sirt3 promotes chondrogenesis, chondrocyte mitochondrial respiration and the development of high-fat diet-induced osteoarthritis in mice. *J Bone Miner Res* 2022;37(12):2531–2547.
43. Aubourg G, Rice SJ, Bruce-Wootton P, et al. Genetics of osteoarthritis. *Osteoarthritis Cartilage* 2022;30(5):636–649.
44. Boer CG, Hatzikotoulas K, Southam L, et al; arcOGEN Consortium; HUNT All-In Pain; ARGO Consortium; Regeneron Genetics Center. Deciphering osteoarthritis genetics across 826,690 individuals from 9 populations. *Cell* 2021;184(18):4784–4818.e17.
45. Gavile CM, Kazmers NH, Novak KA, et al. Familial clustering and genetic analysis of severe thumb carpometacarpal joint osteoarthritis in a large statewide cohort. *J Hand Surg Am* 2022;47(10):923–933.
46. Juryneć MJ, Gavile CM, Honegger M, et al. NOD/RIPK2 signalling pathway contributes to osteoarthritis susceptibility. *Ann Rheum Dis* 2022;81(10):1465–1473.
47. Juryneć MJ, Sawitzke AD, Beals TC, et al. A hyperactivating proinflammatory RIPK2 allele associated with early-onset osteoarthritis. *Hum Mol Genet* 2018;27(13):2406.
48. Sliz E, Taipale M, Welling M, et al. TUFT1, a novel candidate gene for metatarsophalangeal osteoarthritis, plays a role in chondrogenesis on a calcium-related pathway. *PLoS One* 2017;12(4):e0175474.
49. Styrkarsdóttir U, Stefánsdóttir L, Thorgeirsson G, et al. Meta-analysis of erosive hand osteoarthritis identifies four common variants that associate with relatively large effect. *Ann Rheum Dis* 2023;82(6):873–880.

# Multiparametric Profiling of Circulating Immune Cells Identifies an Expansion of CD25<sup>high</sup> Switched Memory B Cells in Osteoarthritis

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**Objective.** Osteoarthritis (OA) is a chronic, debilitating disease with no available disease-modifying drugs. Biomarker identification in patients with OA has hitherto been limited to serum proteins and bulk epigenomic feature identification.

**Methods.** Peripheral blood mononuclear cells (PBMCs) from 21 healthy donors, 17 patients with OA, and 10 patients with degenerative meniscal tears (DMTs) were immunophenotyped at single-cell resolution by mass cytometry by time-of-flight using a 29-marker panel to identify OA-associated features in the circulating immune cells. Single-cell RNA sequencing was used to discern mechanistic attributes of perturbed immune cell populations in OA.

**Results.** Comparison of the PBMCs of healthy donors and OA patients revealed distinct perturbations in OA. Although subsets of naive B cells were depleted, switched memory B cells were significantly expanded in OA, including a CD25<sup>hi</sup>CXCR5<sup>hi</sup>CD27<sup>+</sup>IgD<sup>+</sup> subpopulation. Single-cell RNA sequencing revealed a dysfunction of interleukin 2/Stat5 and tumor necrosis factor signaling in the CD25<sup>hi</sup> switched memory B cells in OA. A similar expansion of CD25<sup>hi</sup> switched memory B cells was observed in patients with DMT, a population at enhanced risk for OA.

**Conclusion.** A CD25<sup>hi</sup> switched memory B cell population was identified to be a potential cellular biomarker for OA that can be detected in the early stages of OA in the readily accessible circulating blood cells.

## INTRODUCTION

Osteoarthritis (OA) is an age-associated chronic disease that affects one in every five adults aged >60 years, leading to joint dysfunction and persistent adverse effects on quality of life.<sup>1,2</sup> No disease-modifying drugs are available for OA, with treatment options being limited to pain management until the eventual surgery for total joint replacement.<sup>3</sup> Part of the reason why OA disease modification has been challenging is the late identification of the disease making the reversal difficult, along with a lack of biomarkers that can accurately reflect the molecular causation and manifestation of the disease.<sup>4</sup>

Assaying joint tissues, like cartilage and synovium, from patients is difficult because procurement procedures are invasive and can even be detrimental to patients for tissues like cartilage.

Therefore, the joint tissues that are generally available to study the disease are the ones harvested from patients undergoing total joint replacement surgeries, often at advanced stages of OA. Efforts have been made for biomarker identification in longitudinal cohorts of patients with OA using imaging modalities<sup>5</sup> and readily available tissues such as blood or urine specimens.<sup>6,7</sup> However, such studies have hitherto been limited to serum proteins<sup>8</sup> and bulk transcriptomics or epigenomic feature identification.<sup>9</sup> The advent of high-resolution single-cell techniques has made it possible to obtain precise maps of OA patient tissues wherein rare and transitional cell types can be identified.<sup>10,11</sup> Studying the peripheral blood can provide a systemic snapshot of the immune landscape that is readily accessible and noninvasive, providing single-cell datapoints of immune cells as correlative biomarkers.<sup>12–15</sup> Immune cell studies of the OA blood have

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the advantage of allowing larger cohort designs and serial monitoring from the early to late stages of the disease. Besides an increased understanding of OA pathogenesis, such studies can also help in earlier detection and patient stratification strategies, thereby contributing to precision medicine approaches to prevent and treat OA.

OA pathophysiology is distinct from the autoimmune disease rheumatoid arthritis (RA); however, significant trafficking of immune cells is evident in OA joints. Details regarding the immune cell types involved, the tissues that are infiltrated in the joint, the timing of infiltration, and the cellular cross-talk that results in the breakdown of joint homeostasis are still unclear.<sup>16–18</sup> In this study, we used mass cytometry and single-cell RNA sequencing (scRNA-seq) to profile immune populations that are differentially abundant in the peripheral blood of patients with OA compared with healthy donors to identify immune cell correlates associated with OA. We have additionally profiled the peripheral blood of patients with degenerative meniscal tears (DMTs), a population known to be at enhanced risk of OA. The goal is to comprehensively define an immune cell atlas for patients with OA as well as for patients after DMT as a proof-of-concept study to identify meaningful biomarkers for OA that can be extended to larger patient cohorts for earlier detection of OA as well as data-driven patient stratification.

## MATERIALS AND METHODS

**Study design.** The primary objective of the study was to profile immune cells in the peripheral blood of patients with OA to highlight differences in their immune populations compared with healthy individuals and to identify immune cell signatures of OA that can be identified at earlier stages of the disease. The next aim was to investigate whether the immune profile of patients with DMTs, considered at risk of developing OA after injury show immune cell signatures associated with OA. To this end, peripheral blood mononuclear cells (PBMCs) were isolated from clinically diagnosed OA patient cohorts (n = 17), healthy individuals (n = 21) with no known history of OA, and patients with DMT before corrective surgery (Table 1; Supplementary Table 1). An additional cohort of PBMCs from healthy individuals (n = 6) and patients with OA (n = 5) was collected to validate our findings from the initial datasets. PBMCs were profiled by mass cytometry using a 29-marker mass cytometry panel (Human Immune Monitoring Panel Kit, catalog number 201234, Standard Bio Tools). Immune profiles of healthy (n = 21), OA (n = 17), and DMT (n = 10) cohorts were analyzed together to identify differentially abundant populations.

**Cohort description.** *Experimental OA cohort.* The OA cohort consisted of patients clinically diagnosed with OA who

**Table 1.** Social and clinical demographic data of patient samples used in the study and summary statistics\*

| ID    | Type | Age, y | Sex    | BMI, kg/m <sup>2</sup> | Laterality | KL grade |         |    |
|-------|------|--------|--------|------------------------|------------|----------|---------|----|
|       |      |        |        |                        |            | Medial   | Lateral | PF |
| OA1   | OA   | 59     | Male   | 27.43                  | Right      | 4        | 1       | 1  |
| OA2   | OA   | 52     | Female | 23.22                  | Right      | 2        | 1       | 1  |
| OA3   | OA   | 65     | Male   | 25.72                  | Right      | 1        | 1       | 2  |
| OA4   | OA   | 53     | Female | 25.26                  | Left       | 2        | 0       | 0  |
| OA5   | OA   | 48     | Female | 28.6                   | Right      | 3        | 1       | 3  |
| OA6   | OA   | 57     | Male   | 31.35                  | Right      | 2        | 3       | 2  |
| OA7   | OA   | 70     | Male   | 26.28                  | Right      | 3        | 2       | 2  |
| OA8   | OA   | 66     | Male   | 29.9                   | Right      | 1        | 0       | 0  |
| OA10  | OA   | 60     | Female | 27.72                  | Left       | 2        | 0       | 1  |
| OA11  | OA   | 68     | Male   | 22.95                  | Left       | 1        | 0       | 2  |
| OA12  | OA   | 48     | Female | 24.87                  | Left       | 2        | 0       | 0  |
| OA13  | OA   | 69     | Male   | 43.17                  | Right      | 4        | 3       | 4  |
| OA14  | OA   | 44     | Male   | 30.4                   | Left       | 1        | 1       | 2  |
| OA16  | OA   | 38     | Male   | 23.11                  | Left       | 3        | 1       | 0  |
| OA17  | OA   | 59     | Female | 24.89                  | Right      | 1        | 0       | 1  |
| OA15  | OA   | 56     | Female | 22.92                  | Right      | 0        | 0       | 1  |
| OA18  | OA   | 33     | Female | 28.24                  | Left       | 1        | 1       | 1  |
| DMT1  | DMT  | 47     | Male   | 44                     | Right      | 1        | 0       | 1  |
| DMT2  | DMT  | 55     | Female | 36.15                  | Left       | 1        | 0       | 1  |
| DMT3  | DMT  | 30     | Male   | 41.18                  | Left       | 1        | 0       | 1  |
| DMT4  | DMT  | 69     | Male   | 28.13                  | Right      | 1        | 0       | 0  |
| DMT5  | DMT  | 48     | Male   | 25.18                  | Left       | 0        | 0       | 0  |
| DMT6  | DMT  | 59     | Male   | 37.24                  | Left       | 2        | 1       | 1  |
| DMT7  | DMT  | 60     | Male   | 32.94                  | Left       | 2        | 1       | 1  |
| DMT8  | DMT  | 54     | Male   | 40.9                   | Right      | 0        | 0       | 0  |
| DMT10 | DMT  | 38     | Male   | 23.62                  | Right      | 1        | 0       | 0  |
| DMT11 | DMT  | 25     | Male   | 24.64                  | Right      | 0        | 0       | 0  |

\* BMI, body mass index; DMT, degenerative meniscal tear; KL, Kellgren-Lawrence; OA, osteoarthritis; PF, patellofemoral.

were either undergoing total knee surgery (OA 3, 4, and 5) or were symptomatic patients with early to moderate knee OA being treated with injection therapy. OA patients undergoing injection therapy were symptomatic with pain scores of at least 4 of 10, Kellgren-Lawrence (KL) radiographic grade 1–2 (osteophytes and/or <50% joint space narrowing), or KL grade 0 (normal radiographs) with magnetic resonance imaging (MRI) scan and/or arthroscopic evidence of cartilage degeneration or loss in at least one joint. KL is a global joint grading system established in 1957, which is scored from 0 to 4, and although only tibio-femoral compartments are typically assessed, recent clinical data include patellofemoral (PF) KL scores for a more comprehensive joint OA assessment.<sup>19,20</sup>

The KL scores of most patients were  $\geq 2$  in at least one region (ie, medial, lateral, and PF regions of the joint [0 being normal]) except for a subset (OA 8,15,17,18, 19, 20, 21, and 22) of patients with early OA with normal-appearing radiographs and joint degeneration verified by MRI scan or arthroscopy. Patients are a mix of unilateral or bilateral OA. The exclusion criteria included inflammatory arthritis, gout or recurrent pseudogout, or symptomatic OA of other lower extremity joints.

Reference samples and the DMT cohort. The “healthy” cohort consisted of samples from anonymous individuals ( $n = 21$ ) with no known history of OA, sourced from the Stanford Blood Center, for whom radiographic OA status is unknown. The DMT cohort consisted of patients undergoing arthroscopic surgery for DMT. The KL grades for these patients were mostly normal (0 or 1) for the medial, lateral, and PF compartments, except for those of two patients (DMT 6 and 7) who had a KL score of 2 for the medial compartment.

**Validation cohort.** The validation cohort was a mix of healthy patients, patients with early OA, and patients with moderate OA used to validate the B cell markers identified to be associated with OA. It consisted of four patients with early OA (OA 19, 20, 21, and 22) who were radiographically normal but had pain (>4/10) and arthroscopically detected cartilage damage; one patient with OA (OA 23) had radiographic OA. It also included six healthy patients (H 32–37) from the Blood Center with no known history of OA.

**Peripheral blood collection and PBMC isolation.** The whole blood of patients diagnosed with either OA or DMT were collected by clinicians. The protocols, policies, and human sample collection methods were approved by the Stanford University Institutional Review Board (IRB-31943), and informed consent was obtained from all participants. The whole blood of healthy donors was procured from the Stanford Blood Center. All blood samples were collected in EDTA vacutainer tubes and processed to isolate PBMCs within 6 hours of blood draw. PBMCs were isolated by Ficoll-based density gradient centrifugation. Briefly, whole blood was centrifuged at 200g for 10 minutes at room temperature to remove plasma in the supernatant. Plasma-deficient

blood was diluted with an equal volume of phosphate-buffered saline free of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions and layered over Ficoll-Paque Plus (density 1.077 g/mL; GE Healthcare). Density gradient centrifugation of blood layered over Ficoll was conducted at 1,000g for 15 minutes with minimum acceleration and zero deceleration settings at room temperature. The straw-colored buffy coat was carefully collected and treated with ACK lysing buffer (Gibco) to remove red blood cells. PBMCs were washed thrice with phosphate-buffered saline and centrifuged and the PBMC pellet was frozen in fetal bovine serum +20% DMSO and stored in liquid nitrogen until ready for staining for mass cytometry.

**PBMC staining and mass cytometry.** All staining and barcoding steps were performed using buffers, reagents, and protocols provided by Fluidigm. Briefly, frozen PBMCs were thawed in a 37°C water bath, and approximately  $3 \times 10^6$  cells per sample were stained with 10  $\mu\text{M}$  cisplatin to stain dead cells for 5 minutes at room temperature. Cisplatin was quenched using a serum-containing RPMI medium and washed in Maxpar cell-staining buffer. PBMCs were fixed in Fix I buffer and permeabilized in 1× barcode perm buffer before barcoding using cell-ID 20-plex barcoding kit (Fluidigm) per the manufacturer’s instructions. Once barcoded, the PBMCs from all samples were combined into one tube and stained for 29 surface markers using metal-conjugated antibodies provided in the Maxpar human immune cell monitoring panel kit (catalog no. 210324; Fluidigm) following the manufacturer’s guidelines. Finally, stained PBMCs were labeled with cell-ID intercalator (dilution 1:1,000; Fluidigm) to stain DNA. To normalize signal over runtime, the PBMCs were diluted in EQ four-element calibration beads (Fluidigm) diluted with water (1:10) before injection into cytometry by time-of-flight (cyTOF) 2 mass cytometer using the supersampler, housed at the Shared FACS facility at Beckman Center, Stanford University. As only 20 samples could be barcoded and run at a time, four batches of barcoding were performed and run at different times in the same mass cytometer. One common sample was included in all four batches to normalize batch effects.

#### Data cleaning, normalization, and de-barcoding.

Data were acquired in fcs files. Signal normalization over runtime for each run was performed using Normalizer (version 0.3; <https://github.com/nolanlab/bead-normalization/wiki/Installing-the-Normalizer>). All fcs files were concatenated and de-barcoded using the ‘Premessa’ package in R software (<https://github.com/ParkerICI/premessa>). Channel data was arcsine transformed and batch normalized across three runs with respect to the common sample using the BatchAdjust() command-line application in R (<https://github.com/CUHIMSR/CytofBatchAdjust>). Files were then uploaded into cloud-based platform Cytobank for gating, clustering, and visualization.

### Traditional phenotyping, hierarchical clustering by FlowSOM, and uniform manifold approximation and projection projections.

CD45<sup>+</sup>CD66b<sup>-</sup> cell population gated on cisplatin-negative viable cells were classified into major immune populations and their subtypes using the manual gating strategy provided by Fluidigm (Supplementary Figure 1). Unsupervised hierarchical clustering by FlowSOM (Cytobank) was used to identify subpopulations of major immune cell types from gated parent populations with optimized metacluster and cluster inputs, namely: (1) CD45<sup>+</sup>CD66b<sup>-</sup>CD14<sup>-</sup>CD3<sup>+</sup> CD8<sup>-</sup>CD4<sup>+</sup> population CD4T cell subpopulations (20 metaclusters and 64 clusters); (2) CD45<sup>+</sup>CD66b<sup>-</sup>CD14<sup>-</sup>CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> population CD8T cell subpopulations (10 metaclusters and 25 clusters); (3) CD45<sup>+</sup>CD66b<sup>-</sup>CD14<sup>-</sup>CD16<sup>-</sup>CD161<sup>-</sup>CD3<sup>-</sup>CD19<sup>+</sup> population B cell subpopulations (10 metaclusters and 25 clusters); and (4) CD45<sup>+</sup>CD66b<sup>-</sup>CD20<sup>-</sup>CD19<sup>-</sup>CD3<sup>-</sup>CD14<sup>+</sup> population monocyte subpopulations (5 metaclusters and 9 clusters). All events (cells) per sample were used for FlowSOM analyses, and abundances were represented as the percentage of total cells analyzed per sample. Uniform manifold approximation and projection (UMAP) of manually gated populations and FlowSOM clusters were created using equal down-sampled files in the Cytobank platform with a perplexity of 60 and  $\theta$  of 0.5.

**B cell isolation.** PBMC samples from patients with OA and healthy donors ( $n = 6$  per group) that were previously profiled using cyTOF were enriched for B cells using the magnetic-activated cell sorting bead isolation kit (Human Pan B cell isolation kit 130-101-638; Miltenyi Biotec) according to manufacturer's instructions. Isolated B cell samples were prepared for downstream scRNA library preparation.

**scRNA library preparation, sequencing, and analyses.** Cells were fixed and complementary DNA libraries were generated using the Evercode WT Mini v1 kit (Parse Biosciences) according to the manufacturer's instructions. Three samples each were pooled to obtain two single-cell complementary DNA libraries per group. Sequencing files were processed using the Parse Biosciences Pipeline (v1.0.3) and aligned to the human GRCh38 genome. The count matrix was loaded into R (v4.3.0) using the Seurat package (v4.3.0.1) to cluster, visualize, and analyze the dataset. A total of 4,018 cells with a median of 7,861 transcripts (and 2,706 genes) per cell were successfully recovered from sequencing after filtering out low-quality reads. Although the median mitochondrial read percentage was 3.2%, we filtered out cells with >15% mitochondrial reads. Further cells with <5,000 features and 20,000 transcripts were retained for downstream analysis. Detailed analysis procedures are made available in the code uploaded. scRNA-seq data were deposited in the GEO data repository and can be accessed using accession number GSE253539.

**Statistics.** The Spearman correlation was performed across demographic data and immune cell populations to identify significant correlations ( $P < 0.01$ ). Statistical differences between the means of immune cell populations and cluster frequencies were first controlled for age- and sex-associated effects by nonparametric Quade test using SPSS software. The Wilcoxon rank sum test was used to measure significance when comparing two groups. When more than two groups were compared, pairwise comparisons were calculated by the Dunnett test for groups with unequal variances after correcting for multiple hypotheses by the Dunnett T3 test. Graphical data and heatmaps were created using RStudio software (<http://www.r-project.org>). Half boxplots were used for graphical representation of cyTOF data using gghalves and ggplot2 libraries in RStudio. Dots in the half boxplots represent the distribution of individual samples in a cohort. The line inside the boxplot is the median at the 50th percentile, and upper and lower whiskers denote data within 1.5 times the interquartile range under the 75th and 25th percentiles, respectively. Correlation plots were generated using 'corrplot,' principal component analysis (PCA) using 'factoextra,' and heatmaps using the 'pheatmap' package in R.

**Data availability statement.** All data needed to evaluate the results are in the paper or the supplementary materials. Raw fcs files acquired from the cyTOF2 mass cytometer and subsequent gating and FlowSOM results are stored in Cytobank and will be shared upon request. scRNA-seq data were deposited in the GEO data repository and can be accessed using accession number GSE253539.

## RESULTS

### Traditional immunophenotyping identifies significant differences between healthy and OA PBMCs.

PBMCs were isolated from symptomatic patients with OA, as described in the Methods ( $n = 17$ ; aged 33–70 years), and healthy individuals with no known history of OA ( $n = 21$ ; aged 42–76 years) (Table 1). The mean  $\pm$  SD age of the OA cohort,  $55.59 \pm 10.81$  years, was comparable to the mean  $\pm$  SD age of the healthy cohort at  $57.29 \pm 10.31$  years. To identify whether any immune cell populations were differentially populated in the circulating blood of patients with OA, we used cyTOF, a mass spectrometry-based high-dimensional method for single-cell detection of isotope-labeled antibodies. PBMCs were isolated and stained with a 29-marker antibody panel (Fluidigm) to identify major immune cell populations by cyTOF (Figure 1A). Before using algorithmic analyses, we employed a traditional manual gating strategy (Supplementary Figure 1) for immunophenotyping and identifying known immune populations in healthy individuals and those with OA (Figure 1A; Supplementary Figure 1). Correlations between the individual subtypes of major immune populations and demographic data (sex, age, and body mass index

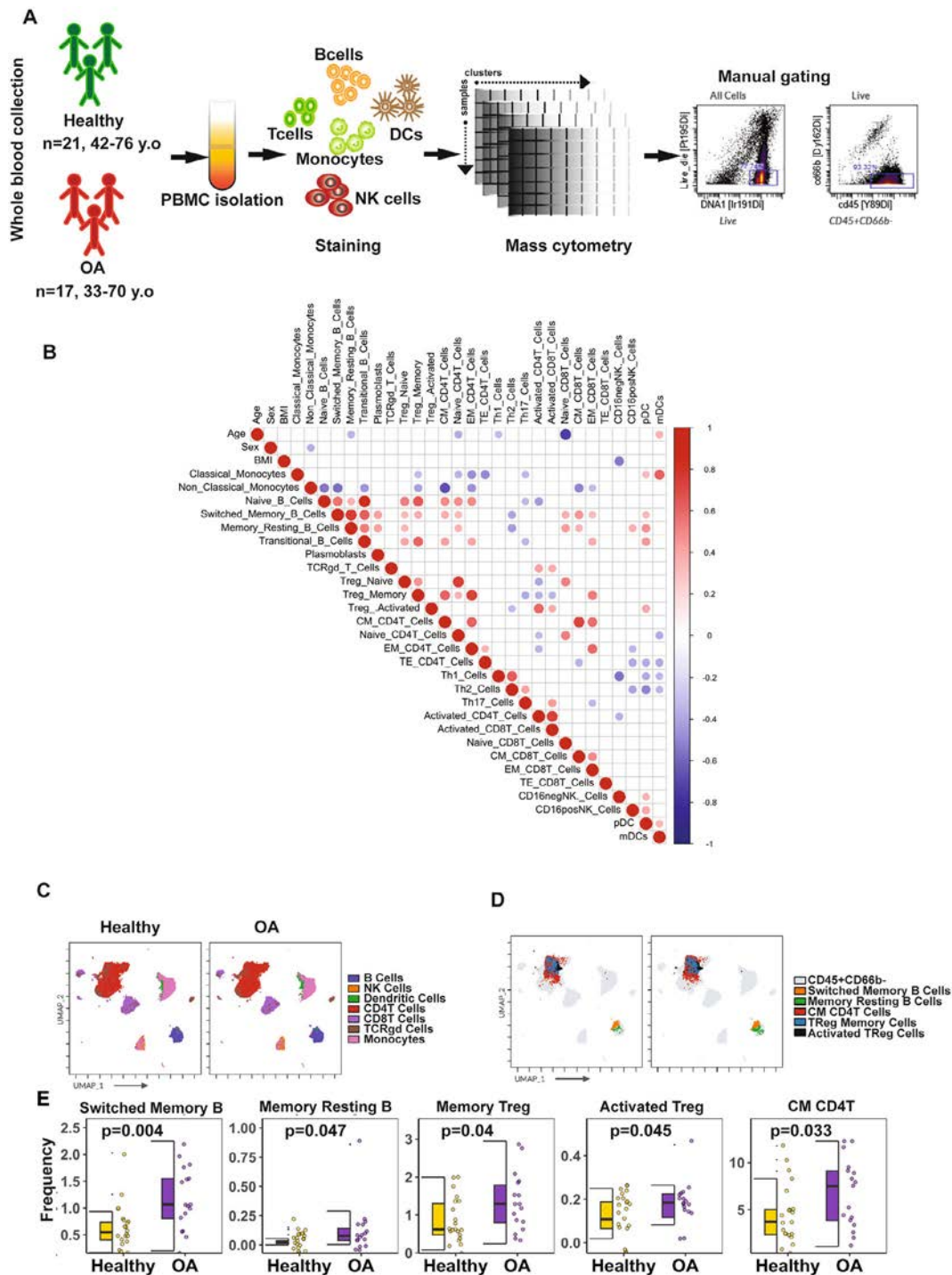


**Figure 1.** Traditional immunophenotyping identifies significant differences between healthy and OA PBMC. (A) Schematic representation of experimental design. Briefly, whole blood PBMCs of healthy donors ( $n = 21$ ) and patients with OA ( $n = 17$ ) were isolated and labeled with metal isotope-conjugated antibodies followed by mass cytometry and analysis by traditional phenotyping. (B) Spearman correlation matrix demonstrating significant correlations ( $P < 0.05$ ) between the immune population frequencies and social and clinical demographics of healthy and OA cohorts. (C) Major immune populations identified by traditional hierarchical gating from parent CD45+CD66b- population are visualized by uniform manifold approximation and projection plots in healthy and OA samples. (D) Uniform manifold approximation and projection highlighting significantly different immune subpopulations between healthy and OA cohorts. (E) Half boxplots of significantly different immune cell subtypes of major populations. Dots in the half boxplots represent the distribution of individual samples in a cohort. The line inside the boxplot is the median at the 50th percentile, and the upper and lower whiskers denote data within 1.5 times the interquartile range under the 75th and 25th percentiles, respectively. Statistical significance was calculated by the nonparametric Quade test to control for age and sex, represented by  $P$  values. BMI, body mass index; CM, central memory; DC, dendritic cell; NK, natural killer; OA, osteoarthritis; PBMC, peripheral blood mononuclear cell; TCRgd,  $\gamma\delta$  T cell receptor.

[BMI]) were explored to identify any evident trends (Figure 1B; Table 1). Age and sex information was available for all individuals in the healthy and OA cohorts (as summarized in Table 1); clinical joint pathology and pain information is included for the OA and DMT cohorts.

Naive subsets of CD8T and CD4T, along with Th1 cells, myeloid dendritic cells, and memory resting B cells were shown to be negatively correlated with age; myeloid dendritic cells were positively correlated. There was no significant correlation between immune cells and BMI save a negative correlation between CD16<sup>+</sup> natural killer cells and BMI. Given the observed correlations between multiple immune cells and either age or sex, we controlled for age- and sex-associated effects by statistical correction for age (nonparametric Quade test) in our analyses. All major immune populations and their subtypes resolved into distinct populations by UMAP (Figure 1C). No significant differences were noted between healthy and OA PBMCs in the proportion and frequency of major immune cell populations, namely CD14<sup>+</sup> monocytes, natural killer cells, dendritic cells, CD4T cells, CD8T cells, and B cells (Supplementary Figure 2). However, the mean frequencies of switched memory B cells (CD20<sup>+</sup>CD27<sup>+</sup>IgD<sup>+</sup>), resting memory B cells, memory Treg cells (CD25<sup>+</sup>CD127<sup>+</sup>CCR4<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup>), activated Treg and central memory CD4T cells (CD4<sup>+</sup>CCR7<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup>) were found to be significantly expanded in the OA cohort compared with the healthy cohort (Figure 1E).

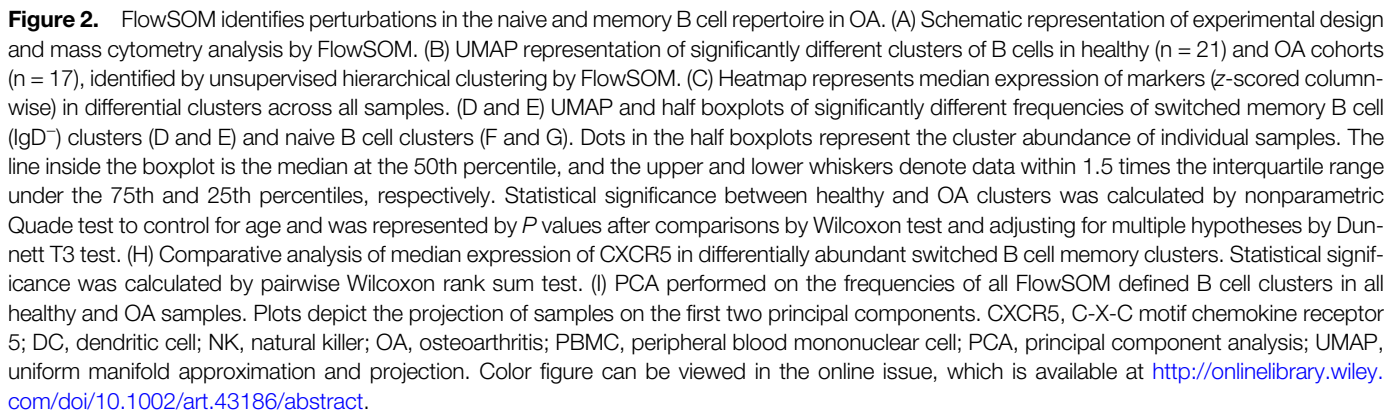
**Differential abundance of T cell repertoire in OA.** A comprehensive analysis by way of unsupervised hierarchical clustering by the FlowSOM algorithm identified distinct immune cell subpopulations that could not be distinguished by traditional markers used in manual gating (Supplementary Figure 3A). With FlowSOM analyses, fluctuations in the classical monocyte clusters were observed in OA, where CD127<sup>+</sup> classical monocyte cluster 3 was expanded but CD14<sup>low</sup> and CD25<sup>+</sup> classical monocyte clusters 2 and 6, respectively, were depleted (Supplementary Figure 3B and C). Cluster 1, belonging to central memory CD8T cell, was expanded whereas cluster 7 of the naive CD8T cell population was depleted in OA (Supplementary Figure 3D–F).

FlowSOM analysis of the CD4T cell population identified differentially abundant clusters of naive (CD4<sup>+</sup>CCR7<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup>), effector memory (EM) phenotypes (CD4<sup>+</sup>CCR7<sup>+</sup>CD45RO<sup>+</sup>CD45RA<sup>+</sup>) and T helper subsets in patients with OA when compared with the healthy cohort. Naive CD4T cell clusters 4 and 14 and EM CD4T cell clusters 37, 41, 42, 51, and 58 were expanded in OA (Supplementary Figure 4A–D). On the other hand, Th2 cluster 26, naive CD4T clusters 15 and 21, and EM CD4T clusters 43 and 8 were depleted in OA (Supplementary Figure 4E–G). Interestingly, we note that CD56 was expressed by all the naive and EM CD4T cell clusters that were depleted in the OA cohort compared with those that were expanded (Supplementary

Figure 4C). Although these differences were interesting, PCA demonstrated that the healthy and OA cohorts overlapped in the respective CD4T repertoires, hence the CD4T subsets could not explicitly distinguish OA from healthy cohorts (Supplementary Figure 4H and I).

**FlowSOM identifies perturbations in the naive and memory B cell repertoire in OA.** FlowSOM revealed distinct B cell clusters with perturbed frequencies in OA compared with the healthy cohort across all B cells (Figure 2A and B). The differential clusters were identified as naive and switched memory B cell phenotypes (Figure 2C). CD27<sup>+</sup>IgD<sup>+</sup> switched memory B cell clusters 6, 7, and 11 were significantly expanded in OA after controlling for age and sex by Quade test (Figure 2D and E). This observation is consistent with our results from immunophenotyping by manual gating in which the switched memory B cells subtype was found to be significantly expanded in OA vs in the healthy cohort (Figure 1G). Within the switched memory B cells, cluster 6 was demarcated by the higher expression of CD25 and CD24, hence we termed it as the CD25<sup>hi</sup> switched memory B cell cluster. Among the naive B cells, clusters 15 and 21, marked by high CD38 expression, were significantly depleted in OA (Figure 2F and G). Higher expression of CD56 differentiated naive B cell cluster 21 from cluster 15 (Figure 2C). Interestingly, C-X-C motif chemokine receptor 5 (CXCR5) was significantly enriched in switched memory B cell clusters 6, 7, and 11, which were expanded in OA compared with the healthy donors (Figure 2H). Contrary to the PCA of CD4T cell clusters in OA and healthy donors (Supplementary Figure 4H and I), we noted a greater separation of OA from healthy individuals in the PCA analyses in the B cell repertoire (Figure 2I).

**Expansion of a CD25<sup>hi</sup> switched memory B cell population in OA.** Because the cyTOF data identified multiple B cell clusters that could differentiate between healthy and OA PBMCs, we performed scRNA-seq on a subset of healthy (n = 6) and OA (n = 6) samples to validate the results. Isolated PBMCs were enriched for B cells using a pan B cell magnetic-activated cell sorting kit, and scRNA libraries were constructed and sequenced. The Seurat package (v4.3.0.1) was used to cluster, visualize, and analyze the dataset (see Methods for details). The enriched B cells clustered into 14 unique clusters (Supplementary Figure 5A), 7 of which had high expression for B cell markers including CD19, MSA1, PAX5, and CD79A (Supplementary Figure 5B and C). Automated clustering algorithms also identified these as B cells (and their subsets) when compared with the MonocleImmuneData transcriptomics reference dataset of sorted human immune cells<sup>21</sup> (Figure 3A; Supplementary Figure 5D). The proportion of naive B cells was reduced (log2 fold change = -0.16) and that of switched memory B cells was expanded (log2 fold change = 0.60) in OA samples (Figure 3B and C), consistent with the cyTOF data.



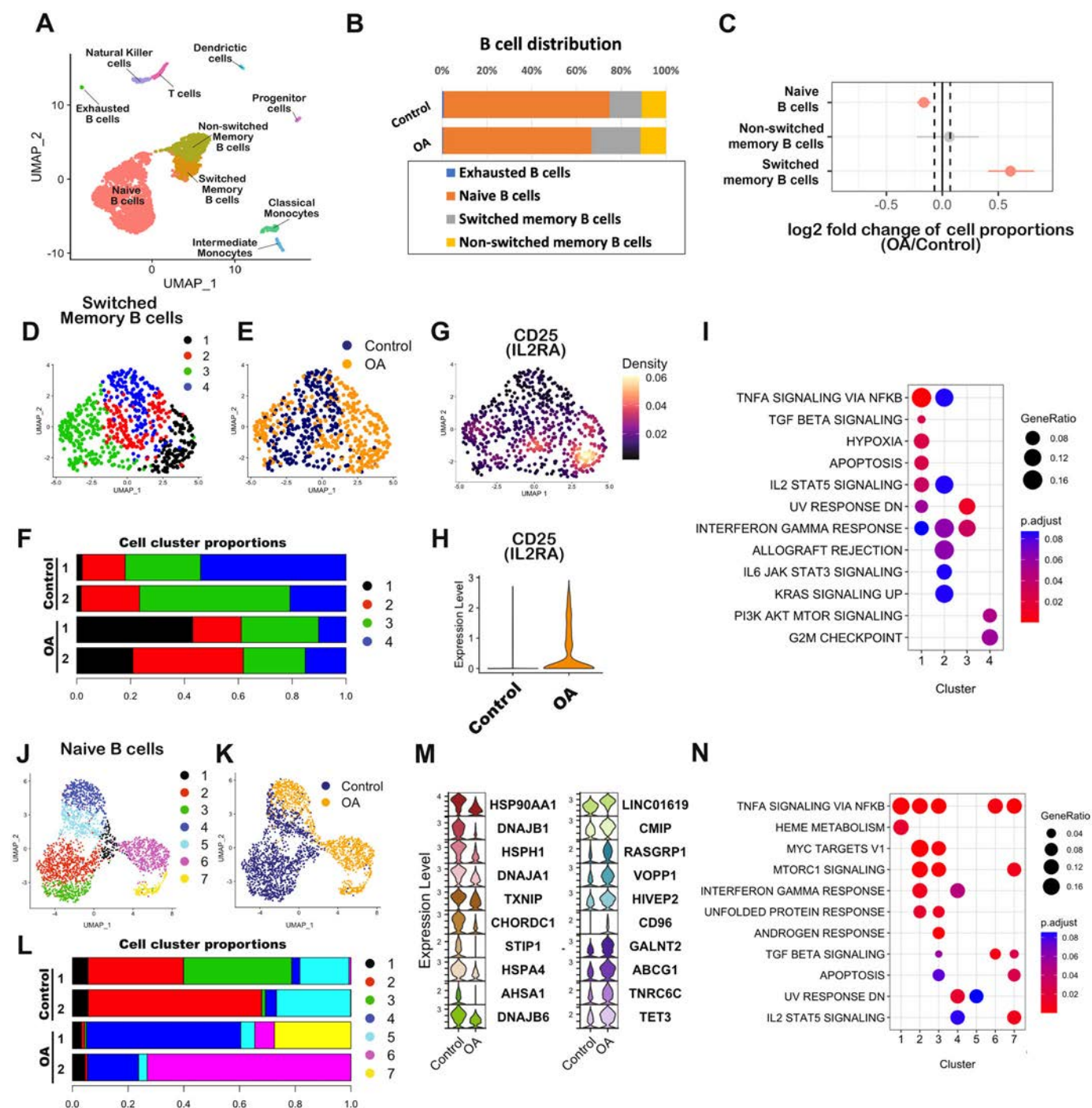


**Figure 3.** Expansion of a CD25<sup>hi</sup> switched memory B cell population in OA. (A) UMAP visualization of cell clusters from the single-cell RNA sequencing analysis depicting the different immune cell types after B cell enrichment. (B) Distribution of the major types of B cells within each group. (C) Proportion testing plot using 'scProportiontest' depicting the log2 fold change of proportions of different B cell subtypes (dashed line indicates a cutoff of 5% change in proportion, and red dots represent changes that are statistically significant). (D) Cell cluster UMAP plots representing switched memory B cell types with distinct gene expression profiles and (E) distribution across control and OA samples. (F) Bar plot representing the proportions of SMB cell clusters within each sample. (G) Density plot of CD25 (IL2RA) gene expression within the switched memory B cell population and (H) violin plot of CD25 across control and OA samples. (I) Enriched pathways for each switched memory B cell subtype. (J) Cell cluster UMAP plots representing naive B cell types with distinct gene expression profiles and (K) distribution across control and OA samples. (L) Bar plot representing the proportions of naive B cell clusters within each sample. Violin plots representing (M) the top 10 down-regulated and top 10 up-regulated genes, respectively, in OA within naive B cells. (N) Pathway analysis representing enriched pathways for each naive B cell subtype. IL2RA, interleukin-2 receptor  $\alpha$ ; mTOR, mammalian target of rapamycin; NF $\kappa$ B, nuclear factor  $\kappa$ B; OA, osteoarthritis; PI3K, phosphoinositide 3-kinase; TGF $\beta$ , transforming growth factor  $\beta$ ; TNF, tumor necrosis factor; UMAP, uniform manifold approximation and projection. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43186/abstract>.

Single-cell clusters of switched memory (total 625 cells) and naive B cells (total 2,461 cells) were extracted and re-examined independently. Switched memory B cells grouped into four subclusters, wherein clusters 1 and 2 were disproportionately higher in OA (Figure 3D–F). In agreement with the findings of the cyTOF analyses, a CD25<sup>hi</sup> cluster (cluster 1) was expanded in OA samples (Figure 3G and H). CD25 denotes the interleukin-2 receptor  $\alpha$  (IL2RA), and genes in cluster 1 were accordingly enriched for the IL2-STAT5 signaling pathway (Figure 3I). Other pathways associated with the CD25<sup>hi</sup> cluster were the tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) signaling via the nuclear factor  $\kappa$ B and the transforming growth factor  $\beta$  signaling pathway, which are also associated with OA pathology in the joint tissues (ie, cartilage, bone, and synovium). The IL2-STAT and TNF $\alpha$  pathways were also overrepresented in the genes expressed in cluster 2 along with the IL6-JAK-STAT pathway. Consistent with the cyTOF data, the CD25<sup>hi</sup> switched memory cell cluster also showed an enrichment for the CXCR5 gene (Supplementary Figure 6C).

Naive B cells grouped into seven subclusters with surprisingly little overlap of clusters between healthy control and OA samples (Figure 3J–L). Although the transcript for CD56 (NCAM1) was not widely expressed in any of these clusters, CD38 gene expression was most concentrated in cluster 1 and was not significantly different between groups, unlike the cyTOF analyses where CD38-expressing clusters were depleted in OA. However, CD38 was found to be a negative marker for OA-enriched clusters 6 and 7, implying that it was significantly underrepresented in these subsets of cells (data not shown). A large proportion of differentially expressed genes belonged to the heat shock protein family, and almost all of these were down-regulated in the naive B cells of patients with OA (Figure 3M). Interestingly, IL2-STAT5 signaling again emerged as a significant pathway in clusters 4 and 7, both of which are disproportionately higher in OA (Figure 3N).

**Validation of CD25<sup>hi</sup> switched memory B cell expansion in an independent cohort.** To confirm our findings from the discovery cohort of patients, we conducted a comprehensive 29 immune cell marker cyTOF experiment in an independent, validation cohort of five patients with OA and six healthy controls, distinct from our original dataset (Supplementary Figure 7A; Supplementary Table 2). FlowSOM clustering of B cells identified 25 distinct clusters as well as 8 metaclusters, which distributed between three naive (IgD<sup>+</sup>CD27<sup>−</sup>CD38<sup>+/lo</sup>), two switched memory (IgD<sup>+</sup>CD27<sup>+</sup>), two unswitched memory (IgD<sup>+</sup>CD27<sup>−</sup>), and one double negative (IgD<sup>−</sup>CD27<sup>−</sup>) B cell metacluster (Supplementary Figure 7C). Similar to our initial findings, we found significantly altered B cell subpopulations in our validation cohort. Notably, CD25<sup>hi</sup> switched memory B cell metaclusters were expanded, and two large naive cell metaclusters were depleted in OA compared with healthy PBMCs as observed previously (Supplementary Figure 7D). We consolidated these eight

metaclusters into the four main B cell subtypes identified based on marker expression patterns (Figure 3A and B) and assigned the 25 FlowSOM clusters to these cell types (Supplementary Figure 7C). Nine of the 25 FlowSOM clusters had significantly altered abundances in OA versus healthy PBMCs. Remarkably, we found two expanded clusters that were higher in their median CD25 expression compared with the entire B cell population: clusters 16 and 22 (Supplementary Figure 7E). Cluster 22 belongs to the switched memory B cell metacluster and had the highest median expression of CD25, whereas cluster 16 was assigned to the double negative B cell subpopulation. All depleted clusters belonged to the naive B cell metaclusters (Supplementary Figure 7F).

**The immune landscape of patients with DMT resembles OA.** Joint injuries have been implicated in increasing the risk of developing OA, with posttraumatic OA accounting for 12% of the overall burden of disease.<sup>2</sup> DMTs are identified as one such injury-related risk factor of OA.<sup>22,23</sup> To investigate whether correlations exist between the DMT and OA immune landscapes, we explored the immune landscape in PBMCs of the whole blood of patients with DMT obtained before any surgical interventions were made ( $n = 10$ ; aged 25–69 years). The DMT cohort was relatively younger with a mean  $\pm$  SD age of  $48.50 \pm 13.91$  years compared with the mean  $\pm$  SD age of the OA cohort,  $55.59 \pm 10.81$  years and of the healthy cohort,  $57.29 \pm 10.31$  years. Interestingly though, the mean  $\pm$  SD BMI of the DMT cohort was higher at  $33.40 \pm 7.60$  kg/m<sup>2</sup> compared with that of the OA cohort at  $27.41 \pm 4.87$  kg/m<sup>2</sup>. The blood PBMCs isolated from the blood of the patients with DMT were stained using the same 29-marker cyTOF panel. All statistical measurements were conducted by controlling for age by nonparametric Quade test as before. Immunophenotyping by manual gating for defined immune populations revealed significant expansion of the switched memory B cells in DMT, similar to patients with OA when compared with healthy donors (Figure 4A and B).

To further discern OA-like perturbations in the DMT cohort, we selected the FlowSOM clusters that were significantly different between the healthy and OA cohorts as a framework to compare the frequencies of the same clusters in the DMT cohort. FlowSOM analyses revealed significant differences in the frequencies of immune clusters belonging to B cells and CD4T cell populations. Switched memory B cell cluster 6, identified previously as CD25<sup>hi</sup> switched memory B cell phenotype, was significantly expanded in the DMT cohort whereas naive B cell cluster 21 was significantly depleted (Figure 4C–E). Meanwhile, among the CD4T cell clusters that were significantly different between the healthy and OA cohorts, naive CD4T cluster 14 and EM CD4T cell cluster 51 were significantly elevated in the DMT cohort when compared with the healthy cohort (Figure 4F–H). We note that the trend in frequencies of the clusters that were different in OA versus healthy cohorts were maintained in DMT versus healthy comparisons.

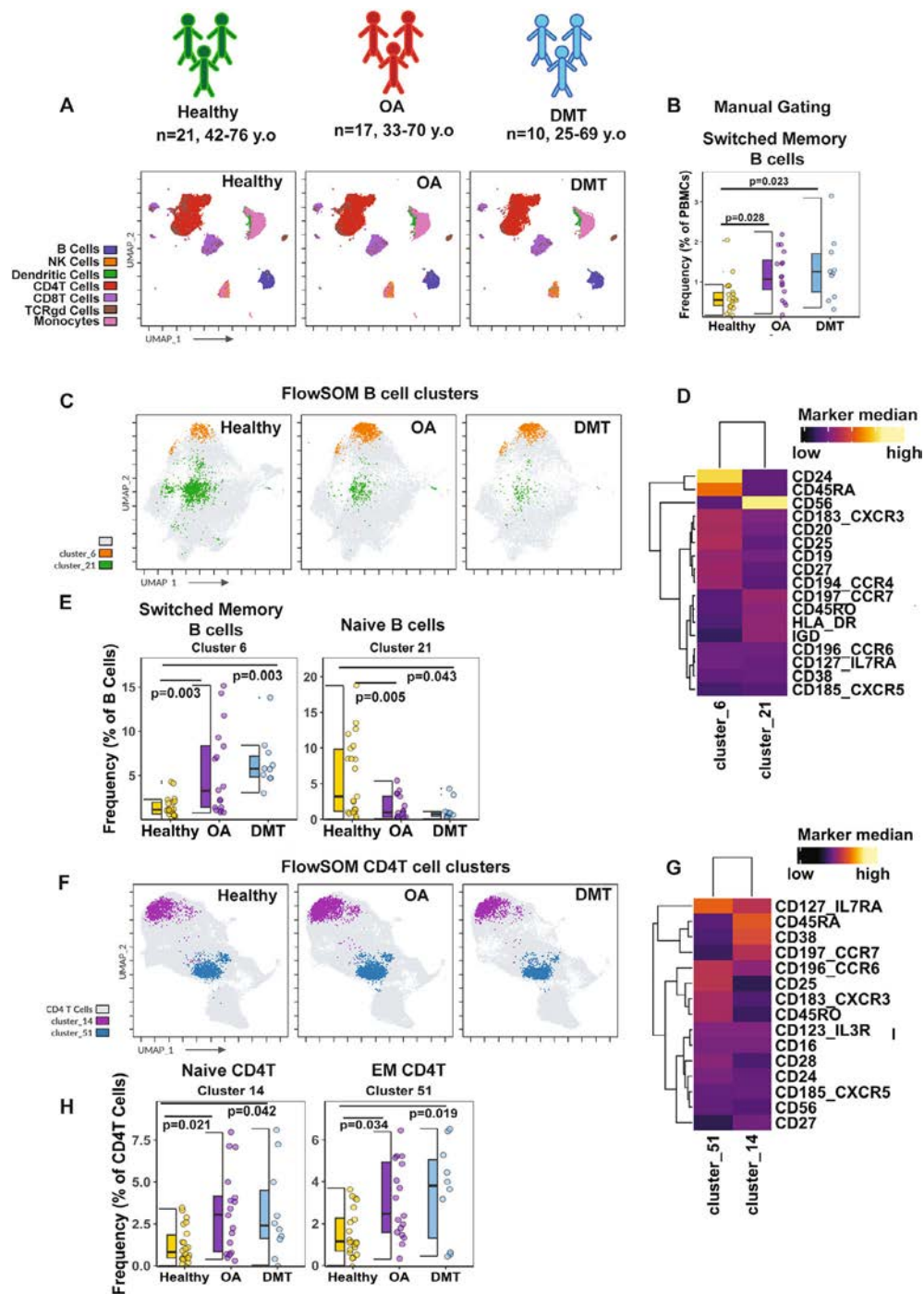


**Figure 4.** The immune landscape of patients with DMT resembles OA. (A) UMAP of major immune cell types identified by traditional phenotyping in healthy (n = 21), OA (n = 17) and DMT (n = 10) PBMCs. (B) Half boxplot of statistically significant switched memory B cell frequency among healthy, OA, and DMT samples derived by manual gating. (C) UMAP of significantly different FlowSOM-identified clusters of B cells and (D) the overall median expression of markers in the differential clusters across samples. (E) Half boxplots of the abundance of significantly different switched memory and naive B cell clusters between healthy, OA, and DMT samples. (F) UMAP of significantly different CD4T cell clusters defined by FlowSOM and (G) identified by overall marker expression across all samples. (H) Half boxplots of significantly different frequencies of CD4T cell clusters in healthy, OA, and DMT cohorts. Dots in the half boxplots represent the cluster abundance of individual samples. The line inside the boxplot is the median at 50th percentile, and the upper and lower whiskers denote data within 1.5 times the interquartile range under the 75th and 25th percentiles, respectively. Statistical significance between healthy, OA, and DMT clusters was calculated by nonparametric Quade test to control for age and represented by *P* values after pairwise comparisons by Dunnett test for unequal variances. CXCR3, C-X-C motif chemokine receptor 3; DMT, degenerative meniscal tear; NK, natural killer; OA, osteoarthritis; PBMC, peripheral blood mononuclear cell; TCRgd,  $\gamma\delta$  T cell receptor; UMAP, uniform manifold approximation and projection. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43186/abstract>.

Also, the frequencies of the aforementioned clusters were not significantly different between the OA and DMT cohorts, suggesting that the specific perturbations in the B and CD4T cell compartments in the OA cohorts were present in the DMT cohort as well.

**Specific immune correlates of OA are already evident in the early stages of disease.** We next evaluated the correlations among the demographics and abundances of FlowSOM-identified differential immune clusters in all our datasets including the healthy, OA, and DMT cohorts (Figure 5A). No correlation between age or sex and immune cluster abundances was noted. BMI, however, was anticorrelated with switched memory B cell cluster 11 (Figure 5A). Among the FlowSOM-identified immune subpopulations that were significantly different in

abundance in OA compared with healthy, hierarchical clustering showed that switched memory B cell clusters 6, 7, and 11, in addition to classical monocyte cluster 3 and central memory CD8T cell cluster 1, clustered separately from other differential cell populations among the patients with OA and DMT (Figure 5B), suggesting agreement between OA and DMT profiles for this set of immune subpopulations. Switched memory B cell types expanded in OA (clusters 6, 7, and 11) were similarly enriched for CXCR5 expression in most patients with DMT (Figure 5C), aligning with the trend observed in the OA versus healthy cohorts (Figure 2H). CD25 expression was uniformly high in all the patients with DMT, consistent with the expansion of the CD25<sup>hi</sup> switched memory B cells in the blood of patients with early and late OA. This is a proof-of-concept study with a limited number of

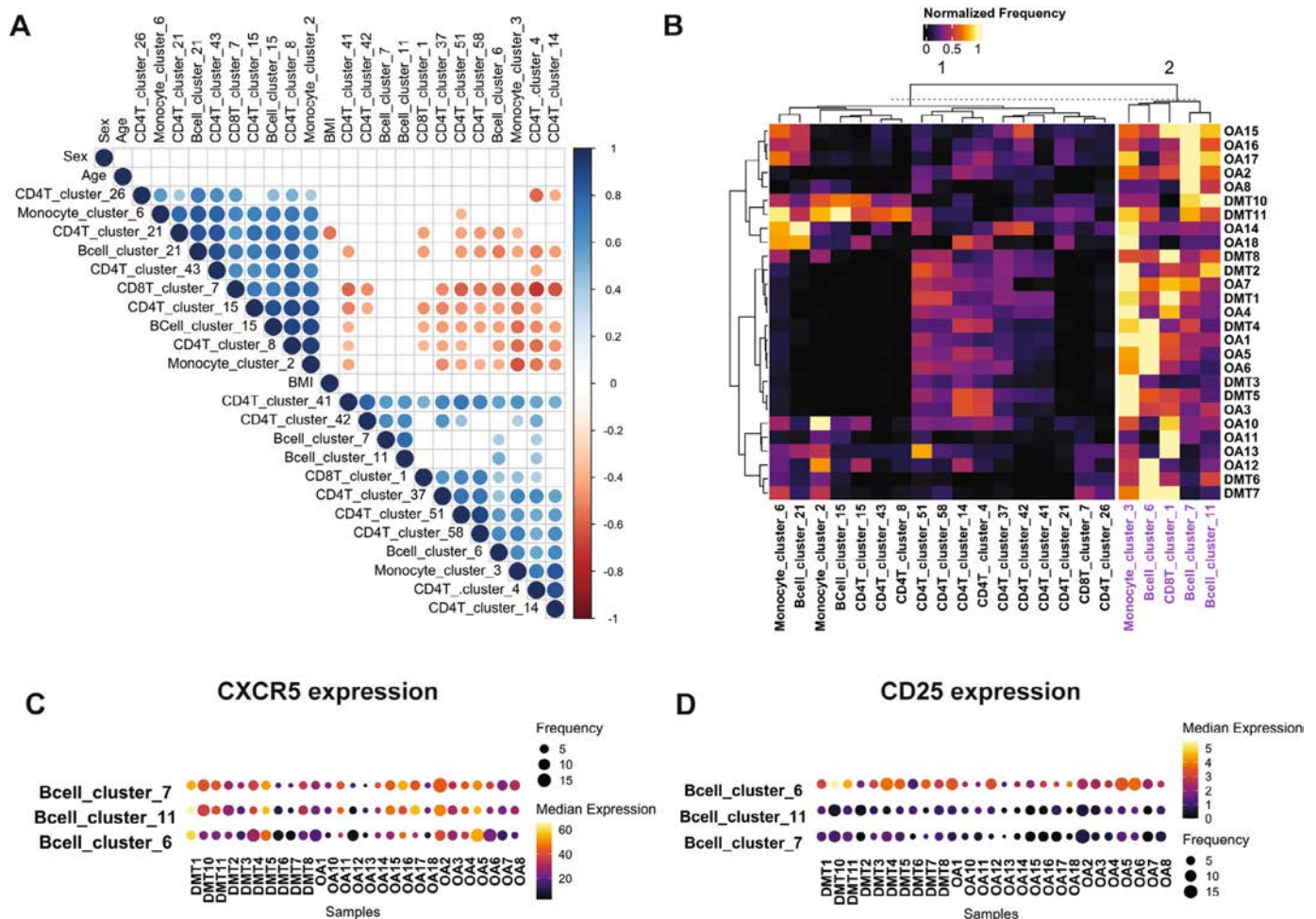


**Figure 5.** Specific immune correlates of OA are already evident in the early stages of the disease. (A) Spearman correlation matrix of significantly different FlowSOM-identified immune clusters between healthy and OA cohorts, and donor demographic information such as age and sex along with Kellgren-Lawrence scores for three different regions of the medial, lateral, and patellofemoral areas of the knee in those with OA. Only statistically significant correlations ( $P < 0.01$ ) are depicted as dots. (B) Heatmap of the cluster frequencies in individual OA and DMT samples wherein each cluster was differential in the OA cohort compared with healthy. Hierarchical clustering was performed on the dataset by using cosine similarity as the distance metric and Ward minimum variance method (ward.D2). (C) Dot plot of C-X-C motif chemokine receptor 5 expression across individual OA and DMT samples. Each dot represents the frequency of each cluster in every sample whereas the median expression of C-X-C motif chemokine receptor 5 per sample per cluster is represented by the color gradient. (D) Dot plot of CD25 expression across individual OA and DMT samples. Each dot represents the frequency of each cluster in every sample whereas the median expression of CD25 per sample per cluster is represented by the color gradient. BMI, body mass index; DMT, degenerative meniscal tear; OA, osteoarthritis. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43186/abstract>.

patients that warrants a follow up with a larger study given the significant findings.

## DISCUSSION

Recent studies have highlighted the role of memory B cells in inflammation, pathogenesis, and relapse of various diseases, including RA,<sup>24,25</sup> prompting the exploration of B cell depletion approaches for therapeutic intervention in their pathogenesis.<sup>26</sup> However, the role of B cells in OA pathogenesis is relatively less explored, although a flow cytometry-based analysis reported a skewing of immune cells in the B cell compartment of OA blood.<sup>27</sup> A remarkable observation in our study is that of a significant expansion of a switched memory B cell subpopulation (CD25<sup>hi</sup>CD24<sup>hi</sup>CD27<sup>+</sup>IgD<sup>-</sup>) in the circulating immune cells of the peripheral blood. This CD25<sup>hi</sup> switched memory B cell population was found to be enriched in OA by two independent approaches, cyTOF and scRNA-seq, validating the observation. These data highlight the power and significance of using higher-resolution techniques like mass cytometry as the precise differences in B cell repertoire could not be detected using flow cytometry with a limited number of cell-surface markers.<sup>28</sup>

Expansion of the CD25<sup>hi</sup>CD24<sup>hi</sup> switched memory B cell population in OA blood warrants further studies in the role of ILR2A/CD25 gene and this memory B cell's population in OA pathogenesis. Recent studies<sup>29,30</sup> have reported perturbations in preswitched and switched memory B cells in the blood, synovium, and synovial fluid of patients diagnosed with RA as well. Interestingly, the ILR2A/CD25 gene has been identified as a susceptibility locus for juvenile idiopathic arthritis and implicated in other autoimmune diseases; however, its function in regulatory T cells rather than B cells has been in focus.<sup>31</sup> RNA-seq data also validated that the cluster of switched memory B cells with enriched CD25 (IL2RA) expressed genes associated with the IL2 STAT5 signaling pathway. Data also revealed TNF $\alpha$  signaling via nuclear factor  $\kappa$ B and the transforming growth factor  $\beta$  signaling pathway to be enriched in the CD25<sup>hi</sup> switched memory B cells. These pathways play important roles in OA pathogenesis in the multiple cells constituting the joints, namely cartilage, bone, and synovium.<sup>32</sup>

Contrary to the expansion of memory B cells, we noted fewer frequencies of naive B cell populations in the peripheral blood of patients with OA. RNA-seq data demonstrated stark differences in the naive B cell populations between normal and OA samples (Figure 3K). Although naive B cell clusters 2, 3, and 5 were largely depleted in OA; clusters 4 and 6 were expanded. Interestingly, the IL2-STAT pathway was operational in naive B cell cluster 4 like in the CD25<sup>hi</sup> switched memory B cell that is also expanded in the OA samples. An imbalance in the T cell repertoire was also prominent in the OA landscape of immune cells even though the T cell signature did not completely distinguish between healthy patients and those with OA when assessed by cyTOF. In the CD4T cell

repertoire, there was an expansion of naive CD4T cell clusters (cluster 4 and 14) and five distinct EM CD4T cell clusters, whereas two CD56<sup>+</sup> clusters of each of these subsets were depleted. A circulating Th2 cell cluster was depleted in the OA peripheral blood, consistent with a previous study that showed a loss of an IL-4-producing Th2 subset in OA peripheral blood.<sup>33</sup>

Patients with OA are clinically screened and defined by joint space narrowing detected by x-ray along with patient-reported outcomes on pain.<sup>3</sup> Joints of patients with early knee OA with knee pain can appear radiographically normal, even when cartilage damage is evident arthroscopically or on MRI images. In this study, we have included PBMCs from patients with early OA that were symptomatic for OA pain and clinically indicated for injection therapy in addition to patients with moderate OA that is radiographically evident. The clinical attributes of OA pathogenesis can, however, be reached through multiple molecular routes, including joint trauma, mechanical instabilities in gait, inflammation, and metabolic disorders. It is becoming increasingly clear that the identification of OA biomarkers and potential stratification of patients based on their molecular characteristics is required. Such a stratification would be helpful in risk identification as well as clinical treatments in patients, thereby taking the first steps toward a precision medicine approach.<sup>34</sup> The quest for OA biomarkers so far has not used single-cell approaches, with the focus being on bulk analyses of blood cells or proteomic analyses of cell-free blood and urine samples.<sup>7-9</sup> Our recent cyTOF studies of OA cartilage also identified distinct patient subtypes based on the abundance of cell populations as well as drug response<sup>10,35</sup>; however, such an approach for prospective patient stratification before a clinical trial, for example, is not feasible. Immune cell profiling of easily accessible peripheral blood, on the other hand, can be greatly informative for patient stratification independently or in conjunction with clinical features like pain, BMI, and radiologic imaging in a larger cohort of patients with OA. In this proof-of-concept study in a small cohort, we demonstrate that the identification of an OA-specific signature of immune cell features through the single-cell data can be a potential approach to define OA biomarkers.

Another major bottleneck in OA treatment is the relatively late detection of the disease, which makes it difficult to reverse the pathogenesis and is the main reason for the lack of a disease-modifying OA drug in the clinic. Earlier detection of OA, before the irreversible clinical symptoms are apparent, would be a significant clinical breakthrough that will accelerate drug discovery efforts as well as help effective clinical trials for candidate drugs in early or moderate OA rather than late-stage OA. Although it takes many years for OA to manifest, it is known that patients with DMTs are at a much higher risk for OA development.<sup>2</sup> Novel methods in MRI have been applied for visualizing and quantifying changes in the cartilage before the advent of irreversible damage.<sup>36,37</sup> However, high interpatient variability in cartilage extracellular matrix composition may hinder the accuracy of

predicting early OA based on MRI alone, especially in patients for whom initial matrix changes are not apparent.

Our study, therefore, highlights the potential of using patient-specific immune cell features either as a standalone or in combination with advanced MRI methods in the future to improve on the current clinical methods for OA prognosis. On profiling the immune cell landscape in these populations known to be at risk for OA, we observed that there were significant similarities in the immune landscape of the DMT cohort with the OA cohort, although the healthy cohort was distinct from both. These analyses suggest that the immune cell features associated with OA might be present and detectable in the early stages of OA as well and can be used for earlier detection of the disease. One limitation of our study is that although the healthy reference samples had no known history of OA because they were sourced from blood bank donors, their radiographic joint status was not available. Additionally, immunophenotyping reflects a difference in the composition but does not establish a causal relationship between immune correlates of OA and its onset or progression, which would be the next area of exploration. In summary, our data show the promise and potential of future large cohort single-cell proteomic and transcriptomic studies in patients to generate maps for the whole spectrum of OA progression to provide novel biologic insights as well as clinical tools for OA detection and prognosis.

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## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Bhutani confirms that all authors have provided the final approval of the version to be published and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

- Martel-Pelletier J, Barr AJ, Cicuttini FM, et al. Osteoarthritis. *Nat Rev Dis Primers* 2016;2:16072.
- Brown TD, Johnston RC, Saltzman CL, et al. Posttraumatic osteoarthritis: a first estimate of incidence, prevalence, and burden of disease. *J Orthop Trauma* 2006;20(10):739–744.
- Katz JN, Earp BE, Gomoll AH. Surgical management of osteoarthritis. *Arthritis Care Res (Hoboken)* 2010;62(9):1220–1228.
- Bedson J, Croft PR. The discordance between clinical and radiographic knee osteoarthritis: a systematic search and summary of the literature. *BMC Musculoskelet Disord* 2008;9:116.
- Kraus VB, Collins JE, Charles HC, et al; OA Biomarkers Consortium. Predictive validity of radiographic trabecular bone texture in knee osteoarthritis: the Osteoarthritis Research Society International/ Foundation for the National Institutes of Health Osteoarthritis Biomarkers Consortium. *Arthritis Rheumatol* 2018;70(1):80–87.
- Kraus VB, Hargrove DE, Hunter DJ, et al. Establishment of reference intervals for osteoarthritis-related soluble biomarkers: the FNIH/OARSI OA Biomarkers Consortium. *Ann Rheum Dis* 2017;76(1):179–185.
- Kraus VB, Collins JE, Hargrove D, et al; OA Biomarkers Consortium. Predictive validity of biochemical biomarkers in knee osteoarthritis: data from the FNIH OA Biomarkers Consortium. *Ann Rheum Dis* 2017;76(1):186–195.
- Zhou K, Li YJ, Soderblom EJ, et al. A “best-in-class” systemic biomarker predictor of clinically relevant knee osteoarthritis structural and pain progression. *Sci Adv* 2023;9(4):eabq5095.
- Dunn CM, Sturdy C, Velasco C, et al. Peripheral blood DNA methylation-based machine learning models for prediction of knee osteoarthritis progression: biologic specimens and data from the Osteoarthritis Initiative and Johnston County Osteoarthritis Project. *Arthritis Rheumatol* 2023;75(1):28–40.
- Grandi FC, Baskar R, Smeriglio P, et al. Single-cell mass cytometry reveals cross-talk between inflammation-dampening and inflammation-amplifying cells in osteoarthritic cartilage. *Sci Adv* 2020;6(11):eaay5352.
- Knights AJ, Farrell EC, Ellis OM, et al. Synovial fibroblasts assume distinct functional identities and secrete R-spondin 2 in osteoarthritis. *Ann Rheum Dis* 2023;82(2):272–282.
- Fonseka CY, Rao DA, Raychaudhuri S. Leveraging blood and tissue CD4+ T cell heterogeneity at the single cell level to identify mechanisms of disease in rheumatoid arthritis. *Curr Opin Immunol* 2017;49:27–36.
- Rao DA, Gurish MF, Marshall JL, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* 2017;542(7639):110–114.
- Krieg C, Nowicka M, Guglietta S, et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat Med* 2018;24(2):144–153.
- Good Z, Sarno J, Jager A, et al. Single-cell developmental classification of B cell precursor acute lymphoblastic leukemia at diagnosis reveals predictors of relapse. *Nat Med* 2018;24(4):474–483.
- Felson DT, Niu J, Neogi T, et al; MOST Investigators Group. Synovitis and the risk of knee osteoarthritis: the MOST Study. *Osteoarthritis Cartilage* 2016;24(3):458–464.
- Moradi B, Rosshirt N, Tripel E, et al. Unicompartamental and bicompartamental knee osteoarthritis show different patterns of mononuclear cell infiltration and cytokine release in the affected joints. *Clin Exp Immunol* 2015;180(1):143–154.
- Sun F, Zhou JL, Peng PJ, et al. Identification of disease-specific hub biomarkers and immune infiltration in osteoarthritis and rheumatoid arthritis synovial tissues by bioinformatics analysis. *Dis Markers* 2021;2021:9911184.
- Kellgren JH, Lawrence JS. Radiological assessment of osteoarthrosis. *Ann Rheum Dis* 1957;16(4):494–502.
- van Middelkoop M, Bennell KL, Callaghan MJ, et al. International Patellofemoral Osteoarthritis Consortium: consensus statement on the diagnosis, burden, outcome measures, prognosis, risk factors and treatment. *Semin Arthritis Rheum* 2018;47(5):666–675.
- Monaco G, Lee B, Xu W, et al. RNA-seq signatures normalized by mRNA abundance allow absolute deconvolution of human immune cell types. *Cell Rep* 2019;26(6):1627–1640 e1627.
- Katz JN, Martin SD. Meniscus--friend or foe: epidemiologic observations and surgical implications. *Arthritis Rheum* 2009;60(3):633–635.

23. Chu CR, Williams AA, Coyle CH, Bowers ME. Early diagnosis to enable early treatment of pre-osteoarthritis. *Arthritis Res Ther* 2012;14(3):212.
24. Leandro MJ, Cambridge G, Ehrenstein MR, et al. Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. *Arthritis Rheum* 2006;54(2):613–620.
25. Kristyanto H, Blomberg NJ, Slot LM, et al. Persistently activated, proliferative memory autoreactive B cells promote inflammation in rheumatoid arthritis. *Sci Transl Med* 2020;12(570):eaaz5327.
26. Lee DSW, Rojas OL, Gommerman JL. B cell depletion therapies in autoimmune disease: advances and mechanistic insights. *Nat Rev Drug Discov* 2021;20(3):179–199.
27. Xie X, Doody GM, Shuweihi F, et al. B-cell capacity for expansion and differentiation into plasma cells are altered in osteoarthritis. *Osteoarthritis Cartilage* 2023;31(9):1176–1188.
28. Ponchel F, Burska AN, Hensor EM, et al. Changes in peripheral blood immune cell composition in osteoarthritis. *Osteoarthritis Cartilage* 2015;23(11):1870–1878.
29. Souto-Carneiro MM, Mahadevan V, Takada K, et al. Alterations in peripheral blood memory B cells in patients with active rheumatoid arthritis are dependent on the action of tumour necrosis factor. *Arthritis Res Ther* 2009;11(3):R84.
30. Guo X, Xu T, Zheng J, et al. Accumulation of synovial fluid CD19+ CD24hiCD27+ B cells was associated with bone destruction in rheumatoid arthritis. *Sci Rep* 2020;10(1):14386.
31. Hinks A, Ke X, Barton A, et al; UK Rheumatoid Arthritis Genetics Consortium; British Society of Paediatric and Adolescent Rheumatology Study Group. Association of the IL2RA/CD25 gene with juvenile idiopathic arthritis. *Arthritis Rheum* 2009;60(1):251–257.
32. Yao Q, Wu X, Tao C, et al. Osteoarthritis: pathogenic signaling pathways and therapeutic targets. *Signal Transduct Target Ther* 2023;8(1):56.
33. Ishii H, Tanaka H, Katoh K, et al. Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis. *Osteoarthritis Cartilage* 2002;10(4):277–281.
34. Angelini F, Widera P, Mobasheri A, et al. Osteoarthritis endotype discovery via clustering of biochemical marker data. *Ann Rheum Dis* 2022;81(5):666–675.
35. Sahu N, Grandi FC, Bhutani N. A single-cell mass cytometry platform to map the effects of preclinical drugs on cartilage homeostasis. *JCI Insight* 2022;7(20):e160702.
36. Culvenor AG, Øiestad BE, Hart HF, et al. Prevalence of knee osteoarthritis features on magnetic resonance imaging in asymptomatic uninjured adults: a systematic review and meta-analysis. *Br J Sports Med* 2019;53(20):1268–1278.
37. Chu CR, Williams AA, Erhart-Hledik JC, et al. Visualizing pre-osteoarthritis: integrating MRI UTE-T2\* with mechanics and biology to combat osteoarthritis-The 2019 Elizabeth Winston Lanier Kappa Delta Award. *J Orthop Res* 2021;39(8):1585–1595.

# Neutrophil Extracellular Traps–Associated RNA Impedes CD4<sup>+</sup> Treg Differentiation by TLR7–IRF7 Axis in Ankylosing Spondylitis

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**Objective.** Our objective was to investigate the role of neutrophil extracellular traps (NETs) in the pathogenesis of inflammatory disorders in ankylosing spondylitis (AS).

**Methods.** Local and circulating NETs levels were determined by immunofluorescence (IF) and myeloperoxidase (MPO)–DNA quantification in both patients with AS and AS model SKG mice. Flow cytometry (FCM) was performed to detect the effect of NETs on CD4<sup>+</sup> subpopulation differentiation. The therapeutic effects of the neutrophil elastase inhibitor sivelestat (SVT) and the peptidylarginine deiminase 4 (PAD4) inhibitor CI–amidine were evaluated in SKG mice. The localization of NETs and their ability to impede CD4<sup>+</sup> Treg cell differentiation were evaluated via IF, FCM, and Western blotting. RNA sequencing and specific inhibitors were used to clarify the detailed mechanism by which NETs inhibit CD4<sup>+</sup> Treg differentiation.

**Results.** The NETs levels were elevated locally and systemically in both patients with AS and SKG mice, which impeded the differentiation of CD4<sup>+</sup> Treg cells. Blocking NETs formation via SVT or CI–amidine restored the CD4<sup>+</sup> Treg ratio and subsequently alleviated inflammation in SKG mice. NETs were internalized by CD4<sup>+</sup> T cells, and their associated RNA activated the Toll-like receptor 7 (TLR7)–interferon regulatory factor 7 (IRF-7) axis, which then inhibited Treg differentiation. Inhibiting CD4<sup>+</sup> T cells endocytosis, removing the bound RNA component, or blocking the TLR7–IRF-7 axis abrogated the negative effect of NETs on CD4<sup>+</sup> Treg differentiation.

**Conclusion.** Elevated NETs impeded CD4<sup>+</sup> Treg differentiation by activating the TLR7–IRF-7 axis via their associated RNA in AS, and targeting NETs may be a novel treatment strategy for AS and related inflammatory disorders.

## INTRODUCTION

Ankylosing spondylitis (AS) is a major type of spondyloarthritis, with a global prevalence of 0.32% to 1.4%. AS, which is characterized by chronic inflammatory disorders and ectopic ossification, preferentially affects the axial skeleton and results in ankylosis.<sup>1,2</sup> Previous studies have demonstrated that immune dysfunction, such as a decreased proportion of CD4<sup>+</sup> Treg cells, an immunosuppressive subpopulation that restricts excessive

immune responses, leads to the development of chronic inflammation in AS.<sup>3,4</sup> However, the detailed mechanism requires further investigation.

Neutrophil extracellular traps (NETs) are extracellular web-like structures composed of decondensed chromatin and proteins that are released from activated neutrophils.<sup>5</sup> Extensive evidence suggests that NETs are involved in the pathogenesis of many inflammation-associated diseases by affecting the number and function of T cells.<sup>6,7</sup> A recent study revealed that NETs could

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be used as a biomarker of disease activity and were related to new bone formation in AS.<sup>8</sup> However, whether and how NETs play a role in AS inflammation are still ambiguous.

Toll-like receptors (TLRs), a family of highly conserved pattern recognition receptors, are critical for both the innate and adaptive immune systems. Membrane TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) and intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) recognize different components of pathogen-associated molecular patterns, such as histones and nucleic acids, which subsequently regulate cell differentiation and functions and contribute to the inflammatory response *in vivo*.<sup>9</sup> Specifically, TLR2 was recently reported to recognize NETs and their histones to promote Th17 cell differentiation.<sup>10</sup> Considering the importance of TLRs in AS pathogenesis,<sup>11</sup> it is likely that TLRs are involved in T cell dysfunction mediated by NETs in AS.

In this study, we demonstrated that the NETs levels were elevated in both the peripheral blood and diseased joint tissue of patients with AS and AS model animals, SKG mice. Inhibiting NETs formation with sivelestat (SVT) or Cl-amidine mitigated disease progression in SKG mice. Mechanistically, NETs impeded CD4<sup>+</sup> Treg differentiation by activating the TLR7–IRF-7 axis via NETs-bound RNA. Our study provides a new understanding of NETs in AS inflammation and suggests that NETs may be potential therapeutic targets for AS.

## MATERIALS AND METHODS

**Study approval, patients, and related controls.** The animal experiments were performed under protocols approved by the Animal Care and Use Committee of Sun Yat-Sen University. The collection of human samples and the corresponding experiments were approved by the Ethics Committee of the Eighth Affiliated Hospital of Sun Yat-Sen University. Peripheral blood samples were collected from 15 patients with AS diagnosed according to the 1984 Revised New York Diagnostic Criteria and 15 age- and sex-matched healthy donors. Spine enthesitis tissue was obtained from three patients with AS and three patients with spine fracture who did not have AS or other rheumatic diseases, infectious diseases, or tumors. Informed consent was obtained from all the participants. The detailed information is shown in Supplemental Tables 1 and 2.

**Mice.** The mice were housed in a specific pathogen-free barrier facility. To induce arthritis in SKG mice (Shanghai Model Organism), eight-week-old female SKG mice were intraperitoneally injected with 3 mg of curdlan in 200  $\mu$ L of phosphate buffered saline (PBS). The arthritis score was assessed weekly, as described in our previous study.<sup>12</sup> For the treatment of arthritis, each mouse was intraperitoneally injected with 50 mg/kg neutrophil elastase (NE) inhibitor SVT (3535/10, Tocris) in 200  $\mu$ L of PBS or 75 mg/kg peptidylarginine deiminase 4 (PAD4) inhibitor

Cl-amidine (506282, Millipore) in 200  $\mu$ L of PBS or an equal volume of PBS every other day.

To observe the effect of NETs on Treg differentiation *in vivo*, eight-week-old female C57BL/6 mice (GemPharmatech-GD) were given anti-CD3 antibodies (0.1  $\mu$ g/g, 100339; Biolegend) intravenously on day 0. Twenty-four hours later, each mouse was given 200  $\mu$ L of NETs derived from mouse bone marrow–polymorphonuclear leukocytes (BM-PMNs) induced with phorbol 12-myristate 13-acetate (PMA) (50 nM, 70-CS0001; Multi-Sciences) or an equal volume of PBS through tail vein injection.

**Enzyme-linked immunosorbent assay.** The level of myeloperoxidase (MPO)–DNA in the plasma was detected by capture enzyme-linked immunosorbent assay, as previously described.<sup>13</sup> Briefly, whole-blood samples were collected via a plasma separation tube. The plasma was isolated by 10 minutes of centrifugation at 1,000  $\times$  *g* at 4°C. A total of 5  $\mu$ g/mL anti-MPO monoclonal antibody (0400-0002, ABD Serotec) was added to the 96-well plates overnight at 4°C as the capture antibody. After blocking in 1% bovine serum albumin, plasma together with a peroxidase-labeled anti-DNA monoclonal antibody (11774425001, Roche) was added, and the mixture was incubated at room temperature for two hours. The plate was washed with PBS three times. Thereafter, peroxidase substrate (11774425001, Roche) was added, and the mixture was incubated at 37°C for 40 minutes. Finally, the optical density was measured at 405 nm using a Thermo Varioskan lux instrument. To identify the MPO–DNA complex in SKG plasma, a goat anti-myeloperoxidase antibody (AF3667, R&D) was used as the capture antibody.

### Human neutrophil isolation and NETs preparation.

Neutrophils were isolated from peripheral blood by density gradient centrifugation using Ficoll solution (TBD Science, no. LDS1075), followed by erythrocyte lysis using red blood cell lysis buffer (R1010-500, Solarbio). The isolated neutrophils were resuspended in RPMI 1640 culture medium supplemented with 10% fetal bovine serum.

For NETs preparation, neutrophils were seeded in six-well plates at  $4 \times 10^6$  per well and stimulated with 50 nM PMA for four hours. The supernatants were removed carefully, and NETs adhering to the well were washed with precooled PBS. Thereafter, 500  $\mu$ L of MNase (2 U/mL; N863776-10KU, Macklin) was added to digest the NETs for 10 minutes at 37°C. The mixture containing NETs was collected and centrifuged for 10 minutes at 1,000  $\times$  *g* to remove cell debris. For the indicated experiments, NETs were treated with RNase A (10  $\mu$ g/mL, EN0531; Thermo Fisher) or DNase I (10 U/mL, 4716728001; Roche) to digest NETs-bound RNA or NETs-DNA. To obtain NETs-DNA, a MicroElute DNA Clean Up Kit (D6296-02, Omega) was used following the manufacturer's instructions. For visualization of NETs *in vitro*, neutrophils were seeded in confocal dishes

coated with  $2 \times 10^6$  cells per dish (354240, Corning) and stimulated with 2.5 nM PMA for 4 hours or without PMA for 4 or 12 hours for spontaneous NETosis.

**Human CD4<sup>+</sup> T cell isolation, differentiation, and stimulation.** Human peripheral blood samples were subjected to density gradient separation as described previously. The peripheral blood mononuclear cell (PBMC) layer was subsequently extracted, and human CD4 microbeads (Miltenyi, 130-045-101) were used to isolate CD4<sup>+</sup> T cells. To visualize NETs-derived proteins in CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells were stimulated with NETs for 24 hours and then transferred to confocal dishes coated with Cell-Tak. To assess the effect of NETs on CD4<sup>+</sup> T cell differentiation, CD4<sup>+</sup> T cells were seeded in 48-well plates at  $0.5 \times 10^6$  cells per well and cultured with anti-CD3 (1  $\mu$ g/mL, 05121-25-500; Biogems) and anti-CD28 (1  $\mu$ g/mL, 10311-25-500; Biogems) antibodies. CD4<sup>+</sup> T cells were then induced for differentiation as follows: Th1, interleukin-2 (IL-2) (10 U/mL, CSBSJ-IL-2; Prosperich), IL-12 (10 ng/mL, 200-12; Peprotech), and anti-IL-4 (10  $\mu$ g/mL, 81121-25; Biogems); Th2, IL-2 (10 U/mL), IL-4 (5 ng/mL, 200-04; Peprotech), and anti-interferon- $\gamma$  (anti-IFN- $\gamma$ ) (10  $\mu$ g/mL, 80821-25; Biogems) antibodies; Th17, IL-2 (10 U/mL), transforming growth factor  $\beta$  (TGF $\beta$ ) (10 ng/mL, 100-21C; Peprotech), IL-6 (10 ng/mL, 200-06; Peprotech), IL-23 (20 ng/mL, 200-23; Peprotech), anti-IFN- $\gamma$  (10  $\mu$ g/mL), and anti-IL-4 (10  $\mu$ g/mL) antibodies; and Treg, IL-2 (10 U/mL), and TGF $\beta$  (2.5 ng/mL) antibodies.

For the indicated experiments, CD4<sup>+</sup> T cells were cultured with 200  $\mu$ L of NETs or NETs treated with RNase A or DNase I. In some experiments, CD4<sup>+</sup> T cells were treated with pitstop 2 (S9670, Selleck), chloroquine (CQ) (C6628, Sigma-Aldrich), synthetic RNA40 (tlrl-rna40, Invivo), or TLR inhibitors for two hours before stimulation with NETs. The TLR inhibitors used in the study were as follows: the TLR7-selective inhibitor IRS661 (5'-TGCTTGCAAGCTTGCAAGCA-3', IGE Biotechnology), the TLR7/9 inhibitors IRS954 (5'-TCGATGACGTTCTCTGATGCT-3', IGE Biotechnology), and control oligodeoxynucleotide (cODN, 5'-TCCTGCAGGTTAAGT-3', IGE Biotechnology), the TLR3-selective inhibitor CU-CPT 4a (TLR3-IN-1, MedChemExpress), the TLR8-selective inhibitor CU-CPT 9a (inh-cc9a, InvivoGen), and the TLR9-specific inhibitor G-type-iODN (5'-CTCCTATTGGGGTTTCCTAT-3', ALX-746-252-C100; Enzo).

**Mouse PBMC isolation.** Mouse whole blood was obtained via pericardial puncture. After being diluted in PBS, whole blood was loaded onto Ficoll solution for density gradient centrifugation. The PBMC layer was carefully transferred into another tube and washed with PBS.

**Isolation of mouse BM-PMNs and preparation of NETs.** The femurs of wild-type C57BL/6 mice were dissected, and the bone marrow cavity was rinsed with PBS. The cell

suspension was centrifuged, and the red blood cells were removed using lysis buffer (00-4300-54, Invitrogen). BM-PMNs were subsequently isolated via a mouse neutrophil isolation kit (130-097-658, Miltenyi Biotec) following the manufacturer's instructions. NETs were obtained from mouse BM-PMNs as described previously for further experiments.

**NETosis assay.** Neutrophils were seeded in 96-well plates at  $0.5 \times 10^5$  per well and cultured for 4 or 12 hours for spontaneous NETosis or stimulated with 2.5 nM of PMA for 4 hours to induce NETosis. SYTOX Green nucleic acid stain (S7020, Invitrogen) was used to detect extracellular DNA. SYTOX Green was added to a final concentration of 0.2  $\mu$ M, and the mixture was cocultured with the cells for 30 minutes. The fluorescence intensity was quantified using a Thermo Varioskan lux instrument.

**Statistical analysis.** All the statistical analyses were performed via GraphPad Prism 8.0 software. The Shapiro-Wilk normality test was used to assess the normality of the data. Two-tailed Student's *t*-tests were performed to analyze differences between two groups of samples. One-way analysis of variance (ANOVA) was used to analyze differences among three or more groups. To analyze the differences in the arthritis scores, two-way repeated-measures ANOVA was performed. The number of samples is indicated in the relevant figure legends. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001 indicate statistical significance, and "ns" indicates no statistical significance.

**Data availability.** The RNA sequencing data generated in this study have been deposited in the NCBI BioProject database under accession code PRJNA1169677. All other data supporting the findings of this study are available within the article and its supplementary data. Additional methods are included in the Supplemental Materials and Methods.

## RESULTS

**Elevated NETs levels involved in AS inflammatory disorder.** The amount of NETs infiltrating spine enthesitis tissues was greater in patients with AS than in control individuals (Figure 1A). In addition, the level of MPO-DNA, a marker of circulating NETs, was significantly greater in the plasma samples of patients with AS than in those of healthy donors (Figure 1B). The plasma NETs levels were positively related to the C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) in patients with AS (Supplemental Figure 1A). Similar results were observed in the ankle joints and peripheral blood of SKG mice, an AS model animal (Figure 1C and D). Although the number of infiltrating neutrophils was greater in both the spine enthesitis tissues of patients with AS (Supplemental Figure 1B) and the ankle

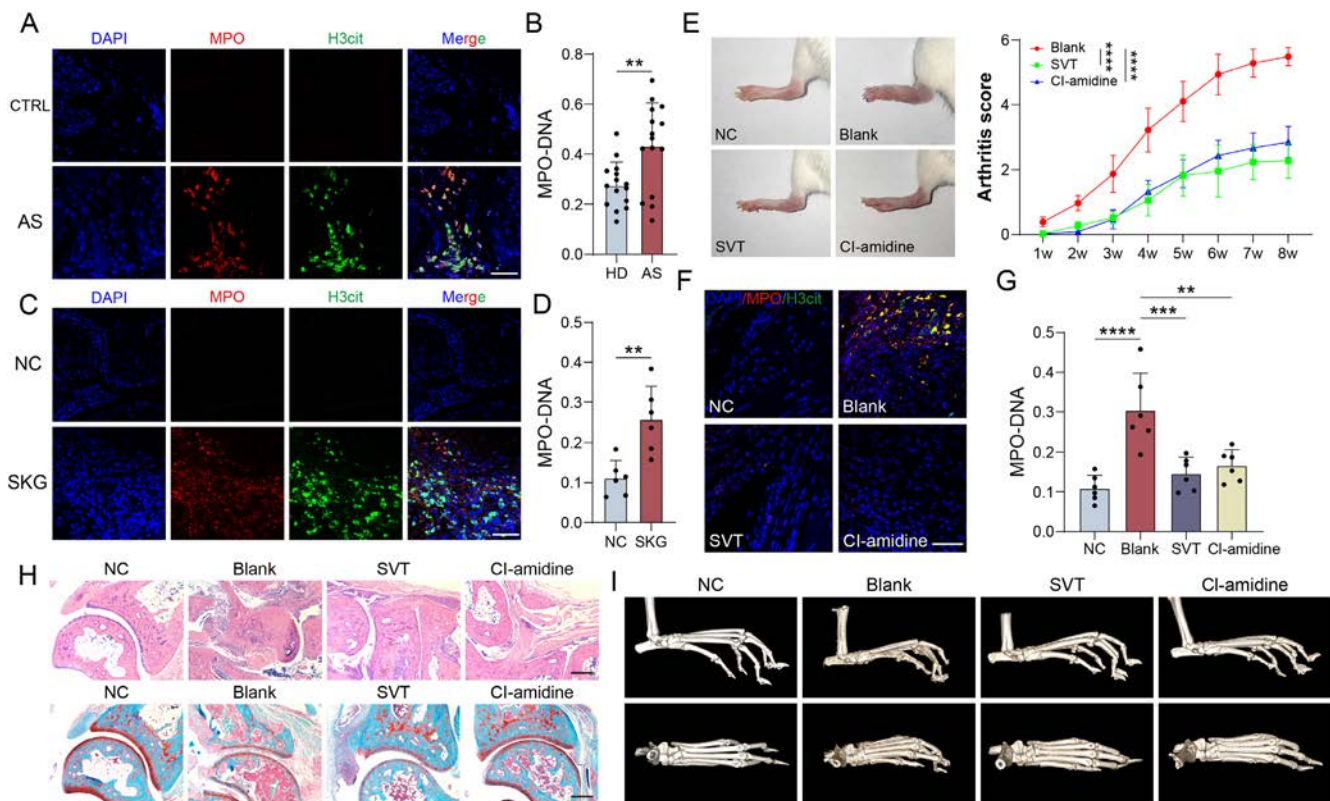


**Figure 1.** Elevated NETs level was involved in AS inflammatory disorder. (A) NETs infiltration in spine entheses tissues from patients with AS and matched CTRLs. Scale bar = 50  $\mu$ m. (B) The optical density of MPO-DNA in the plasma of patients with AS ( $n = 15$ ) and healthy donors ( $n = 15$ ). (C) NETs infiltration in entheses from SKG mice and corresponding normal controls without arthritis. Scale bar = 50  $\mu$ m. (D) The optical density of MPO-DNA in plasma from SKG mice ( $n = 6$ ) and normal controls ( $n = 6$ ). (E) Ankle joints and arthritis scores of induced SKG mice without SVT treatment (Blank) and induced SKG mice with SVT treatment ( $n = 6$ ) or Cl-amidine treatment ( $n = 6$ ). (F) NETs infiltration in the ankles of SKG mice in the four groups. NC indicates uninduced SKG mice. Scale bar = 50  $\mu$ m. (G) The optical density of MPO-DNA in the plasma of SKG mice in the four groups ( $n = 6$ ). (H) Hematoxylin and eosin staining and safranin O-fast green staining of the ankles of the SKG mice in the four groups ( $n = 6$ ). Scale bar = 200  $\mu$ m. (I) 3D reconstructions of the ankles of the SKG mice in the three groups ( $n = 6$ ). The data are presented as means  $\pm$  SDs. \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ; ns,  $P > 0.05$ . AS, ankylosing spondylitis; Cl, clathrin inhibitor; CTRL, control; DAPI, 4',6-diamidino-2-phenylindole; MPO, myeloperoxidase; NC, normal control; NET, neutrophil extracellular trap; ns, not significant; SVT, sivelestat.

joints of SKG mice (Supplemental Figure 1C), the ability of neutrophils to form NETs spontaneously or after PMA stimulation was the same between healthy donors and patients with AS, as determined by immunofluorescence and SYTOX Green staining to detect extracellular DNA levels (Supplemental Figure 1D–G). These results indicated that the elevated NETs levels in patients with AS resulted from the increased quantity of neutrophils rather than their potential to generate NETs.

We further applied the neutrophil elastase inhibitor SVT and the PAD4 inhibitor Cl-amidine to suppress NETs formation and detected its therapeutic effect on SKG mice. After treatment with SVT or Cl-amidine, ankle swelling was relieved, and the arthritis score decreased (Figure 1E). The number of NETs infiltrating the ankle joints and the number of NETs in the peripheral blood were significantly reduced after SVT or Cl-amidine treatment (Figure 1F and G). Hematoxylin and eosin staining and safranin O-fast green staining revealed that immune cell infiltration and articular cartilage

degradation in the ankle joints were alleviated after SVT or Cl-amidine treatment (Figure 1H). Ectopic ossification was also ameliorated after treatment with SVT or Cl-amidine (Figure 1I). Taken together, these results suggest that elevated NETs levels are involved in AS-related inflammation and that targeting NETs could have a therapeutic effect on AS.

#### CD4<sup>+</sup> Treg differentiation impeded by AS-NETs. CD4<sup>+</sup>

T cells dysfunction is one of the pathologic features leading to chronic inflammation in AS,<sup>14</sup> and the differentiation potential of CD4<sup>+</sup> T cells is reportedly affected by NETs.<sup>15</sup> To further investigate the effect of NETs on AS, we evaluated the subpopulation proportion of CD4<sup>+</sup> T cells treated with NETs derived from neutrophils isolated from patients with AS (AS-NETs) in vitro. AS-NETs stimulation led to a significant decrease in the proportion of CD4<sup>+</sup> Treg cells, and the inhibitory effect was positively correlated with the concentration of AS-NETs (Figure 2A). The

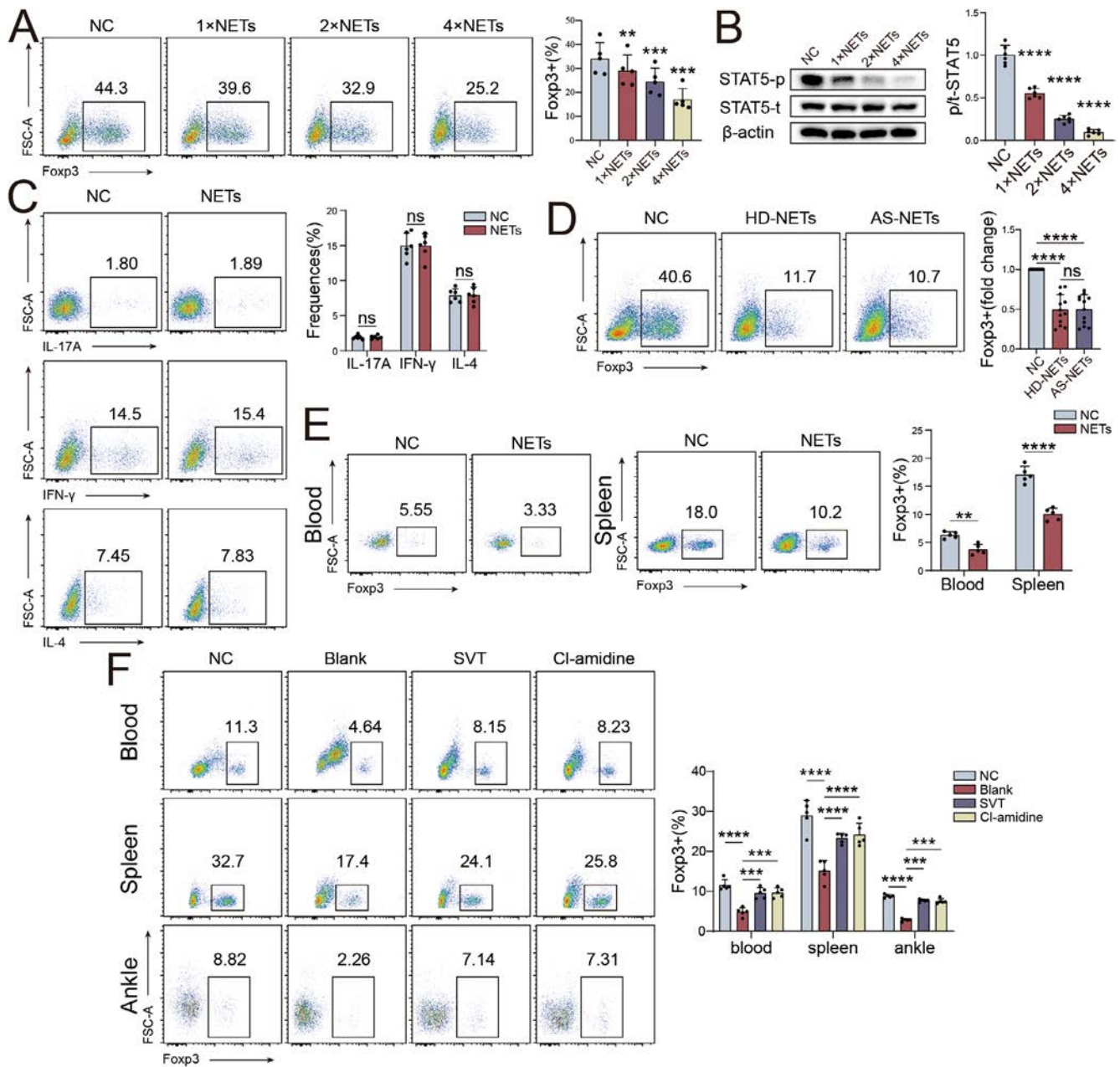


**Figure 2.** AS-NETs impeded CD4<sup>+</sup> Treg differentiation. (A) CD4<sup>+</sup> T cells were cultured with or without different concentrations of AS-NETs for five days, and the proportion of Treg cells among CD4<sup>+</sup> T cells was measured by flow cytometry ( $n = 5$ ). (B) Phosphorylation of STAT5 in CD4<sup>+</sup> T cells stimulated with or without different concentrations of NETs for 24 hours ( $n = 6$ ). (C) Proportions of Th1, Th2, and Th17 cells among CD4<sup>+</sup> T cells cultured in the presence or absence of NETs under corresponding polarization conditions ( $n = 6$ ). (D) Proportions of Treg cells among CD4<sup>+</sup> T cells from HDs cultured in the presence or absence of AS-NETs or HD-NETs for five days ( $n = 12$ ). Treg proportion is presented as fold change in the number of stimulated CD4<sup>+</sup> T cells over the number of NC-treated cells. (E) Proportion of Treg cells among CD4<sup>+</sup> T cells in the peripheral blood mononuclear cells (PBMCs) or spleens of C57BL/6 mice treated with or without bone marrow–polymorphonuclear leukocytes–derived NETs ( $n = 5$ ). (F) Proportions of Treg cells among CD4<sup>+</sup> T cells in the PBMCs, spleen, or ankle tissue from the animal experiments mentioned in Figure 1 ( $n = 5$ ). The data are presented as means + SDs. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ; ns,  $P > 0.05$ . AS, ankylosing spondylitis; HD, healthy donor; NC, negative control; NET, neutrophil extracellular trap; ns, not significant; SVT, sivelestat.

phosphorylation of STAT5, a key transcription factor that regulates FoxP3 expression and Treg differentiation, was also inhibited by AS-NETs (Figure 2B). However, AS-NETs had no effect on the differentiation of Th1, Th2, or Th17 cells (Figure 2C). No significant difference in the potential to prevent Treg differentiation

was detected between equal amounts of AS-NETs and NETs obtained from healthy donor neutrophils (Figure 2D). We also investigated the effect of NETs on Treg differentiation in vivo. After receiving mouse-derived NETs, the proportions of CD4<sup>+</sup> Treg cells in the peripheral blood and spleens of the mice were

significantly decreased (Figure 2E). We further detected the proportions of CD4<sup>+</sup> Treg in the peripheral blood, spleen, and joint tissues of SKG mice treated with SVT or CI-amidine. After the induction of arthritis, the proportion of CD4<sup>+</sup> Treg cells was significantly decreased in the peripheral blood, spleen, and ankle joints, which was abrogated by SVT or CI-amidine treatment (Figure 2F). These results suggest that AS-NETs impeded CD4<sup>+</sup> Treg cells differentiation both in vivo and in vitro.

**CD4<sup>+</sup> Treg differentiation impeded by internalization of NETs components.** Confocal microscopy revealed that NETs components were present in the cytoplasm of CD4<sup>+</sup> T cells cocultured with NETs (Figure 3A). Western blot

analysis also confirmed that NETs components MPO and H3cit were internalized by CD4<sup>+</sup> T cells (Figure 3B). We pretreated CD4<sup>+</sup> T cells with clathrin inhibitor (CI) before NETs stimulation to inhibit clathrin-mediated endocytosis, which accounts for 95% of the total endocytosis in mammalian cells. The negative regulation of CD4<sup>+</sup> Treg differentiation by NETs was significantly inhibited after CI treatment (Figure 3C). Similarly, the levels of phosphorylated STAT5 were restored by CI treatment (Figure 3D). In addition, MPO and H3cit internalization decreased after CI treatment (Figure 3E and F). These results suggest that the negative regulation of CD4<sup>+</sup> Treg cells differentiation by NETs depends on the endocytosis of NET components by CD4<sup>+</sup> T cells.

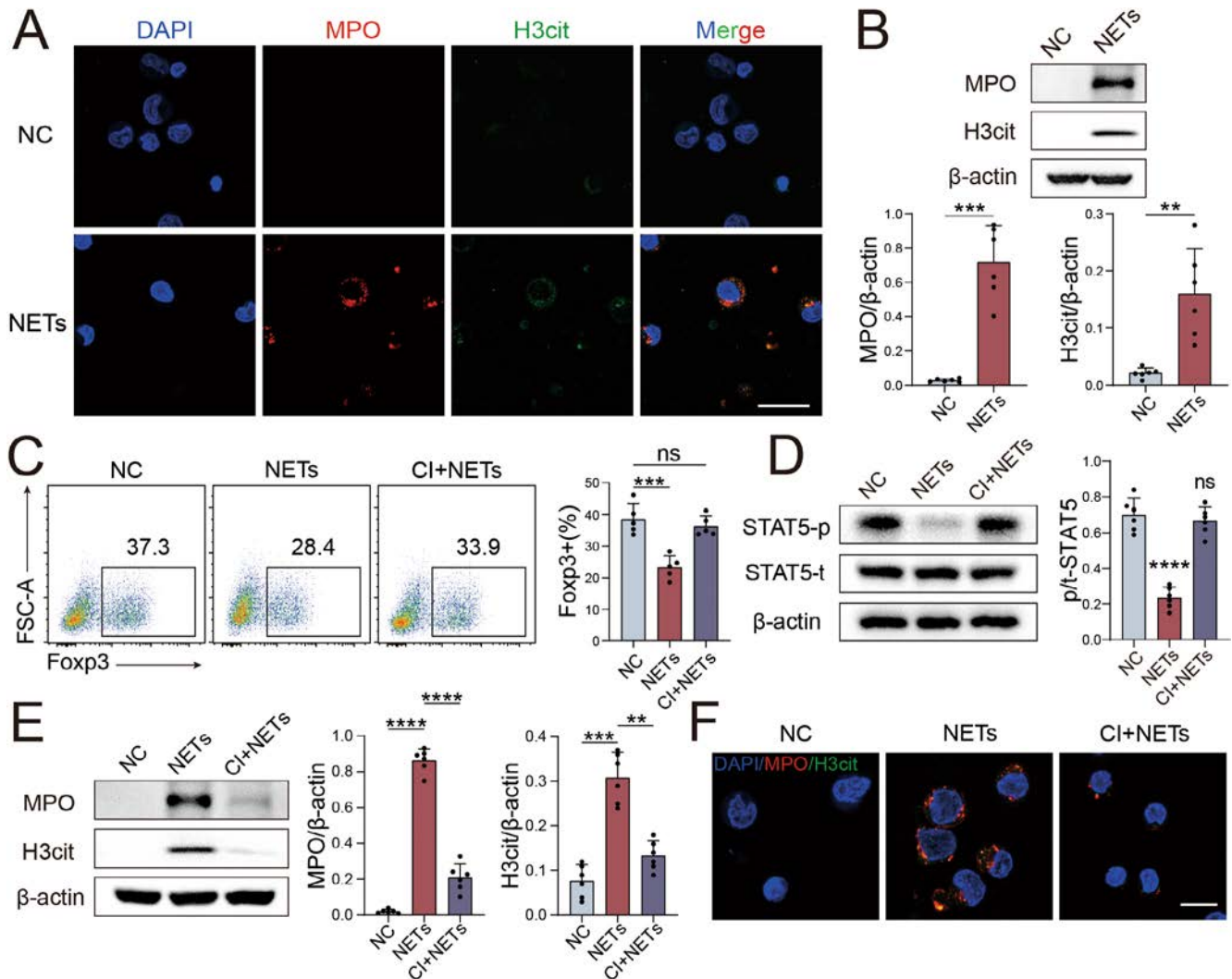


**Figure 3.** Internalization of NETs components by CD4<sup>+</sup> T cells impedes Treg differentiation. (A and B) CD4<sup>+</sup> T cells were cultured with or without NETs for 24 hours. Thereafter, the NETs components H3cit and MPO were detected in CD4<sup>+</sup> T cells by (A) immunofluorescence and (B) Western blot analysis. (C–F) Pitstop 2, a CI, was applied to inhibit the endocytosis of NETs by CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were pretreated with or without CI and subsequently stimulated with NETs. (C) The proportion of Treg cells among CD4<sup>+</sup> T cells was analyzed via flow cytometry (n = 5). (D) The levels of phosphorylated STAT5 in CD4<sup>+</sup> T cells (n = 5). (E) H3cit and MPO levels in CD4<sup>+</sup> T cells were analyzed via Western blotting (n = 5). (F) Visualization of H3cit and MPO in CD4<sup>+</sup> T cells via immunofluorescence staining. Scale bar = 50 μm. The data bars represent means + SDs. \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001; ns, *P* > 0.05. CI, clathrin inhibitor; DAPI, 4',6-diamidino-2-phenylindole; MPO, myeloperoxidase; NC, negative control; NET, neutrophil extracellular trap; ns, not significant.

**CD4<sup>+</sup> Treg differentiation impeded by the NETs associated RNA components.** NETs contain various components that may participate in disease development through different mechanisms.<sup>16</sup> Nucleic acid components in NETs have been reported to regulate inflammatory responses, and the sensors for nucleic acids are distributed mainly in the cytoplasm.<sup>17</sup> Therefore, we used DNase I or RNase A to digest the DNA or RNA components of NETs. Compared with that in the untreated NETs group, the negative regulatory effect on CD4<sup>+</sup> Treg differentiation was significantly weakened in the RNase A-treated NETs group, whereas no significant difference was detected in the DNase I-treated NETs group (Figure 4A). The level of phosphorylated STAT5 was consistent (Figure 4B). In addition, NET DNA alone did not affect CD4<sup>+</sup> Treg differentiation or phosphorylated STAT5 levels (Supplemental Figure 2A and B). Using SYTO RNaselect Green fluorescent cell stain, we showed that NETs carried a large amount of RNA, which was removed after RNase treatment (Supplemental Figure 2C). NET-bound RNA labeled with SYTO RNaselect Green was present in the cytoplasm of CD4<sup>+</sup> T cells and was reduced when CD4<sup>+</sup> T cells were stimulated with RNase

A-treated NETs or pretreated with CI before NETs stimulation (Figure 4C). Furthermore, neither synthetic RNA (RNA40) nor RNase-treated NETs (NETs-scaffolds) alone inhibited CD4<sup>+</sup> Treg differentiation, whereas synthetic RNA combined with NETs-scaffolds had a negative regulatory effect similar to that of NETs (Figure 4D). The changes in phosphorylated STAT5 levels were consistent (Figure 4E). These results indicate that the negative regulation of CD4<sup>+</sup> Treg differentiation depends on the endocytosis of NETs-bound RNA by CD4<sup>+</sup> T cells, and that the NETs-bound RNA sequence is not specific but needs the synergistic effect of other NET components.

**CD4<sup>+</sup> Treg differentiation impeded by the activated TLR7 signaling pathway.** RNA sequencing was performed, and a total of 137 differentially expressed genes (DEGs) were identified between CD4<sup>+</sup> T cells cultured with or without NETs, with 107 up-regulated genes and 30 down-regulated genes (Figure 5A). The TLR signaling pathway was enriched according to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and gene set enrichment analysis (GSEA) (Figure 5B and C). After

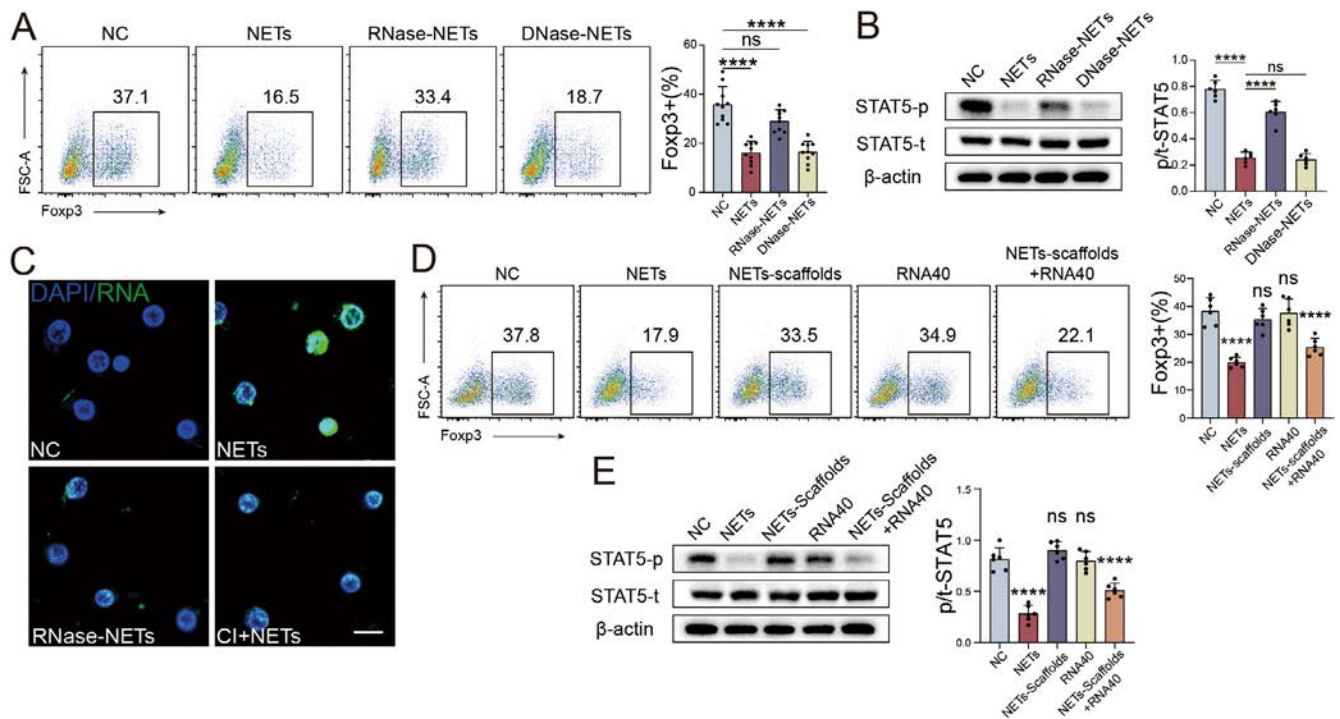


**Figure 4.** NETs impeded CD4<sup>+</sup> Treg differentiation by the associated RNA components. (A) NETs induced by phorbol myristate acetate *in vitro* were treated with DNase I or RNase A. CD4<sup>+</sup> T cells were stimulated with or without NETs, RNase-NETs, or DNase-NETs for five days, and the proportion of Treg cells in CD4<sup>+</sup> T cells was analyzed via flow cytometry ( $n = 10$ ). (B) Phosphorylated STAT5 levels in CD4<sup>+</sup> T cells treated with different types of NETs for 24 hours ( $n = 6$ ). (C) NETs-bound RNA was labeled with SYTO RNaselect Green and then treated with RNase A or not. CD4<sup>+</sup> T cells were pretreated with or without a CI for 2 hours and subsequently stimulated with labeled NETs or RNase-NETs for 24 hours. The distribution of labeled RNA (green) in CD4<sup>+</sup> T cells was visualized via confocal microscopy. Scale bar = 50  $\mu$ m. (D) Proportions of Treg cells among CD4<sup>+</sup> T cells stimulated with or without NETs, NETs-scaffolds (RNase-NETs), synthetic RNA (RNA40), or RNA40 combined with NETs-scaffolds for five days ( $n = 6$ ). (E) Phosphorylated STAT5 levels in CD4<sup>+</sup> T cells in the presence or absence of NETs, NETs-scaffolds (RNase-NETs), RNA40, or RNA40 combined with NETs-scaffolds for 24 hours ( $n = 6$ ). The data are presented as means + SDs. \*\*\*\* $P < 0.0001$ ; ns,  $P > 0.05$ . CI, clathrin inhibitor; DAPI, 4',6-diamidino-2-phenylindole; NC, negative control; NET, neutrophil extracellular trap; ns, not significant.

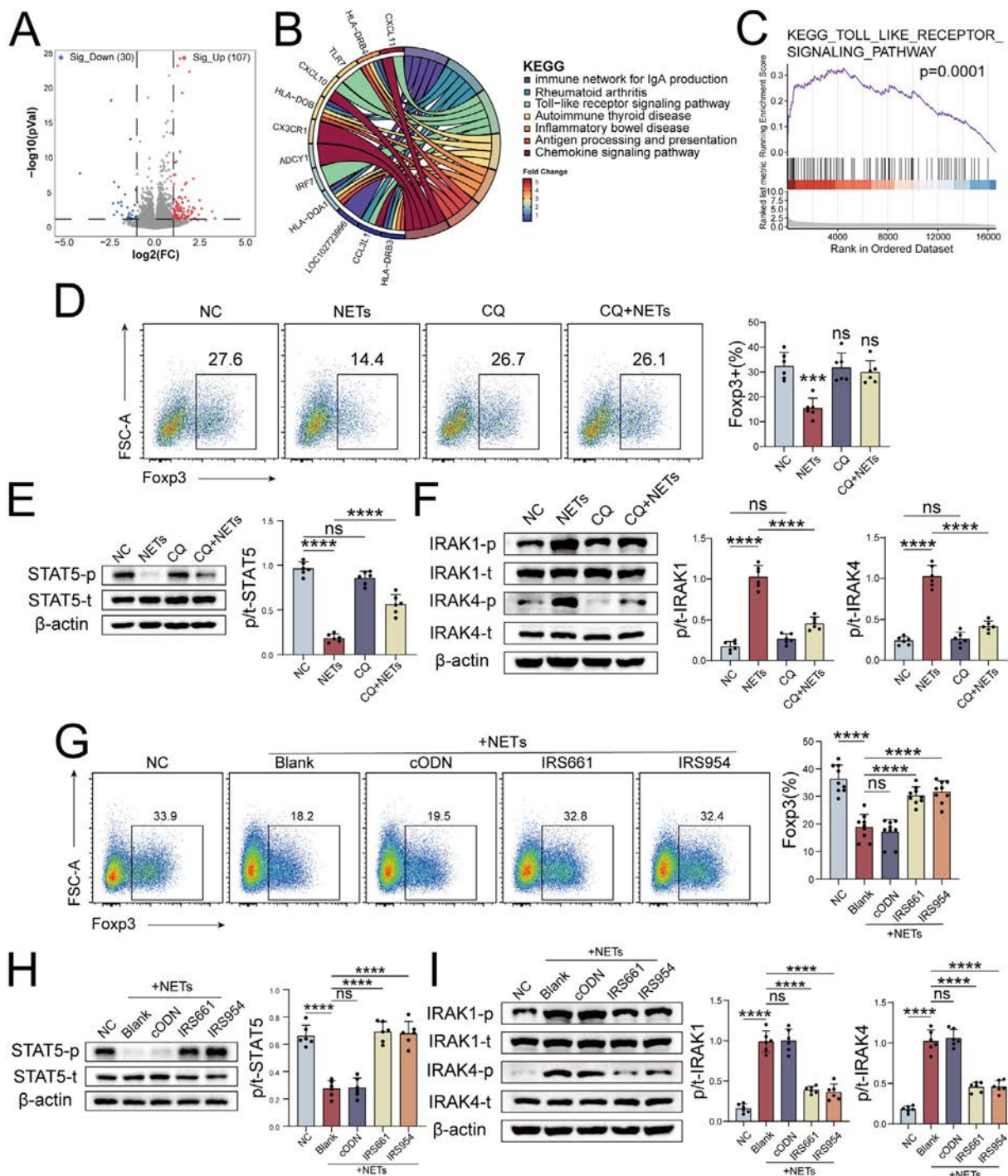


**Figure 5.** NETs impeded CD4<sup>+</sup> Treg differentiation through the Toll-like receptor 7 (TLR7) signaling pathway. (A) Volcano plots showing differentially expressed genes between the NETs group and NC group. (B) KEGG analysis of NETs group versus NC group. (C) Gene set enrichment analysis of TLR signaling pathway in NETs group versus NC group. (D–F) CD4<sup>+</sup> T cells were pretreated with or without the intracellular TLR inhibitor CQ for two hours and subsequently stimulated with NETs or not ( $n = 6$ ). (D) Proportion of Treg cells among CD4<sup>+</sup> T cells stimulated with NETs for five days. (E) Phosphorylated STAT5 levels in CD4<sup>+</sup> T cells stimulated with NETs for 24 hours. (F) Phosphorylated IRAK1 and IRAK4 levels in CD4<sup>+</sup> T cells stimulated with NETs for 24 hours. (G–I) CD4<sup>+</sup> T cells were pretreated with or without ODNs (IRS 661, IRS 954 and cODN) for two hours. IRS 661 to inhibit TLR7 and IRS 954 to inhibit TLR7 and TLR9 were used, and cODN was used as inactive control, followed by stimulation with NETs. (G) Proportion of Treg cells among CD4<sup>+</sup> T cells cultured for five days ( $n = 9$ ). (H) Phosphorylated STAT5 levels in CD4<sup>+</sup> T cells stimulated with NETs for 24 hours ( $n = 6$ ). (I) Phosphorylated IRAK1 and IRAK4 levels in CD4<sup>+</sup> T cells stimulated with NETs for 24 hours ( $n = 6$ ). The data are presented as means  $\pm$  SDs. \*\*\*\* $P < 0.0001$ ; ns,  $P > 0.05$ . cODN, control oligodeoxynucleotide; CQ, chloroquine; KEGG, Kyoto Encyclopedia of Genes and Genomes; IRAK1, interleukin-1 receptor–associated kinase 1; IRS, immunoregulatory sequence; NC, negative control; NET, neutrophil extracellular trap; ns, not significant.

CD4<sup>+</sup> T cells were cultured with NETs, the levels of phosphorylated interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4, two key proteins in the TLR pathway, were significantly increased, indicating the activation of the TLR pathway (Supplemental Figure 3). Moreover, this NET activation effect was partially blocked by RNase or CI treatment (Supplemental Figure 3). After pretreatment with the intracellular TLR inhibitor CQ, the inhibitory effect of NETs on CD4<sup>+</sup> Treg differentiation was significantly weakened (Figure 5D). Similarly, the effect of NETs on STAT5 phosphorylation was inhibited after CQ pretreatment (Figure 5E). CQ pretreatment also reduced the levels of phosphorylated IRAK1 and IRAK4 in response to NETs stimulation (Figure 5F). Taken together, these results indicate that NETs impeded CD4<sup>+</sup> Treg differentiation by activating the TLR pathway, which depended on the endocytosis of NETs-bound RNA by CD4<sup>+</sup> T cells.

Intracellular TLRs include TLR3, which recognizes double-stranded RNA mainly from viruses or bacteria; TLR7/8, which recognizes single-stranded RNA; and TLR9, which recognizes unmethylated cytosine phosphate guanosine DNA. TLR7 was identified as one of the top 10 DEGs (Figure 5B). To further confirm which TLRs mediate the effect of NETs on CD4<sup>+</sup> Treg differentiation, we used specific inhibitors of TLR7/9, including immunoregulatory sequence (IRS) 661, a TLR7-specific inhibitor; IRS 954, a TLR7/9 inhibitor; G-type-iODN, a TLR9-specific inhibitor; and cODN, a negative control. In addition, we also applied small-molecule selective inhibitors of TLR3 (CU-CPT 4a) and TLR8 (CU-CPT9a). Compared with cODN pretreatment, pretreatment with IRS 661 or IRS 954 blocked the negative regulatory effect of NETs on CD4<sup>+</sup> Treg differentiation (Figure 5G). However, pretreatment with CU-CPT4a (TLR3i), CU-CPT9a (TLR8i), or G-type iODN (TLR9i) had no effect on the negative regulatory effect of NETs (Supplemental Figure 4A). Similar results were shown for the STAT5 phosphorylation levels (Figure 5H, Supplemental Figure 4B). The phosphorylation levels of IRAK1 and IRAK4 were also restored by IRS 661 (TLR7i) or IRS 954 (TLR7/9i) pretreatment (Figure 5I). These results indicate that NETs-bound RNA impedes CD4<sup>+</sup> Treg differentiation through the TLR7 signaling pathway.

**CD4<sup>+</sup> Treg differentiation impeded by NETs - bound RNA via the TLR7-IRF-7 axis.** We further examined the phosphorylation levels of several key proteins downstream of TLR7 after NETs stimulation and found that the phosphorylation level of IRF7 significantly increased, whereas the phosphorylation levels of IRF-5, c-Fos, and NF- $\kappa$ B did not change (Figure 6A). IRF-7 is mainly distributed in the cytoplasm in the resting state and is translocated from the cytoplasm to the nucleus once activated.<sup>18</sup> Confocal microscopy revealed that IRF-7 signals in the nuclei of CD4<sup>+</sup> T cells significantly increased after NETs stimulation (Figure 6B). Moreover, the ability of NETs to activate IRF-7 was restored by RNase, IRS 661, or IRS 954 treatment (Figure 6C, Supplemental Figure 5). We further constructed an IRF-7-knockdown lentivirus (Figure 6D) and demonstrated that

knocking down IRF-7 inhibited the negative regulatory effect of NETs on CD4<sup>+</sup> Treg differentiation as well as the phosphorylation of STAT5 (Figure 6E, Supplemental Figure 6). These results suggest that NETs impeded CD4<sup>+</sup> Treg differentiation through the TLR7-IRF-7 axis.

## DISCUSSION

AS is a common type of rheumatic disease characterized by chronic inflammation and ectopic ossification.<sup>19</sup> In recent years, novel therapies that reduce inflammation, such as biologic disease-modifying antirheumatic drugs, have been developed and have provided substantial benefit in terms of achieving remission in patients with AS.<sup>20</sup> However, the clinical response rate of even the most effective treatment is approximately 60% to 70%, as determined by the Assessment of Spondyloarthritis International Society for 20% improvement,<sup>21,22</sup> indicating that the pathogenesis of AS inflammation remains unknown.

Neutrophils are among the most common but also the most neglected immune cells. As the first line of host defense against microorganisms, neutrophils play important roles in the development of chronic inflammation and the progression of rheumatic diseases.<sup>23,24</sup> Previous studies demonstrated that the ratio of neutrophils to lymphocytes was positively related to markers of inflammation in patients with AS<sup>25</sup> and revealed the contribution of neutrophils to AS pathogenesis.<sup>26,27</sup> However, the detailed mechanism by which neutrophils are involved in AS needs further investigation. Although NETs formation constitutes one of the critical strategies by which neutrophils neutralize invading microbes, neutrophils exacerbate inflammation under pathologic conditions through the excessive formation of NETs.<sup>5</sup> Recently, neutrophil-derived NETs have been reported to contribute to the development and progression of rheumatic diseases such as rheumatoid arthritis.<sup>28</sup> In this study, we first demonstrated that the local and systemic levels of NETs were significantly increased in both patients with AS and SKG mice. In addition, the increased levels of NETs in the peripheral blood samples of patients with AS were positively correlated with the levels of the inflammatory biomarkers CRP and ESR, indicating a strong association between NETs levels and disease activity in patients with AS.

NETs affect the differentiation and function of various immune cells, among which T cells have been widely reported to play important roles in AS pathogenesis.<sup>29,30</sup> Abnormal T cell subpopulation proportions, manifested by a higher Th17:Treg ratio, are a feature of patients with AS and suggest aberrant immune system activation.<sup>3,31</sup> Therefore, we investigated whether AS-derived NETs impact T cell differentiation. Although AS-NETs did not alter the differentiation of Th1, Th2, or Th17 cells, they significantly impeded CD4<sup>+</sup> Treg differentiation in vitro, and this effect increased with increasing AS-NET concentration. Moreover, increasing the number of circulatory NETs markedly decreased the proportion of CD4<sup>+</sup> Treg cells in both the peripheral

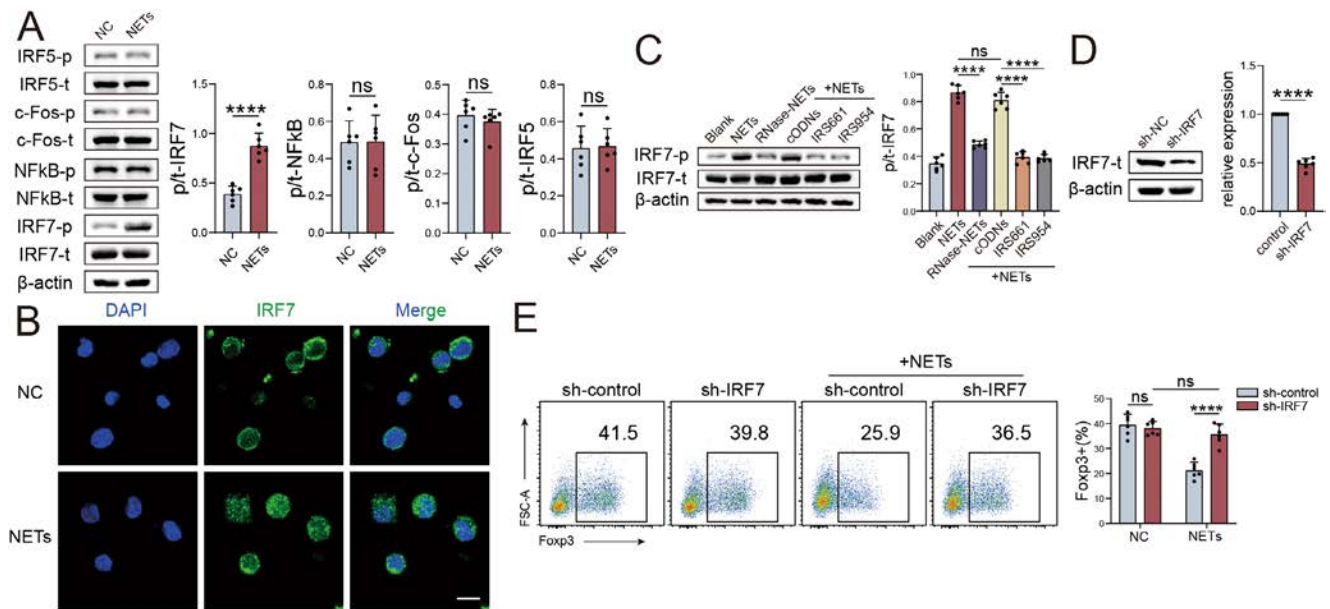


**Figure 6.** NETs-bound RNA impeded CD4<sup>+</sup> Treg differentiation via the Toll-like receptor 7 (TLR7)–IRF-7 axis. (A) Levels of phosphorylated IRF-5, c-Fos, NF-κB, and IRF-7 in CD4<sup>+</sup> T cells stimulated with or without NETs for 24 hours (n = 6). (B) Distribution of IRF-7 in CD4<sup>+</sup> T cells stimulated with or without NETs for 24 hours. Scale bar = 50 μm. (C) CD4<sup>+</sup> T cells were pretreated with cODN, IRS 661 (TLR7) or IRS 954 (TLR7/9) for 2 hours and then stimulated with NETs or RNase-NETs for 24 hours. The level of phosphorylated IRF-7 in CD4<sup>+</sup> T cells was analyzed via Western blotting (n = 6). (D) Efficiency of IRF-7 knockdown in CD4<sup>+</sup> T cells via lentivirus transfection (n = 6). (E) CD4<sup>+</sup> T cells were transfected with an IRF-7-knockdown lentivirus or control vector; subsequently, the cells were cultured in the presence or absence of NETs. The proportion of Treg cells among CD4<sup>+</sup> T cells was analyzed by flow cytometry (n = 6). The data are presented as means + SDs. \*\*\*\**P* < 0.0001; ns, *P* > 0.05. cODN, control oligodeoxynucleotide; IRF-7, interferon regulatory factor 7; IRS, immunoregulatory sequence; NC, negative control; NET, neutrophil extracellular trap; ns, not significant.

blood and the spleen. We suggest that elevated NETs levels may be one of the critical reasons for the increased Th17:Treg ratio in patients with AS, leading to inflammatory disorders. A recent study reported that NETs from the neutrophils of healthy donors consistently increase the Th17:Treg ratio by promoting Th17 differentiation.<sup>10</sup> This effect on Th17 cells was not observed in our study, potentially because of the different sources and culture systems of neutrophils, and this discrepancy needs further investigation.

A greater amount of neutrophil infiltration in both patients with AS and SKG mice was reported in previous studies<sup>32,33</sup> as well as in our present study. To our surprise, the ability of the same number of neutrophils from patients with AS to form NETs spontaneously or under PMA stimulation was equal to that of neutrophils from healthy donors. In addition, no difference in the potential to impede CD4<sup>+</sup> Treg differentiation was detected between NETs derived from healthy donors and those derived from patients with AS. These results indicated that the increased levels of NETs and their effects leading to a lower Treg proportion were due to the greater number of neutrophils in the local microenvironment of patients with AS rather than the ability of neutrophils to generate NETs or the components of AS neutrophil-derived NETs. A recent study demonstrated that mesenchymal stem cell (MSC)-derived CXCL12-abundant reticular cells recruit many neutrophils to the site of spine entheses,<sup>27</sup> which may be

one of the critical reasons for neutrophil infiltration in the local microenvironment of patients with AS. In addition, these infiltrative neutrophils promote the osteogenesis of MSCs,<sup>27</sup> which was reported to contribute to new bone formation in AS in our previous study.<sup>34</sup> Excessive chronic inflammation and ectopic ossification are two pathologic features of AS.<sup>2</sup> Our results indicate that NETs are simultaneously involved in the development of these two pathologic processes through regulating the MSC and Th17:Treg ratios.

The NETs components are extremely complex and include various kinds of nucleic acids and proteins. Through diverse mechanisms involving their components, NETs regulate the fate and functions of different cells.<sup>13,28,35</sup> Thus, we investigated which NET components impede CD4<sup>+</sup> Treg differentiation and how they interact with CD4<sup>+</sup> T cells. Our results revealed that NETs components were present in the cytoplasm of CD4<sup>+</sup> T cells after coculture, suggesting that NETs were internalized by CD4<sup>+</sup> T cells via endocytosis. Clathrin-mediated endocytosis accounts for approximately 95% of the total endocytosis performed by mammalian cells.<sup>36</sup> We further demonstrated that pitstop 2, a Cl, significantly blocked the internalization of NETs and their subsequent negative regulatory effect on CD4<sup>+</sup> Treg differentiation. These results indicate that the impeding effect on CD4<sup>+</sup> Treg differentiation depends on the internalization of NETs and their

subsequent intracellular regulatory mechanism. Previous studies reported that internalized NETs activate immune cells via their bound RNA.<sup>17,35</sup> In this study, the negative regulatory effect of NETs on CD4<sup>+</sup> Treg differentiation was restored by RNase A treatment but not by DNase I treatment, and DNA extracted from NETs had no effect on CD4<sup>+</sup> Treg differentiation, suggesting that NETs impeded CD4<sup>+</sup> Treg differentiation via their RNA components. Moreover, RNA40 or NETs-scaffolds alone had no effect on Treg differentiation, but RNA40 plus NETs-scaffolds inhibited CD4<sup>+</sup> Treg differentiation, similar to NETs. These results indicate that the negative regulatory effect of NETs-bound RNA on Treg differentiation does not depend on special sequences but relies on the assistance of some components of the NET scaffold, such as the previously reported<sup>17</sup> LL-37.

To investigate the mechanism by which internalized NETs-bound RNA impedes Treg differentiation, RNA sequencing of NETs-stimulated CD4<sup>+</sup> T cells was performed, and the TLR signaling pathway was identified via GSEA and KEGG analysis. Intracellular TLRs, including TLR3, TLR7, TLR8, and TLR9, recognize DNA or RNA engulfed by cells and subsequently regulate their differentiation and function.<sup>37</sup> We showed that CQ, a broad-spectrum inhibitor of intracellular TLRs that modulates the pH of endosomes, significantly restored CD4<sup>+</sup> Treg differentiation as well as the TLR signaling pathway activation inhibited by NETs, which revealed the important role of intracellular TLRs in NETs-mediated inhibition of CD4<sup>+</sup> Treg differentiation. Furthermore, specific inhibitors of TLR7, rather than those of TLR3, TLR8, and TLR9, blocked the inhibitory effect of NETs on CD4<sup>+</sup> Treg differentiation. Because TLR7 was one of the top 10 DEGs identified in our study and was previously reported to negatively regulate CD4<sup>+</sup> Treg differentiation,<sup>38</sup> we propose that internalized NETs-bound RNA activated the TLR7 signaling pathway and then impeded CD4<sup>+</sup> Treg differentiation. The TLR7 signaling pathway was previously reported to be involved in dendritic cell migration and the subsequent control of inflammation in AS.<sup>39</sup> In addition, TLR7 mediates the inflammatory response through a series of transcription factors, such as downstream interferon regulatory factors.<sup>40</sup> Our results demonstrated that the level of phosphorylated IRF-7 was increased by NETs stimulation and that blocking IRF-7 eliminated the inhibitory effect of NETs on CD4<sup>+</sup> Treg differentiation. Therefore, the TLR7–IRF-7 axis is strongly involved in the effects of NETs on Treg cells as well as the pathogenesis of AS.

As the pathologic role of NETs in various diseases has been explored in recent years, an increasing number of therapies targeting NETs have been developed for these diseases.<sup>41</sup> SVT, a neutrophil elastase inhibitor that blocks NETs formation, has shown substantial benefits for several inflammatory diseases in clinical trials.<sup>42,43</sup> In addition, the PAD4 inhibitor CI-amidine restrained NETs formation and exhibited therapeutic effects in a mouse model of rheumatoid arthritis,<sup>7</sup> and it also improved inflammation by inhibiting NETs formation in a mouse model of several diseases, including lupus, atherosclerosis, and traumatic brain

injury.<sup>44–46</sup> To investigate treatments targeting NETs in AS, we evaluated the therapeutic effects of SVT and CI-amidine in SKG mice. Given that local and systematic NETs levels decreased in SKG mice, arthritis scores were significantly lower after SVT or CI-amidine treatment. In addition, both immune cell infiltration and ectopic ossification were reduced by SVT or CI-amidine treatment, and these phenomena were accompanied by a restored proportion of CD4<sup>+</sup> Treg cells in the peripheral blood and ankle joints. From another point of view, these results demonstrated that NETs were involved in both the pathogenesis of chronic inflammation and ectopic ossification in AS and that targeting NETs may have a remarkable therapeutic effect on patients with AS. Clinical trials should be conducted in the future, which may provide novel insight for AS treatment.

In summary, our study demonstrated that elevated NETs in patients with AS impeded CD4<sup>+</sup> Treg differentiation through the activation of the TLR7–IRF-7 axis via their RNA components. Some limitations, such as the upstream factors involved in increased neutrophil infiltration and the effects of NETs on other immune cells in AS, remain to be addressed in the future, which may provide further understanding of the pathologic process of AS and insights into its clinical treatment.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Z. Xie confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

1. Taurog JD, Chhabra A, Colbert RA. Ankylosing spondylitis and axial spondyloarthritis. *N Engl J Med* 2016;374(26):2563–2574.
2. Ranganathan V, Gracey E, Brown MA, et al. Pathogenesis of ankylosing spondylitis - recent advances and future directions. *Nat Rev Rheumatol* 2017;13(6):359–367.
3. Lai NL, Zhang SX, Wang J, et al. The proportion of regulatory T Cells in patients with ankylosing spondylitis: a meta-analysis. *J Immunol Res* 2019;2019:1058738.
4. Wang M, Liu C, Bond A, et al. Dysfunction of regulatory T cells in patients with ankylosing spondylitis is associated with a loss of Tim-3. *Int Immunopharmacol* 2018;59:53–60.
5. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* 2018;18(2):134–147.
6. Lambert S, Hambro CA, Johnston A, et al. Neutrophil extracellular traps induce human Th17 cells: effect of psoriasis-associated TRA-F3IP2 genotype. *J Invest Dermatol* 2019;139(6):1245–1253.
7. Papadaki G, Kambas K, Choulaki C, et al. Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation. *Eur J Immunol* 2016;46(11):2542–2554.

8. Papagoras C, Chrysanthopoulou A, Mitsios A, et al. IL-17A expressed on neutrophil extracellular traps promotes mesenchymal stem cell differentiation toward bone-forming cells in ankylosing spondylitis. *Eur J Immunol* 2021;51(4):930–942.
9. Fitzgerald KA, Kagan JC. Toll-like receptors and the control of immunity. *Cell* 2020;180(6):1044–1066.
10. Wilson AS, Randall KL, Pettitt JA, et al. Neutrophil extracellular traps and their histones promote Th17 cell differentiation directly via TLR2. *Nat Commun* 2022;13(1):528.
11. Roozbehkia M, Mahmoudi M, Aletaha S, et al. The potent suppressive effect of beta-d-mannuronic acid (M2000) on molecular expression of the TLR/NF- $\kappa$ B signaling pathway in ankylosing spondylitis patients. *Int Immunopharmacol* 2017;52:191–196.
12. Xie Z, Yu W, Zheng G, et al. TNF- $\alpha$ -mediated m<sup>6</sup>A modification of ELMO1 triggers directional migration of mesenchymal stem cell in ankylosing spondylitis. *Nat Commun* 2021;12(1):5373.
13. Yang L, Liu Q, Zhang X, et al. DNA of neutrophil extracellular traps promotes cancer metastasis via CCDC25. *Nature* 2020;583(7814):133–138.
14. Yuanyuanxu, Qipeng, Qingqingma, et al. scRNA + TCR-seq revealed the dual TCR pTh17 and Treg T cells involvement in autoimmune response in ankylosing spondylitis. *Int Immunopharmacol* 2024;135:112279.
15. Chen W, Chen H, Yang ZT, et al. Free fatty acids-induced neutrophil extracellular traps lead to dendritic cells activation and T cell differentiation in acute lung injury. *Aging (Albany NY)* 2021;13(24):26148–26160.
16. Wang Y, Du C, Zhang Y, et al. Composition and function of neutrophil extracellular traps. *Biomolecules* 2024;14(4):416.
17. Herster F, Bittner Z, Archer N K, et al. Neutrophil extracellular trap-associated RNA and LL37 enable self-amplifying inflammation in psoriasis. *Nat Commun* 2020, 11(1):105.
18. Ma W, Huang G, Wang Z, et al. IRF7: role and regulation in immunity and autoimmunity. *Front Immunol* 2023;14:1236923.
19. Sieper J, Poddubnyy D. Axial spondyloarthritis. *Lancet* 2017;390(10089):73–84.
20. Ramiro S, Nikiphorou E, Sepiano A, et al. ASAS-EULAR recommendations for the management of axial spondyloarthritis: 2022 update. *Ann Rheum Dis* 2023;82(1):19–34.
21. van der Heijde D, Gensler LS, Deodhar A, et al. Dual neutralisation of interleukin-17A and interleukin-17F with bimekizumab in patients with active ankylosing spondylitis: results from a 48-week phase IIb, randomised, double-blind, placebo-controlled, dose-ranging study. *Ann Rheum Dis* 2020;79(5):595–604.
22. Deodhar A, Reveille JD, Harrison DD, et al. Safety and efficacy of golimumab administered intravenously in adults with ankylosing spondylitis: results through week 28 of the GO-ALIVE study. *J Rheumatol* 2018;45(3):341–348.
23. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013;13(3):159–175.
24. Carmona-Rivera C, Kaplan MJ, O'Neil LJ. Neutrophils in inflammatory bone diseases. *Curr Osteoporos Rep* 2024;22(2):280–289.
25. Xu S, Ma Y, Wu M, et al. Neutrophil lymphocyte ratio in patients with ankylosing spondylitis: a systematic review and meta-analysis. *Mod Rheumatol* 2020;30(1):141–148.
26. Rosenzweig HL, Vance EE, Asare-Konadu K, et al. Card9/neutrophil signalling axis promotes IL-17A-mediated ankylosing spondylitis. *Ann Rheum Dis* 2024;83(2):214–222.
27. Feng X, Wang C, Ji B, et al. CD<sub>99</sub> G1 neutrophils modulate osteogenic differentiation of mesenchymal stem cells in the pathological process of ankylosing spondylitis. *Ann Rheum Dis* 2024;83(3):324–334.
28. O'Neil LJ, Oliveira CB, Wang X, et al. Neutrophil extracellular trap-associated carbamylation and histones trigger osteoclast formation in rheumatoid arthritis. *Ann Rheum Dis* 2023;82(5):630–638.
29. Taifour T, Attalla SS, Zuo D, et al. The tumor-derived cytokine Chi3l1 induces neutrophil extracellular traps that promote T cell exclusion in triple-negative breast cancer. *Immunity* 2023;56(12):2755–2772.e8.
30. Yi K, Jo S, Song W, et al. Analysis of single-cell transcriptome and surface protein expression in ankylosing spondylitis identifies OX40-positive and glucocorticoid-induced tumor necrosis factor receptor-positive pathogenic Th17 cells. *Arthritis Rheumatol* 2023;75(7):1176–1186.
31. Xueyi L, Lina C, Zhenbiao W, et al. Levels of circulating Th17 cells and regulatory T cells in ankylosing spondylitis patients with an inadequate response to anti-TNF- $\alpha$  therapy. *J Clin Immunol* 2013;33(1):151–161.
32. Jiang J, Zhan X, Qu H, et al. Upregulated of ANXA3, SORL1, and neutrophils may be key factors in the progression of ankylosing spondylitis. *Front Immunol* 2022;13:861459.
33. Nakamura A, Zeng F, Nakamura S, et al. Macrophage migration inhibitory factor drives pathology in a mouse model of spondyloarthritis and is associated with human disease. *Sci Transl Med* 2021;13(616):eabg1210.
34. Xie Z, Wang P, Li Y, et al. Imbalance between bone morphogenetic protein 2 and noggin induces abnormal osteogenic differentiation of mesenchymal stem cells in ankylosing spondylitis. *Arthritis Rheumatol* 2016;68(2):430–440.
35. Blanco LP, Wang X, Carlucci PM, et al. RNA externalized by neutrophil extracellular traps promotes inflammatory pathways in endothelial cells. *Arthritis Rheumatol* 2021;73(12):2282–2292.
36. Charpentier JC, King PD. Mechanisms and functions of endocytosis in T cells. *Cell Commun Signal* 2021;19(1):92.
37. Lasker MV, Nair SK. Intracellular TLR signaling: a structural perspective on human disease. *J Immunol* 2006;177(1):11–16.
38. Alzabin S, Kong P, Medghalchi M, et al. Investigation of the role of endosomal Toll-like receptors in murine collagen-induced arthritis reveals a potential role for TLR7 in disease maintenance. *Arthritis Res Ther* 2012;14(3):R142.
39. Ermoza K, Glatigny S, Jah N, et al. Tolerogenic XCR1<sup>+</sup> dendritic cell population is dysregulated in HLA-B27 transgenic rat model of spondyloarthritis. *Arthritis Res Ther* 2019;21(1):46.
40. Carmona-Pérez L, Dagenais-Lussier X, Mai LT, et al. The TLR7/IRF-5 axis sensitizes memory CD4<sup>+</sup> T cells to Fas-mediated apoptosis during HIV-1 infection. *JCI Insight* 2023;8(13):e167329.
41. Li X, Xiao S, Filipczak N, et al. Role and therapeutic targeting strategies of neutrophil extracellular traps in inflammation. *Int J Nanomedicine* 2023;18:5265–5287.
42. Kohira S, Oka N, Inoue N, et al. Effect of the neutrophil elastase inhibitor sivelestat on perioperative inflammatory response after pediatric heart surgery with cardiopulmonary bypass: a prospective randomized study. *Artif Organs* 2013;37(12):1027–1033.
43. Aikawa N, Ishizaka A, Hirasawa H, et al. Reevaluation of the efficacy and safety of the neutrophil elastase inhibitor, sivelestat, for the treatment of acute lung injury associated with systemic inflammatory response syndrome; a phase IV study. *Pulm Pharmacol Ther* 2011;24(5):549–554.
44. Knight JS, Zhao W, Luo W, et al. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Invest* 2013;123(7):2981–2993.
45. Knight JS, Luo W, O'Dell AA, et al. Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res* 2014;114(6):947–956.
46. Shi G, Liu L, Cao Y, et al. Inhibition of neutrophil extracellular trap formation ameliorates neuroinflammation and neuronal apoptosis via STING-dependent IRE1 $\alpha$ /ASK1/JNK signaling pathway in mice with traumatic brain injury. *J Neuroinflammation* 2023;20(1):222.

# Recurrence Risk in Pediatric Noninfectious Uveitis During Adalimumab Tapering: An International Multicenter Retrospective Study

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**Objective.** This study aims to assess the risk of noninfectious uveitis (NIU) relapse in pediatric patients undergoing adalimumab (ADA) tapering, evaluating potential predictors of such risk.

**Methods.** We conducted a multicenter retrospective cohort study involving pediatric patients with NIU who underwent ADA tapering due to inactive uveitis. Cox proportional hazards regression was used to analyze risk factors for NIU recurrence.

**Results.** The study cohort comprised 114 patients (65 girls; 57%). Most commonly, patients presented with juvenile idiopathic arthritis–associated uveitis (JIA-U) (52 of 114; 46%) or idiopathic uveitis (46 of 114; 40%). At ADA tapering, 46% of patients (53 of 114) experienced NIU recurrence after an overall median time of 30 weeks (interquartile range [IQR] 15–58 weeks) from the start of ADA tapering. Patients without recurrences were observed for a median of 70 weeks (IQR 48–98 weeks). Multivariate Cox regression analysis showed that a slower ADA tapering schedule was associated with a lower recurrence rate during the waning (hazard ratio [HR] 0.40, 95% confidence interval [CI] 0.21–0.74;  $P < 0.01$ ). Subgroup analysis of patients with JIA-U indicated that beginning ADA tapering after at least two years of disease inactivity significantly reduced recurrence risk (HR 0.65, 95% CI 0.43–0.95;  $P = 0.05$ ). Among 59 patients (52%) who discontinued ADA, recurrence rates were similar between fast- and slow-tapering groups (21% vs 33%;  $P = 0.6$ ), but median time to recurrence was shorter with fast tapering (10 weeks vs 37 weeks;  $P = 0.05$ ).

**Conclusion.** This study highlights the significant clinical impact of ADA tapering on uveitis recurrence risk, recommending a gradual, slow-tapering approach with close monitoring.

## INTRODUCTION

Noninfectious pediatric uveitis (NIU) is an inflammatory disorder affecting the uveal tract. It prevails in developed countries, with a childhood prevalence in the United States

estimated at 29 per 100,000 persons and a worldwide prevalence of 3 per 10,000 children.<sup>1</sup> The majority of childhood uveitis is chronic anterior uveitis (CAU). Juvenile idiopathic arthritis (JIA), an umbrella term for a group of childhood arthropathies, is the most frequent systemic disorder accompanying

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CAU.<sup>2–4</sup> However, up to 40% of children with CAU never develop JIA.

Persistently active uveitis can result in various complications, including synechiae, glaucoma, cataract, band keratopathy, macular edema, and hypotony.<sup>3,4</sup> Prompt diagnosis and early implementation of steroid-sparing agents may reduce the risk of uveitis-related damage. Current treatment guidelines advocate for the early use of disease-modifying antirheumatic drugs (DMARDs) with methotrexate (MTX) as the first option.<sup>5,6</sup> Adalimumab (ADA), a fully humanized monoclonal antibody against tumor necrosis factor (TNF)  $\alpha$ , is frequently administered to pediatric patients with NIU who do not adequately respond to MTX or cannot tolerate it.<sup>7,8</sup> Most patients achieve remission on ADA without experiencing recurrences while on treatment,<sup>7,8</sup> and the number of patients treated with ADA has progressively increased. The possibility of tapering and even withdrawing ADA in pediatric patients experiencing a prolonged period of persistent remission has been suggested.<sup>9</sup> Safety considerations, cost implications, and children's quality of life make this option rather appealing.<sup>10,11</sup>

However, there is a need for further insights about how to discontinue ADA in NIU, as available information is limited and derived from small cohorts.<sup>12–18</sup> This study aims to assess the risk of NIU relapse in pediatric patients undergoing ADA tapering, evaluating potential predictors of such risk.

## MATERIALS AND METHODS

We conducted a multicenter retrospective cohort study involving 16 international tertiary centers located in various geographic regions: Europe (4 centers), North Africa (3 centers), the Middle East (2 centers), Asia (3 centers), Central America (1 center), South America (2 centers), and North America (1 center). Adherence to the Health Insurance Portability and Accountability Act of 1996 and the principles outlined in the Declaration of Helsinki were ensured. Approval was obtained from the institutional review boards (IRBs) of all authors' centers, following the requirements set forth by each center (approval IRB number of the leading center A-2022-042).

Patients were included based on the following criteria: a diagnosis of NIU before the age of 18 years, treatment with ADA for active NIU, ADA tapering due to persistently inactive uveitis, at least 26 weeks of quiescence, and a negative screening for infectious diseases such as hepatitis B and C, syphilis, and tuberculosis. At the initiation of ADA treatment, active NIU was

characterized by at least one of the following: anterior chamber cell grade  $\geq 1$ , one active inflammatory chorioretinal or retinal vascular lesion, or vitreous haze grade<sup>19</sup>  $\geq 1$ . Exclusion criteria included incomplete data with loss to follow-up before ADA tapering; the need for weekly ADA to control uveitis, media opacity, or eye conditions hampering the assessment of inflammation activity; ADA tapering not being based on uveitis status; previous use of other biologic DMARDs; and side effects that forced ADA tapering or suspension despite active NIU.

Medical records from the rheumatology and ophthalmology departments from 2012 to 2022 were retrospectively reviewed. Data regarding demographic details (age at NIU onset, sex, race), diagnosis of underlying systemic disease, disease onset and duration, NIU anatomic classification, presenting best corrected visual acuity, clinical characteristics, and history of concomitant therapy other than ADA were collected.

ADA was administered subcutaneously every two weeks, and the dose was determined by the patient's weight (20 mg for patients  $<30$  kg; 40 mg otherwise). Patients were deemed inactive when the anterior chamber cell grade and vitreous haze quality were  $\leq 0.5$ , in the absence of active inflammatory chorioretinal or retinal vascular lesions or any topical steroid use. Patients with bilateral uveitis should fulfill requirements for inactivity in both eyes to be classified as inactive. In case of persistently inactive uveitis, ADA was tapered progressively by increasing the time between injections (spacing) according to the treating physician's clinical judgment. All enrolled participants began the ADA tapering after 2012. As reported in Table 1, 10 different tapering modalities were identified based on how frequently the interval between ADA injections was increased. For example, the interval between ADA injections could be increased by one week every month (class 1, the fastest tapering modality) or by one week every year (class 10, the slowest tapering modality). Given such wide heterogeneity, patients were subclassified into two groups based on the modality of ADA tapering: "fast tapering" (classes 1–4) and "slow tapering" (classes 5–10) (Table 1). The threshold was determined using a receiver operating characteristic curve analysis based on the Youden index. No change in the tapering class is anticipated. The goal of the taper is to discontinue the medication within two years, based on the treating physician's assessment.

Uveitis recurrence was defined as an increase in anterior chamber cell grade, vitreous haze, or new active inflammatory chorioretinal or retinal lesions compared to the visit when ADA tapering was started, either in one or both eyes during the

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**Table 1.** Tapering schedules of adalimumab

| Tapering class<br>(from fastest<br>to slowest) | Tapering schedule <sup>a</sup> | Patients,<br>n (%) | Patients with<br>recurrences<br>(n = 53), n (%) <sup>b</sup> |
|------------------------------------------------|--------------------------------|--------------------|--------------------------------------------------------------|
| Fast tapering                                  |                                |                    |                                                              |
| 1                                              | 1 week every mo*               | 1 (1)              | 1 (100)                                                      |
| 2                                              | 1 week every 2 mo              | 2 (2)              | 1 (50)                                                       |
| 3                                              | 1 week every 3 mo              | 50 (44)            | 27 (54)                                                      |
| 4                                              | 2 weeks every 4 mo             | 1 (1)              | 1 (100)                                                      |
| Slow tapering                                  |                                |                    |                                                              |
| 5                                              | 1 week every 4 mo              | 4 (3)              | 1 (25)                                                       |
| 6                                              | 1 week every 5 mo              | 5 (4)              | 3 (60)                                                       |
| 7                                              | 4 weeks every 6 mo             | 1 (1)              | 0 (0)                                                        |
| 8                                              | 2 weeks every 6 mo             | 2 (2)              | 1 (50)                                                       |
| 9                                              | 1 week every 6 mo              | 24 (21)            | 8 (33)                                                       |
| 10                                             | 1 week every 12 mo             | 24 (21)            | 10 (42)                                                      |

<sup>a</sup> The interval between adalimumab injections is increased by.

<sup>b</sup> The denominator is the number of patients in each tapering class.

\* mo, months.

observation period. The date of NIU recurrence was recorded. The observation period of the study began with the first visit when ADA taper was initiated for the initial patients and ended at the latest visit for the most recently included patient.

This cohort included some patients who had also been enrolled in the Humira in Ocular Inflammations Taper study, in which only 28.4% of recruited patients were below 18 years of age. However, no specific subanalysis was conducted on children, thus preventing the drawing of definite conclusions about the impact of ADA tapering in the pediatric age group.<sup>20</sup>

**Statistical analysis and data availability.** Continuous variables were described as means  $\pm$  SDs or medians and interquartile ranges (IQRs), whereas categorical variables were reported as percentages. Variations in continuous variables based on recurrence status were evaluated using the Mann-Whitney nonparametric test. As appropriate, relationships between categorical variables were examined by applying either the chi-square test or Fisher's exact tests.

To assess the incidence of NIU recurrence after the initiation of ADA tapering, Kaplan-Meier survival curves were drawn to plot the time from the beginning of ADA tapering to the first uveitis relapse. Patients without recurrences were censored at their latest follow-up visit. Risk factors for NIU recurrence were analyzed with Cox proportional hazards regression. Hazard ratios (HRs) and 95% confidence intervals (CIs) were computed. The assumptions of the proportional hazards model were validated by analyzing Schoenfeld residuals.  $P \leq 0.05$  was considered statistically significant. Missing data were imputed using multiple imputation, assuming that the data were missing at random. Analyses were performed with R Studio (version RStudio 2021.09.2+382 for macOS). The data that support the findings of this study are available from the corresponding author (AM) upon reasonable request.

## RESULTS

**Study cohort and demographics.** The study cohort comprised 114 patients (65 girls; 57%) with NIU treated with ADA. Demographic and clinical characteristics of included patients are presented in Table 2. The median age at disease onset was 5.6 years (IQR 3.3–10.2 years). Patients were aged 13 years (IQR 10–15 years) at the start of ADA tapering. White represented the most frequent race (74 of 114 patients; 65%), followed by Asian patients (13 of 114 patients; 10%).

Most commonly, patients presented with juvenile idiopathic arthritis–associated uveitis (JIA-U) (52 of 114; 46%) or idiopathic uveitis (IU) (46 of 114; 40%). Anterior uveitis was documented in half of the patients; panuveitis and intermediate uveitis accounted for 28% of patients (32 of 114) and 18% of patients (21 of 114), respectively. Twenty-two of 114 patients (19%) did not receive any concomitant DMARDs during ADA treatment; concomitant MTX at the beginning of ADA treatment was recorded in 70% of patients (81 of 114). Twenty-one of 114 patients (18%) received a course of systemic glucocorticoids, which was discontinued before ADA tapering began.

The median duration of NIU before the introduction of ADA was 30 weeks (IQR 9–104 weeks); the median duration of disease inactivity before starting the taper was 104 weeks (IQR 52–162 weeks). ADA tapering schedules and the related rates of recurrences are outlined in Table 1.

**Recurrence rates and associated risk factors.** In our cohort, 46% of patients (53 of 114) experienced NIU recurrence after an overall median time of 30 weeks (IQR 15–58 weeks) from the start of ADA tapering. Patients without recurrences were observed for a median of 70 weeks (IQR 48–98 weeks) since ADA tapering was started. In the whole cohort, the 40th percentile of time to recurrence was 55 weeks (Figure 1).

To manage uveitis relapse, the following treatment strategies were successfully implemented (data available for 44 of 53 patients): 12 patients were treated with topical steroids alone without modification of ADA regimen, 23 patients had changes in ADA frequency or resumed ADA, 4 patients underwent modulation of DMARD therapy, 3 patients received oral steroids, and 2 patients were switched to infliximab. Unfortunately, the time for recapture was not recorded.

In 54 patients, a fast-tapering modality was adopted, whereas physicians opted for a slow tapering of ADA in 60 patients. An association between the tapering schedule and the incidence of uveitis relapse was observed, with 56% of recurrences (30 participants) in case of fast tapering compared to 38% (23 participants) in those undergoing slow tapering ( $P = 0.06$ ). ADA dose reduction over time, categorized into fast- and slow-tapering groups, is presented in Figure 2.

A subanalysis was conducted comparing the fast- and slow-tapering groups, revealing differences in diagnoses, time to initiate

**Table 2.** Demographic and clinical characteristics of the cohort\*

| Characteristic                                                   | Overall (N = 114) | No recurrences<br>(n = 61 patients) | Recurrences<br>(n = 53 patients) | P value           |
|------------------------------------------------------------------|-------------------|-------------------------------------|----------------------------------|-------------------|
| Age at onset, median (IQR), yr                                   | 5.6 (3.3–10.2)    | 6.7 (4.0–10.6)                      | 4.6 (2.4–8.1)                    | 0.02 <sup>a</sup> |
| Age at ADA tapering, median (IQR), yr                            | 13.0 (10.0–15.0)  | 12.0 (10.0–16.0)                    | 13.0 (9.0–15.0)                  | 0.81 <sup>a</sup> |
| Sex, n (%)                                                       |                   |                                     |                                  | 0.85 <sup>b</sup> |
| Female                                                           | 65 (57)           | 34 (56)                             | 31 (58)                          |                   |
| Male                                                             | 49 (43)           | 27 (44)                             | 22 (42)                          |                   |
| Race and ethnicity, n (%)                                        |                   |                                     |                                  | 0.04 <sup>c</sup> |
| African                                                          | 7 (6)             | 5 (8)                               | 2 (3)                            |                   |
| African American                                                 | 1 (1)             | 1 (2)                               | 0 (0)                            |                   |
| Arab                                                             | 4 (4)             | 1 (2)                               | 3 (6)                            |                   |
| Asian                                                            | 13 (10)           | 12 (19)                             | 1 (2)                            |                   |
| White                                                            | 74 (65)           | 34 (55)                             | 40 (75)                          |                   |
| Hispanic                                                         | 9 (8)             | 6 (10)                              | 3 (6)                            |                   |
| Mixed                                                            | 2 (2)             | 1 (2)                               | 1 (2)                            |                   |
| Missing                                                          | 4 (4)             | 1 (2)                               | 3 (6)                            |                   |
| Diagnosis, n (%)                                                 |                   |                                     |                                  | 0.51 <sup>c</sup> |
| Behçet disease                                                   | 6 (5)             | 5 (8)                               | 1 (2)                            |                   |
| Idiopathic uveitis                                               | 46 (40)           | 22 (36)                             | 24 (45)                          |                   |
| Juvenile idiopathic arthritis                                    | 52 (46)           | 28 (46)                             | 24 (45)                          |                   |
| Tubulointerstitial nephritis<br>and uveitis syndrome             | 4 (4)             | 3 (5)                               | 1 (2)                            |                   |
| Vogt-Koyanagi-Harada disease                                     | 6 (5)             | 3 (5)                               | 3 (6)                            |                   |
| Uveitis localizations, n (%)                                     |                   |                                     |                                  | 0.98 <sup>b</sup> |
| Anterior                                                         | 58 (51)           | 31 (51)                             | 27 (51)                          |                   |
| Other uveitis                                                    | 56 (49)           | 30 (49)                             | 26 (49)                          |                   |
| DMARDs on adalimumab, n (%)                                      |                   |                                     |                                  | 0.47 <sup>c</sup> |
| Azathioprine                                                     | 4 (4)             | 2 (3)                               | 2 (3)                            |                   |
| Colchicine                                                       | 2 (2)             | 2 (3)                               | 0 (0)                            |                   |
| Mycophenolate mofetil                                            | 5 (4)             | 3 (5)                               | 2 (3)                            |                   |
| Methotrexate                                                     | 79 (68)           | 38 (62)                             | 41 (77)                          |                   |
| Methotrexate + azathioprine                                      | 2 (2)             | 1 (2)                               | 1 (2)                            |                   |
| None                                                             | 22 (20)           | 15 (25)                             | 7 (13)                           |                   |
| Time from diagnosis to adalimumab<br>treatment, median (IQR), mo | 7 (2–24)          | 7 (2–25)                            | 8 (2–15)                         | 0.40 <sup>a</sup> |
| Inactivity before adalimumab tapering,<br>median (IQR), wk       | 104 (52–164)      | 102 (52–261)                        | 104 (54–126)                     | 0.72 <sup>a</sup> |

\* ADA, adalimumab; DMARD, disease-modifying antirheumatic drug; IQR, interquartile range.

<sup>a</sup> Mann-Whitney nonparametric test.

<sup>b</sup> Chi-square test.

<sup>c</sup> Fisher's exact tests.

ADA, and inactivity before starting ADA tapering (see Supplemental Table 1). We then focused on the 59 patients (52%) who discontinued ADA during the study observation period (see Supplemental Table 2) after either a slow-tapering (30 patients) or a fast-tapering regimen (29 patients;  $P = 0.6$ ). The rate of JIA was similar between the fast- and slow-tapering groups (31% vs 47%;  $P = 0.5$ ). The incidence of recurrence was also comparable: 6 patients (21%) in the fast-tapering group and 10 patients (33%) in the slow-tapering group experienced uveitis relapse after ADA discontinuation ( $P = 0.6$ ). Notably, the median time to recurrence was shorter in patients who underwent fast tapering: 10 weeks (IQR 8–14 weeks) in the fast-tapering group compared to 37 weeks (IQR 32–48 weeks) in the slow-tapering group ( $P = 0.05$ ).

In our cohort, Kaplan-Meier survival curve for uveitis relapse did not reach the median (Figure 1A). Survival curves for recurrence based on tapering schedule, duration of disease inactivity

for the entire cohort and patients with JIA, and JIA versus IU are illustrated in Figures 1B–E. Additionally, the Kaplan-Meier curve depicting patients who discontinued the drug based on tapering schedule is shown in Figure 1F. Initiating ADA tapering after at least two years of disease inactivity did not show any significant benefit in terms of uveitis recurrences for the entire group or for the IU subgroup. However, there was a significant difference when considering only patients with JIA (Figure 1D).

Univariate Cox regression analysis of the whole cohort showed that a slower ADA tapering schedule was linked to a lower recurrence rate (HR 0.49, 95% CI 0.28–0.87;  $P = 0.01$ ). To note, the duration of inactivity before commencing ADA tapering, considered either as a continuous variable or categorized (more or less than two years of disease inactivity), did not impact the risk of recurrences in the whole cohort (Table 3). An increase of one year in age at disease onset was associated with a trend toward a lower risk of recurrence (HR 0.94, 95% CI 0.87–1.01;

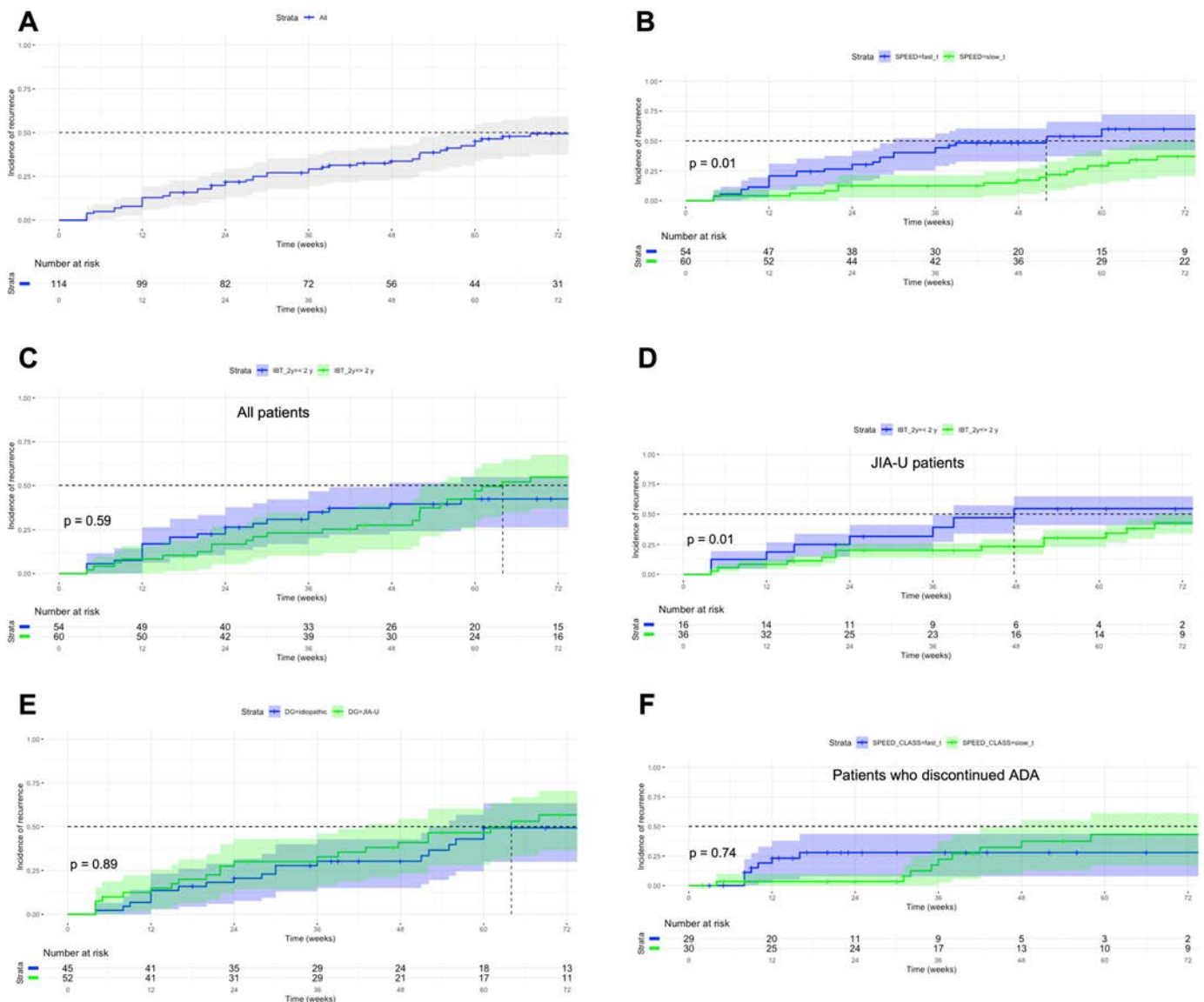


**Figure 1.** Kaplan-Meier survival curves were analyzed for relapse of (A) the whole cohort, based on (B) speed class, (C) idiopathic versus JIA-U, (D) initiating ADA tapering after at least two years of disease inactivity for the whole cohort, (E) for patients with JIA-U, (F) and speed class restricted to patients who discontinued the drug. The long-rank test is showed. ADA, adalimumab; DG, diagnosis; fast\_t, speed classes from 5 to 10 (see Table 1); IBT, inactivity before initiation of adalimumab tapering; JIA-U, juvenile idiopathic arthritis–associated uveitis; slow\_t, speed classes from 1 to 4 (see Table 1); SPEED, class of adalimumab tapering according to the speed.

$P = 0.11$ ). Importantly, in the adjusted model a slower ADA tapering was associated with a 60% lower recurrence risk than a faster tapering (HR 0.40, 95% CI 0.21–0.74;  $P < 0.01$ ) (Figure 3).

**Subgroup analysis: patients with JIA-U and IU.** When a subgroup analysis of patients with IU and JIA-U was conducted, we found no significant differences in the median follow-up period (50 vs 52 weeks;  $P = 0.9$ ), recurrence rates (51% vs 46%;  $P = 0.6$ ), and time to recurrence (30 vs 30 weeks;  $P = 0.7$ ) between the two groups. However, the JIA group had a longer median duration of inactivity before ADA tapering and a higher

rate of slow taper (136 vs 96 weeks,  $P < 0.01$ ; 67% vs 31%,  $P < 0.01$ , respectively).

When Cox regression analysis was performed for patients with JIA-U, it emerged that a slower ADA tapering schedule reduced the recurrence rate by half (HR 0.50, 95% CI 0.34–0.72;  $P < 0.01$ ). This subgroup analysis indicates that with every one-year increase in age at uveitis onset, the risk of uveitis recurrences decreases by 10% (HR 0.90, 95% CI 0.86–0.94;  $P < 0.01$ ). Furthermore, commencing ADA tapering after at least two years of disease inactivity showed a significant reduction in the risk of recurrence (HR 0.47, 95% CI 0.32–0.69;  $P < 0.01$ ).

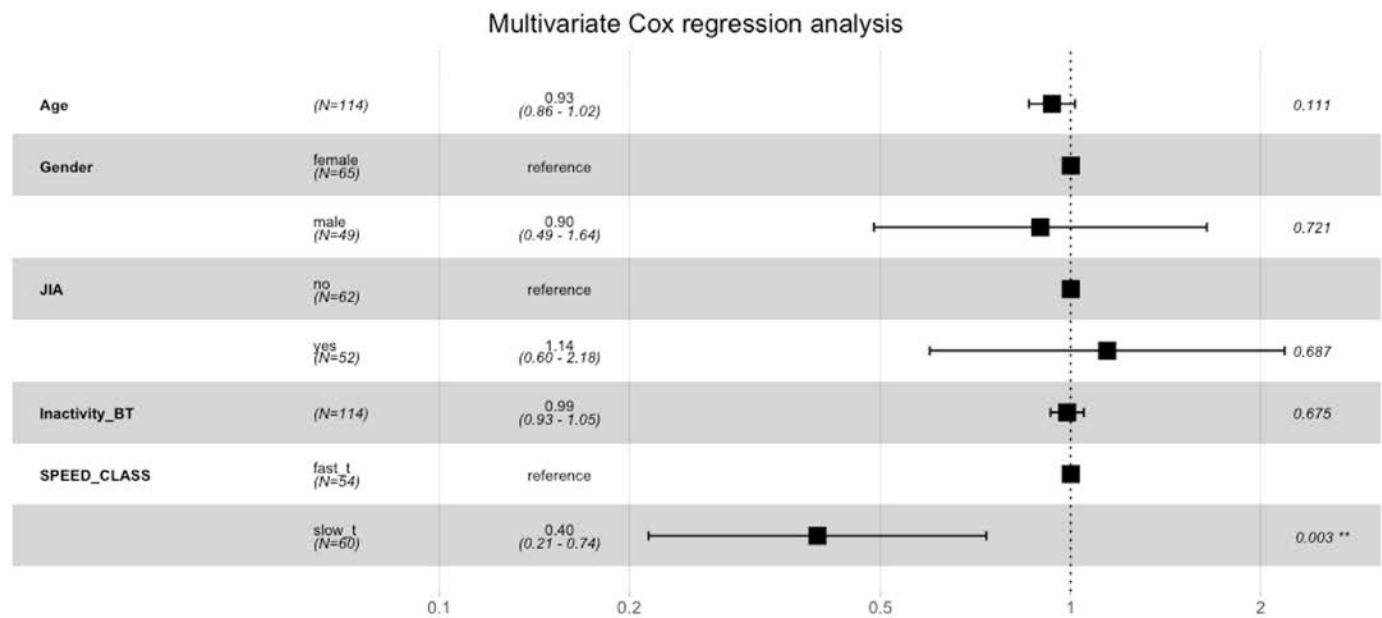


**Figure 2.** Multivariate Cox regression analysis assessing potential predictors of uveitis recurrence. Age: age at uveitis onset. The hazard ratio for Inactivity\_BT reflects the risk associated with a 12-week longer period of disease inactivity before tapering. fast\_t, speed classes from 5 to 10 (see Table 1); Inactivity\_BT, inactivity before adalimumab tapering; JIA, juvenile idiopathic arthritis; slow\_t, speed classes from 1 to 4 (see Table 1); SPEED\_CLASS, class of adalimumab tapering according to the speed.

Multivariate Cox regression analysis revealed a reduced risk of recurrences in patients with JIA-U who began tapering ADA after at least two years of disease inactivity (HR 0.65, 95% CI 0.43–0.95,  $P = 0.05$ ) (Supplemental Table 3). There was a lower risk associated with each one-year increase in age at the onset of uveitis (HR 0.92, 95% CI 0.88–0.96;  $P < 0.01$ ). Adjusted analysis pointed out that a slower ADA tapering reduce the risk of uveitis relapse (HR 0.63, 95% CI 0.43–0.93;  $P = 0.02$ ).

**Table 3.** Univariate Cox regression analysis assessing potential predictors of uveitis recurrence\*

| Characteristic                                                         | Hazard ratio (95% CI) | P value           |
|------------------------------------------------------------------------|-----------------------|-------------------|
| Age at uveitis onset, yr                                               | 0.94 (0.87–1.01)      | 0.11              |
| Age at ADA tapering (for every 10-yr increase), yr                     | 0.79 (0.38–1.67)      | 0.54              |
| Male sex (reference female)                                            | 0.83 (0.48–1.44)      | 0.51              |
| JIA                                                                    | 1.25 (0.72–2.18)      | 0.42              |
| Idiopathic uveitis                                                     | 1.15 (0.66–2.01)      | 0.65              |
| Anterior uveitis                                                       | 1.24 (0.71–2.14)      | 0.45              |
| Methotrexate                                                           | 2.1 (1.07–4.10)       | 0.03 <sup>a</sup> |
| Time from diagnosis to adalimumab treatment (for every 12-wk increase) | 0.87 (0.72–1.06)      | 0.16              |
| Inactivity before adalimumab tapering (for every 12-wk increase)       | 1.02 (0.98–1.05)      | 0.22              |
| At least 2 yr of inactivity before adalimumab tapering                 | 1.16 (0.67–2.01)      | 0.60              |
| Slow tapering (reference fast tapering)                                | 0.49 (0.28–0.87)      | 0.01 <sup>a</sup> |

\* For slow- and fast-tapering schedules, see Table 1. ADA, adalimumab; CI, confidence interval; JIA, juvenile idiopathic arthritis.

<sup>a</sup>  $P < 0.05$ .

In patients with IU, adjusted Cox regression analysis showed that a slower ADA tapering schedule was associated with an 82% lower risk of recurrence compared to faster tapering (HR 0.18, 95% CI 0.06–0.59;  $P < 0.01$ ). No other significant associations were found in the multivariate analysis of the IU group (Supplemental Table 4).

## DISCUSSION

To the best of our knowledge, the present study is the first to investigate the impact of ADA tapering on the risk of uveitis recurrence in a large international cohort of children with NIU who had all been treated with ADA. In particular, approximately half of the patients experienced a recurrence of uveitis at a median time of seven months from the start of ADA tapering.

The rate of uveitis recurrence at ADA tapering we observed falls within the range previously reported in the literature. Indeed, available studies, all retrospective and with a small sample size, reported recurrence rates ranging between 43% and 69% in NIU after stopping immunomodulatory treatment, particularly within the first year after the medication discontinuation.<sup>12–18</sup> Nevertheless, we observed a lower relapse rate after complete ADA discontinuation compared to literature data. Importantly, none of these studies focused on ADA, with the majority assessing the recurrence rate after MTX discontinuation and few studies analyzing the effect of TNF inhibitor (TNFi) withdrawal (mainly infliximab).<sup>12–18</sup> Besides study treatments, the comparison of our results to those emerged in previous studies is also affected

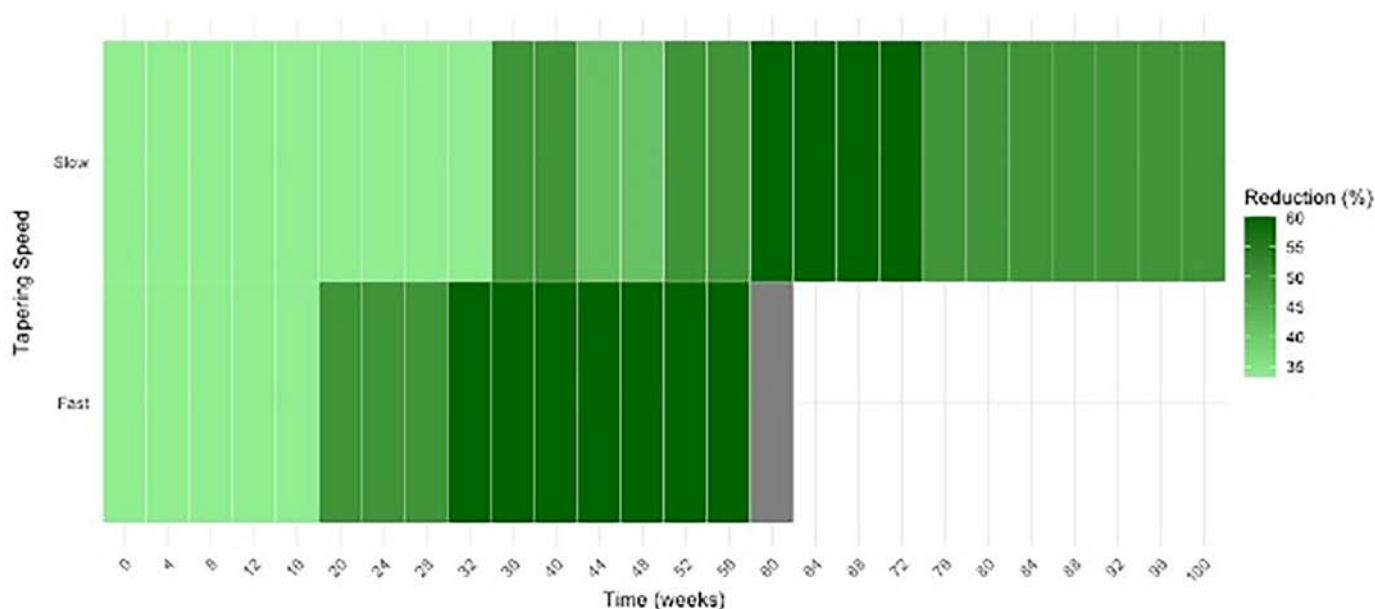


**Figure 3.** Adalimumab dose reduction over time. Notably, 30 patients in the slow taper group and 29 in the fast-taper group discontinued the drug. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43165/abstract>.

by differences in patients' inclusion criteria and length of follow-up.

We believe that the presented data will impact everyday clinical practice, being the first published evidence about the optimal modality to taper and discontinue ADA in NIU. Indeed, even though the recommendations on JIA-U treatment issued by the American College of Rheumatology suggest tapering DMARDs or TNFi after at least two years of well-controlled disease, there is no clear indication of how to pursue tapering.<sup>6,12</sup> Based merely on clinical experience, it is common practice to progressively increase the time between injections rather than abruptly discontinuing treatment.<sup>17</sup> Most importantly, our data provide clear evidence that the modality of tapering impacts the risk of uveitis relapse: a slow-tapering modality (ie, increasing the time between ADA injections by one week at least every four months, or even with a slower approach) was associated with a 60% reduction of the risk of uveitis after adjusting for several variables.

Importantly, we provide solid evidence to support the practical approach usually adopted in clinical practice to taper ADA after at least two years of disease inactivity, with a reduction of the risk of recurrences by more than one-third in patients with JIA-U. This finding highlights the importance of determining the optimal timing for tapering initiation in JIA-U, emphasizing the need for a more cautious approach in these patients.

A wide heterogeneity in predictors of uveitis recurrence has emerged across available studies. In the largest published cohort assessing uveitis relapse after treatment discontinuation including 94 patients with NIU (23 patients previously treated with ADA), variables associated with a reduced risk of relapse were a shorter time to reach the inactivity status (especially for those receiving a TNFi) and a diagnosis of IU rather than JIA-U.<sup>16</sup> The latter finding might

be attributable to the different rates of TNFi use: 56% of patients with IU received TNFi compared to 39% of those with JIA-U. Notably, we found an equal rate of uveitis relapse between patients with JIA-U and IU (45%). Other studies have described several additional predictors of a lower relapse risk of uveitis: earlier onset of treatment, younger age at treatment start, previous use of infliximab rather than ADA, younger age at uveitis onset, use of MTX for more than three years before withdrawal, and two years of inactivity before MTX discontinuation.<sup>12-17</sup> We assessed the impact of age at uveitis onset on recurrence risk and observed a nonsignificant trend toward higher risk in younger patients across the entire cohort. Conversely, when the analysis was restricted to only patients with JIA-U, we found that the risk decreased significantly by 10% with each one-year increase in age at uveitis onset.

In contrast to other studies,<sup>12,20</sup> our analysis of the entire cohort did not find statistically significant evidence that the duration of inactivity before discontinuing ADA treatment affects the risk of uveitis reactivation. Nevertheless, participants who did not experience recurrences had a longer median duration of inactivity before attempting ADA tapering than those who recurred.

The subgroup analysis raises additional points for discussion. Tapering ADA after at least two years of disease inactivity is a significant factor in JIA-U but not in IU. Conversely, when comparing the IU and JIA-U groups, the tapering modality seems to be more important for patients with IU.

About half of the cohort discontinued ADA during the observation period. Among these patients, the rate of recurrences was comparable between fast- and slow-tapering groups. Nevertheless, patients in the slow-tapering group experienced a significantly longer relapse-free period, supporting the beneficial effects of this modality of tapering.

It is worth noting the potential negative impact of uveitis on the success of withdrawing biologic agents in patients with systemic disease. In a recent study involving 220 patients with JIA who discontinued biologic therapy, 54% of participants had to resume treatment after a median time of 4.7 months, and those with a history of uveitis had a more than two-fold higher risk of restarting treatment.<sup>21</sup>

The present study has several limitations that must be acknowledged, *in primis* its retrospective design. Furthermore, the international and multicentric nature of the cohort implies potential differences in the management of NIU among the included centers that, on the other hand, allowed us to compare various modalities of ADA spacing. The participation of tertiary centers could only convey a selection bias as the present cohort might not be representative of the whole spectrum of NIU but only of severe disease. It is important to consider that the tapering modality could affect the frequency of ophthalmologic follow-ups and, consequently, the possibility of detecting a recurrence. Individuals tapering off faster may attend follow-ups more frequently, increasing the likelihood of detecting a uveitis recurrence sooner rather than later. The tapering approach was based on the treating physician's clinical judgment. Some physicians continued treatment at extended intervals, whereas others discontinued therapy earlier.

We understand that the drug exposure time during tapering could be a potential confounding factor, translating into a higher drug exposure. We showed ADA dose reduction over time categorized into fast- and slow-tapering groups in Figure 3. However, the shorter time to recurrence after ADA discontinuation in the fast-tapering group together with the comparable rate of ADA discontinuation across the two groups points toward a true protective effect exerted by the tapering modality.

Despite the aforementioned limitations, this study offers valuable insights into a yet unexplored field. Tapering and discontinuing biologic agents in children with chronic diseases such as NIU represents a key target to limit socioeconomic costs and prevent unnecessary treatment exposition potentially leading to adverse effects, such as infections and malignancies. Although we have a clear understanding of when to initiate biologic agents, determining the optimal time and method for tapering treatment after achieving remission remains uncertain, without any prognostic biomarkers to stratify the risk of recurrence.

Randomized trials conducted in pediatric populations will establish the most effective approach to medication withdrawal. An ongoing trial, the Adalimumab in Juvenile Idiopathic Arthritis-associated Uveitis Stopping Trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03816397) identifier NCT03816397), may provide further insights.<sup>22</sup> This approach has raised solid evidence in adult rheumatology: a systematic Cochrane review analyzed 14 clinical trials, not evincing any significant difference in fixed-dose reduction of TNFi compared to the continuation of standard treatments in patients with rheumatoid arthritis. Moreover, disease activity-guided dose tapering of anti-

TNF resulted in similar remission and disease activity percentages compared to therapy maintenance. On the other hand, patients who discontinued TNFi without tapering exhibited an increasing trend in disease activity and had minimal radiographic progression.<sup>23</sup>

In conclusion, our multicenter study clearly showed that the modality of ADA tapering exerts a great clinical impact on the risk of uveitis recurrence: it is recommended to taper ADA gradually and with a slow schedule, maintaining a strict follow-up given the high rate of relapse. Our data suggest that slow tapering translates into a longer relapse-free time, both during therapy reduction and after complete drug discontinuation. Importantly, we provide solid evidence to taper ADA after at least two years of disease inactivity in patients with JIA-U.

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## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Marino confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

## REFERENCES

1. Thorne JE, Suhler E, Skup M, et al. Prevalence of noninfectious uveitis in the United States: a claims-based analysis. *JAMA Ophthalmol* 2016;134(11):1237–1245.
2. BenEzra D, Cohen E, Maftzir G. Uveitis in children and adolescents. *Br J Ophthalmol* 2005;89(4):444–448.
3. Smith JA, Mackensen F, Sen HN, et al. Epidemiology and course of disease in childhood uveitis. *Ophthalmology* 2009;116(8):1544–1551, 1551e.1.
4. Ferrara M, Eggenschwiler L, Stephenson A, et al. The challenge of pediatric uveitis: tertiary referral center experience in the United States. *Ocul Immunol Inflamm* 2019;27(3):410–417.
5. Foeldvari I, Maccora I, Petrushkin H, et al. New and updated recommendations for the treatment of juvenile idiopathic arthritis-associated uveitis and idiopathic chronic anterior uveitis. *Arthritis Care Res (Hoboken)* 2023;75(5):975–982.
6. Angeles-Han ST, Ringold S, Beukelman T, et al. 2019 American College of Rheumatology/Arthritis Foundation guideline for the Screening, Monitoring, and Treatment of Juvenile Idiopathic Arthritis-Associated Uveitis. *Arthritis Care Res (Hoboken)* 2019;71(6):703–716.
7. Ramanan AV, Dick AD, Jones AP, et al; SYCAMORE Study Group. Adalimumab plus methotrexate for uveitis in juvenile idiopathic arthritis. *N Engl J Med* 2017;376(17):1637–1646.

8. Quartier P, Baptiste A, Despert V, et al; ADJUVITE Study Group. ADJUVITE: a double-blind, randomised, placebo-controlled trial of adalimumab in early onset, chronic, juvenile idiopathic arthritis-associated anterior uveitis. *Ann Rheum Dis* 2018;77(7):1003–1011.
9. Halyabar O, Mehta J, Ringold S, et al. Treatment withdrawal following remission in juvenile idiopathic arthritis: a systematic review of the literature. *Paediatr Drugs* 2019;21(6):469–492.
10. Hughes DA, Culeddu G, Plumpton CO, et al. Cost-effectiveness analysis of adalimumab for the treatment of uveitis associated with juvenile idiopathic arthritis. *Ophthalmology* 2019;126(3):415–424.
11. Marino A, Giani T, Cimaz R. Risks associated with use of TNF inhibitors in children with rheumatic diseases. *Expert Rev Clin Immunol* 2019;15(2):189–198.
12. Kalinina Ayuso V, van de Winkel EL, Rothova A, et al. Relapse rate of uveitis post-methotrexate treatment in juvenile idiopathic arthritis. *Am J Ophthalmol* 2011;151(2):217–222.
13. Saboo US, Metzinger JL, Radwan A, et al. Risk factors associated with the relapse of uveitis in patients with juvenile idiopathic arthritis: a preliminary report. *J AAPOS* 2013;17(5):460–464.
14. Shakoor A, Esterberg E, Acharya NR. Recurrence of uveitis after discontinuation of infliximab. *Ocul Immunol Inflamm* 2014;22(2):96–101.
15. Lerman MA, Lewen MD, Kempen JH, et al. Uveitis reactivation in children treated with tumor necrosis factor alpha inhibitors. *Am J Ophthalmol* 2015;160(1):193–200.el.
16. Simonini G, Bracaglia C, Cattalini M, et al. Predictors of relapse after discontinuing systemic treatment in childhood autoimmune chronic uveitis. *J Rheumatol* 2017;44(6):822–826.
17. Acharya NR, Patel S, Homayounfar G, et al. Relapse of juvenile idiopathic arthritis-associated uveitis after discontinuation of immunomodulatory therapy. *Ocul Immunol Inflamm* 2019;27(4):686–692.
18. McCracken C, Shantha JG, Yeh S, et al. Relapse and remission in children with chronic noninfectious uveitis treated with methotrexate. *J Rheumatol* 2022;49(11):1289–1291.
19. Jabs DA, Nussenblatt RB, Rosenbaum JT; Standardization of Uveitis Nomenclature (SUN) Working Group. Standardization of uveitis nomenclature for reporting clinical data. Results of the first international workshop. *Am J Ophthalmol* 2005;140(3):509–516.
20. Pichi F, Smith SD, Goldstein DA, et al. The Humira in Ocular Inflammations Taper (HOT) study. *Am J Ophthalmol* 2024;258:87–98.
21. Kearsley-Fleet L, Baidam E, Beresford MW, et al. Successful stopping of biologic therapy for remission in children and young people with juvenile idiopathic arthritis. *Rheumatology (Oxford)* 2023;62(5):1926–1935.
22. Acharya NR, Ebert CD, Kelly NK, et al; ADJUST Research Group. Discontinuing adalimumab in patients with controlled juvenile idiopathic arthritis-associated uveitis (ADJUST-Adalimumab in Juvenile Idiopathic Arthritis-associated Uveitis Stopping Trial): study protocol for a randomised controlled trial. *Trials* 2020;21(1):887.
23. Verhoef LM, van den Bemt BJ, van der Maas A, et al. Down-titration and discontinuation strategies of tumour necrosis factor-blocking agents for rheumatoid arthritis in patients with low disease activity. *Cochrane Database Syst Rev* 2019;5(5):CD010455.

# Clinical Factors Associated With Pneumocystis Pneumonia Despite Its Primary Prophylaxis: When to Stop Prophylaxis?

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**Objective.** Although previous studies show that primary prophylaxis against *Pneumocystis jirovecii* pneumonia (PJP) is effective in patients with rheumatic diseases receiving immunosuppressive treatment, there is limited evidence regarding the optimal timing for prophylaxis withdrawal. This study aimed to identify the risk factors for PJP despite prophylaxis and provide evidence for an optimal prophylaxis schedule.

**Methods.** This case-control study included 1,294 prophylactic episodes in 1,148 patients with rheumatic disease who received immunosuppressants and prophylactic trimethoprim-sulfamethoxazole (TMP-SMX). The primary outcome was a one-year incidence of PJP. A Cox proportional hazards model with least absolute shrinkage and selection operator was used to evaluate clinical factors associated with outcomes.

**Results.** During 1,174 person-years of observation, 10 cases of PJP were identified, with an incidence rate of 0.85 per 100 person-years. The mean  $\pm$  SD duration of TMP-SMX prophylaxis was  $181.9 \pm 128.7$  days. Except in one case, PJP occurred after discontinuation of TMP-SMX, with a median (interquartile range [IQR]) interval of 117.0 (86.0–161.0) days. The dose of glucocorticoids at the time of TMP-SMX discontinuation was significantly higher in the PJP group relative to the control group (median [IQR]: 22 [20–40] vs 10 [5–15] mg). Discontinuing TMP-SMX while on a glucocorticoid dose  $>12.5$  mg/day of prednisone equivalent significantly increased the risk of PJP (adjusted hazard ratio: 13.84; 95% confidence interval, 1.71–111.80). There were 63 cases of adverse events during the observation period, and 10 (15.9%) were attributed to TMP-SMX with probable causality.

**Conclusion.** Tapering glucocorticoids with 12.5 mg/day of prednisone equivalent could be a reasonable timepoint to initiate the withdrawal of PJP prophylaxis in patients with rheumatic diseases.

## INTRODUCTION

*Pneumocystis jirovecii* pneumonia (PJP) is a potentially life-threatening opportunistic infection that occurs mainly in immunocompromised patients.<sup>1</sup> Although first identified during the onset of the HIV epidemic in the early 1980s,<sup>2</sup> PJP infection has become more common in non-HIV-immunocompromised patients suffering from various conditions, such as malignancies, solid organ transplants, and autoimmune or inflammatory disease (AIIID).<sup>3,4</sup> Furthermore, PJP in patients without HIV typically exhibits rapid progression with a higher mortality rate than in HIV patients.<sup>3,5,6</sup>

Trimethoprim-sulfamethoxazole (TMP-SMX) is the gold standard for PJP prophylaxis, and its efficacy and safety have

been demonstrated in randomized controlled trials (RCTs) in both HIV-infected and non-HIV-infected immunocompromised patients.<sup>7–9</sup> Although no RCTs have specifically addressed this issue in patients with AIIID, many observational studies have shown that prophylactic TMP-SMX treatment significantly decreases the incidence of PJP in these patients, particularly when they receive prolonged, high-dose glucocorticoid (GC) treatment.<sup>10–13</sup> Based on these results, despite the weak level of evidence and the variation in risk depending on specific diseases and clinical settings, a recent recommendation from EULAR states that PJP prophylaxis should be taken into consideration when patients with AIIID receive daily doses of prednisone exceeding 15 to 30 mg for longer than two to four weeks.<sup>14</sup>

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Despite the established benefits of primary PJP prophylaxis in patients with AILD, the optimal prophylaxis schedule remains uncertain. One of the most important uncertainties is the timing of discontinuation of PJP prophylaxis. TMP-SMX carries some risk of adverse events (AEs), some of which may be severe.<sup>15–18</sup> Therefore, minimizing exposure to TMP-SMX while preserving its optimal prophylactic efficacy is a logical next step in building evidence for the universal recommendation for primary PJP prophylaxis. In patients with HIV, the decision to discontinue primary PJP prophylaxis is guided by CD4 counts and response to antiretroviral therapy (ART). Guidelines recommend the cessation of prophylaxis when CD4 counts have risen to more than 200 cells/mm<sup>3</sup> and are maintained for at least three months in response to ART.<sup>19</sup> However, the correlation between CD4 counts and PJP risk is less clear in patients without HIV, including those with AILD,<sup>20,21</sup> which renders CD4 counts a less reliable surrogate measure to direct prophylaxis. Although previous studies have suggested that PJP mainly occurs when a patient receives >15 mg/day of prednisone or its equivalent, it is uncertain whether this could be a relevant indication to consider the cessation of PJP prophylaxis.<sup>22,23</sup> This study aimed to investigate the risk factors associated with PJP infection despite prophylaxis and to offer insights into establishing an ideal primary prophylaxis regimen for patients with AILD.

## PATIENTS AND METHODS

**Patients.** This study included 1,148 patients who received immunosuppressants and concomitant prophylactic TMP-SMX for the treatment of underlying AILD at a nationwide tertiary referral center in South Korea between January 2010 and December 2022. The patients received TMP-SMX at either a dose of one single-strength tablet daily or a double-strength tablet three times per week. In cases with decreased renal function, TMP-SMX dosing was adjusted according to the estimated glomerular filtration rate (eGFR). Patients were excluded if they had HIV infection, underwent solid organ transplantation, were younger than 18 years of age, or had a follow-up duration of less than 28 days. In this study, none of the patients received prophylactic agents other than TMP-SMX, such as dapsone or pentamidine (Supplementary Figure 1).

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Seoul National University Hospital Institutional Review Board (no. 2009-065-1156). The ethics committee waived the requirement for written informed consent due to the retrospective nature of the study.

**Study design and prophylaxis episodes.** To identify potential risk factors associated with PJP occurrence despite primary prophylaxis, a case-control study design was selected given the low expected incidence of PJP in the study population. As some patients may have undergone multiple courses of

prophylactic TMP-SMX along with immunosuppressive therapy, we examined each episode of prophylaxis (referred to as a prophylaxis episode) within the study population. Episodes in which prophylactic TMP-SMX was administered for less than 14 days were excluded from the analysis of PJP incidence. However, AEs that occurred during these episodes were collected for safety assessment.

The observation scheme for the prophylactic episodes is presented in Supplementary Figure 2. The baseline date was defined as the date of prophylactic TMP-SMX initiation. The index period was designated as four weeks before and after the baseline. The demographics and immunosuppressive agents prescribed during this period were defined as the baseline features of the study population. The observation began from the baseline date and continued until the date of loss to follow-up, occurrence of PJP, or 52 weeks after the baseline date, whichever occurred first. Each prophylaxis was considered a distinct episode if the patient discontinued TMP-SMX and restarted it at least 52 weeks after the baseline date. If a patient had more than one episode, time-varying features such as eGFR, lymphocyte count, and immunosuppressive treatment use at the baseline of each episode were collected.

**Data collection.** The clinical data of the study population were obtained from the electronic medical records database of Seoul National University Hospital. Collected variables included patient demographics, underlying AILD, comorbidities (malignancy diagnosed within five years from the baseline, diabetes mellitus, chronic obstructive pulmonary disease, chronic liver disease, and heart failure), duration of TMP-SMX prescription, and potential risk factors for PJP, such as baseline lymphopenia (lymphocyte <800/ $\mu$ L), azotemia (eGFR <60 mL/min), and interstitial lung disease. In addition, data on the prescribed immunosuppressive therapies (GC, rituximab, cyclophosphamide, mycophenolate mofetil, tacrolimus, azathioprine, JAK inhibitors, and tocilizumab) were collected. Concomitant medications were defined as medications prescribed during the index period. GC pulse therapy was defined as intravenous methylprednisolone administration at a dose of 250 to 1,000 mg/day for a duration of one to five days. To collect data on the underlying AILD, we first collected data on all registered International Classification of Disease, Tenth Revision (ICD-10) codes for each study population. If a patient had more than one ICD-10 code for rheumatic disease, one investigator (JYK) reviewed the patient's medical records to confirm the final diagnosis. We also reviewed the medical records and gathered information on AEs that occurred with the use of prophylactic TMP-SMX. AEs were graded according to the Common Terminology Criteria for Adverse Events version 5.0.<sup>24</sup> Severe AEs were defined as AEs of grade 3 or higher. Two independent investigators (JYK and JWP) assessed the severity and cause of TMP-SMX in each case. Cases with probable or definite causality were defined as having drug reactions related to TMP-SMX.<sup>25</sup>

**PJP detection.** The detection of PJP is based on a combination of clinical, radiologic, and laboratory criteria, which are confirmed based on (1) the presence of clinical and radiographic features suggestive of PJP, and (2) results of the microbiologic test for identification. A two-step algorithm was designed to capture all PJP cases without misclassification.<sup>12</sup> At the outset, we collected all cases that exhibited positive results from direct immunofluorescence staining and/or polymerase chain reaction assays of respiratory specimens, including sputum, induced sputum, and bronchoalveolar lavage, over the observation period. Next, all the medical records of the selected cases were reviewed by two expert investigators (JYK and JWP) to confirm the diagnosis of PJP. Finally, PJP cases were confirmed when both assessors reached a consensus on the diagnosis.

**Statistical analysis.** All clinical variables used for the analysis had <5% missing values; therefore, no imputation was performed in the main analysis. For continuous variables, Student's *t*-test was used when the normality assumption was satisfied according to the Shapiro test, whereas the Wilcoxon rank-sum test was applied when it was not. Fisher's exact test was used for categorical variables. A Cox proportional hazards with least absolute shrinkage and selection operator (LASSO) model was used to identify the risk factors associated with PJP. The LASSO method was employed to prevent overfitting and to identify the most relevant predictive variables among independent clinical factors that exhibited a significant association ( $P < 0.1$ ) in the univariable analysis. Due to multicollinearity among the variables related to decreased renal function (eGFR, azotemia, and hemodialysis status), azotemia was selected for the main multivariable analysis. The chosen variables were used to fit a multivariable Cox proportional hazards regression model, adjusted for intracluster correlation to account for patients with multiple prophylaxis episodes. Firth penalized regression model was used in case of complete separation. Finally, clinical factors that showed a statistically significant association in the multivariable Cox regression analysis with LASSO were identified as risk factors for PJP despite primary prophylaxis.

To investigate the optimal timing for withdrawing PJP prophylaxis, we used the maximally selected rank statistics method to identify the optimal cutoff dose of GC at the time of prophylaxis discontinuation.<sup>26</sup> Subsequently, the association between the determined cutoff dose and cumulative incidence of PJP was evaluated using the Cox proportional hazards model, in which the hazard ratio (HR) was adjusted for the risk factors selected in the final multivariable analysis.

Several sensitivity analyses were conducted. First, the optimal cutoff dose of GC at the time of prophylaxis discontinuation was re-evaluated by excluding patients with recent concomitant malignancies diagnosed within the five years before the baseline date ( $n = 193$ ). Second, the categorical variable "azotemia" was replaced with the continuous variable eGFR, and the analysis

was repeated. Third, multiple imputation was performed using the Multivariate Imputation by Chained Equations (MICE) package in R. Following imputation, a Cox proportional hazards regression model was fitted to the imputed data sets, using the variables selected in the final model in the main analysis. The results from the Cox regression models were pooled using Rubin's rules to obtain combined estimates. Finally, the random survival forest (RSF) model was employed to assess the importance of each clinical variable using the variable importance (VIMP) method. All statistical analyses were performed using R V.3.3.1 software, and  $P < 0.05$  was considered statistically significant.

## RESULTS

**Baseline characteristics.** A total of 1,294 prophylactic episodes were analyzed in this study (Supplementary Figure 1). The most common underlying AIID was systemic lupus erythematosus ( $n = 273$ , 21.1%), followed by antineutrophil cytoplasmic antibody-associated vasculitis (AAV) ( $n = 226$ , 17.5%) and idiopathic inflammatory myositis ( $n = 204$ , 15.8%). The mean  $\pm$  SD duration of TMP-SMX prophylaxis was  $181.9 \pm 128.7$  days. In most episodes ( $n = 1,287$ , 99.5%), patients received TMP-SMX concomitant with GC, with a mean daily dose of  $41.5 \pm 24.2$  mg/day of prednisone or equivalent at the baseline. The most frequently used concomitant immunosuppressant was cyclophosphamide ( $n = 258$ , 19.9%), followed by rituximab ( $n = 168$ , 13.0%), mycophenolate mofetil ( $n = 120$ , 9.3%), azathioprine ( $n = 114$ , 8.8%), tacrolimus ( $n = 64$ , 4.9%), and cyclosporine A ( $n = 63$ , 4.9%). Rituximab was used solely for induction therapy. A combination of two or more non-GC immunosuppressants was administered to 56 (4.3%) patients.

**PJP incidence in the study population.** During the 1,174 person-years (PY) of observation, 10 cases of PJP occurred, with a one-year incidence rate (per 100 PY) of 0.85 (95% confidence interval [CI]: 0.41–1.57). In all cases, patients were evaluated by infection specialists and confirmed PJP. The clinical features of the PJP cases are summarized in Supplementary Table 1. Among these cases, only one occurred as a breakthrough infection during TMP-SMX prophylaxis, whereas the others occurred after the discontinuation of TMP-SMX. In these cases, the median (interquartile range [IQR]) interval between stopping prophylaxis and PJP was 117.0 (86.0–161.0) days. The median (IQR) duration of prophylaxis was 43.0 (22.0–124.0) days, where prophylaxis was stopped due to either AEs ( $n = 3$ ) or the treating physician's discretion ( $n = 6$ ).

The baseline characteristics of the PJP and control groups are presented in Table 1. The PJP group tended to be older, had more lymphopenia and compromised renal function, and had a shorter prophylactic period for TMP-SMX (median [IQR]: 148.0 [68.0–350.0] vs 51.0 [22.0–124.0] days;  $P = 0.007$ ). PJP

**Table 1.** Clinical features of the study population\*

|                                                                      | Control group (n = 1,284) | PJP group (n = 10)    | P value |
|----------------------------------------------------------------------|---------------------------|-----------------------|---------|
| Age, median (IQR), y                                                 | 55.0 (41.0–66.0)          | 70.5 (65.0–76.0)      | 0.001   |
| Female sex, n (%)                                                    | 798 (62.1)                | 6 (60.0)              | 1.000   |
| Smoking, n (%)                                                       |                           |                       | 0.240   |
| Never-smoker                                                         | 842 (78.2)                | 6 (60.0)              |         |
| Ever-smoker                                                          | 235 (21.8)                | 4 (40.0)              |         |
| Underlying rheumatic disease, n (%)                                  |                           |                       |         |
| SLE                                                                  | 272 (21.2)                | 1 (10.0)              | 0.698   |
| AAV                                                                  | 221 (17.2)                | 5 (50.0)              | 0.018   |
| IIM                                                                  | 203 (15.8)                | 1 (10.0)              | 1.000   |
| IgA vasculitis/nephropathy                                           | 185 (14.4)                | 0 (0.0)               | 0.374   |
| IgG4-related disease                                                 | 64 (5.0)                  | 0 (0.0)               | 1.000   |
| AOSD                                                                 | 45 (3.5)                  | 0 (0.0)               | 1.000   |
| RA                                                                   | 41 (3.2)                  | 1 (10.0)              | 0.282   |
| Vasculitides, unclassified                                           | 40 (3.1)                  | 0 (0.0)               | 1.000   |
| Polyarteritis nodosa                                                 | 38 (3.0)                  | 0 (0.0)               | 1.000   |
| Behcet's disease                                                     | 37 (2.9)                  | 1 (10.0)              | 0.259   |
| Systemic sclerosis                                                   | 34 (2.6)                  | 0 (0.0)               | 1.000   |
| Sjögren disease                                                      | 21 (1.6)                  | 0 (0.0)               | 1.000   |
| Large vessel vasculitis                                              | 15 (1.2)                  | 0 (0.0)               | 1.000   |
| Relapsing polychondritis                                             | 15 (1.2)                  | 0 (0.0)               | 1.000   |
| Polymyalgia rheumatica                                               | 12 (0.9)                  | 1 (10.0)              | 0.096   |
| Cryoglobulinemic vasculitis                                          | 5 (0.4)                   | 0 (0.0)               | 1.000   |
| Cutaneous rheumatic disease                                          | 11 (0.9)                  | 0 (0.0)               | 1.000   |
| MCTD/UCTD                                                            | 8 (0.6)                   | 0 (0.0)               | 1.000   |
| Antiphospholipid syndrome                                            | 5 (0.4)                   | 0 (0.0)               | 1.000   |
| Others                                                               | 19 (1.5)                  | 0 (0.0)               | 1.000   |
| Comorbidities, n (%)                                                 |                           |                       |         |
| Malignancy                                                           | 192 (15.0)                | 1 (10.0)              | 1.000   |
| Diabetes mellitus                                                    | 359 (28.0)                | 6 (60.0)              | 0.035   |
| COPD                                                                 | 156 (12.1)                | 2 (20.0)              | 0.350   |
| Interstitial lung disease                                            | 334 (26.0)                | 4 (40.0)              | 0.298   |
| Heart failure                                                        | 77 (6.0)                  | 2 (20.0)              | 0.120   |
| Chronic liver disease                                                | 130 (10.1)                | 0 (0.0)               | 0.611   |
| Azotemia <sup>a</sup>                                                | 400 (31.2)                | 9 (90.0)              | <0.001  |
| Renal replacement therapy                                            | 118 (9.2)                 | 4 (40.0)              | 0.010   |
| Duration of TMP-SMX, median (IQR), d                                 | 148.0 (68.0–350.0)        | 51.0 (22.0–124.0)     | 0.007   |
| eGFR, median (IQR)                                                   | 85.7 (48.6–108.7)         | 21.6 (11.0–46.5)      | <0.001  |
| Lymphocyte count, median (IQR)                                       | 1,280.0 (808.0–1,993.0)   | 571.0 (329.0–1,682.0) | 0.037   |
| Lymphopenia <sup>b</sup> , n (%)                                     | 294 (23.5)                | 7 (70.0)              | 0.003   |
| Treatment at baseline                                                |                           |                       |         |
| Glucocorticoid pulse, n (%)                                          | 136 (10.6)                | 3 (30.0)              | 0.083   |
| Baseline GC dose <sup>c</sup> , median (IQR), mg/day                 | 40.0 (25.0–60.0)          | 50.0 (25.0–62.5)      | 0.431   |
| Rituximab, n (%)                                                     | 163 (12.7)                | 5 (50.0)              | 0.005   |
| Cyclophosphamide, n (%)                                              | 255 (19.9)                | 3 (30.0)              | 0.427   |
| Mycophenolate mofetil, n (%)                                         | 119 (9.3)                 | 1 (10.0)              | 1.000   |
| Cyclosporine, n (%)                                                  | 63 (4.9)                  | 0 (0.0)               | 1.000   |
| Tacrolimus, n (%)                                                    | 64 (5.0)                  | 0 (0.0)               | 1.000   |
| Azathioprine, n (%)                                                  | 113 (8.8)                 | 1 (10.0)              | 0.604   |
| JAK inhibitor, n (%)                                                 | 4 (0.3)                   | 0 (0.0)               | 1.000   |
| Tocilizumab, n (%)                                                   | 20 (1.6)                  | 0 (0.0)               | 1.000   |
| Combination non-GC immunosuppressive treatment, n (%)                | 55 (4.3)                  | 1 (10.0)              | 0.359   |
| GC dose <sup>c</sup> at the end of prophylaxis, median (IQR), mg/day | 10.0 (5.0–15.0)           | 22.5 (20.0–40.0)      | <0.001  |

\* AAV, antineutrophil cytoplasmic antibody-associated vasculitis; AOSD, adult-onset Still disease; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; GC, glucocorticoid; IIM, idiopathic inflammatory myopathies; IQR, interquartile range; MCTD, mixed connective tissue disease; PJP, *Pneumocystis jirovecii* pneumonia; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TMP-SMX, trimethoprim-sulfamethoxazole; UCTD, undifferentiated connective tissue disease.

<sup>a</sup> Defined as a glomerular filtration rate <60 mL/minute.

<sup>b</sup> Defined as <800 lymphocytes per microliter.

<sup>c</sup> Based on the dose of prednisone.

group had a greater proportion of patients who received renal replacement therapy. AAV constituted a significantly higher proportion of the PJP group than other underlying diseases. Regarding concomitant immunosuppressive treatment, the GC dose at baseline was comparable between the two groups. However, the GC dose at the time of discontinuation of TMP-SMX was significantly higher in the PJP group (median [IQR]: 22.5 [20.0–40.0] vs 10.0 [5.0–15.0] mg of prednisone;  $P < 0.001$ ) (Figure 1).

**Risk factors for PJP.** Univariable Cox regression analysis indicated that age, decreased renal function, lymphopenia, diabetes, underlying AAV, polymyalgia rheumatica, and the concomitant use of GC pulse therapy or rituximab were associated with the occurrence of PJP (Table 2). In contrast, baseline GC dose (HR, 1.01; 95% CI, 0.99–1.03) and combination of two or more non-GC immunosuppressants (HR, 2.56; 95% CI, 0.32–20.21) did not increase the risk. Rituximab was administered as maintenance therapy in 95 episodes (7.3%), all within the control group. Rituximab maintenance was not associated with an increased incidence of PJP in this cohort (HR, 0.56; 95% CI, 0.00–4.33). Among the relevant factors discussed, age, impaired renal

function, lymphopenia, and the use of rituximab emerged as significant predictors of increased PJP risk in the multivariable analysis conducted with LASSO.

#### Impact of GC dose at discontinuing TMP-SMX on the incidence of PJP.

Because the GC dose at TMP-SMX discontinuation was significantly higher in the PJP group, we further evaluated its impact on PJP occurrence. Using maximally selected rank statistics, the optimal cutoff GC dose that best distinguished the PJP from the control group was identified as 12.5 mg prednisone equivalent ( $P < 0.001$ ). In fact, 90% (9 of 10) of the patients who developed PJP in the prophylaxis group were taking  $>12.5$  mg/day of prednisone or its equivalent dosage at the time of prophylaxis withdrawal, compared with only 35.1% ( $n = 451$ ) in the control group. When the entire prophylactic episodes were divided into two groups based on the GC dose at the end of prophylaxis ( $>12.5$  mg vs  $\leq 12.5$  mg of prednisone equivalent), the episodes with higher GC dose ( $n = 460$ , 35.5%) at the time of prophylaxis withdrawal showed a significantly higher risk of PJP (HR, 18.55; 95% CI, 2.35–146.42;  $P = 0.006$ ) (Figure 2). The result was consistent in the multivariable Cox regression analysis in which relevant risk factors for PJP were

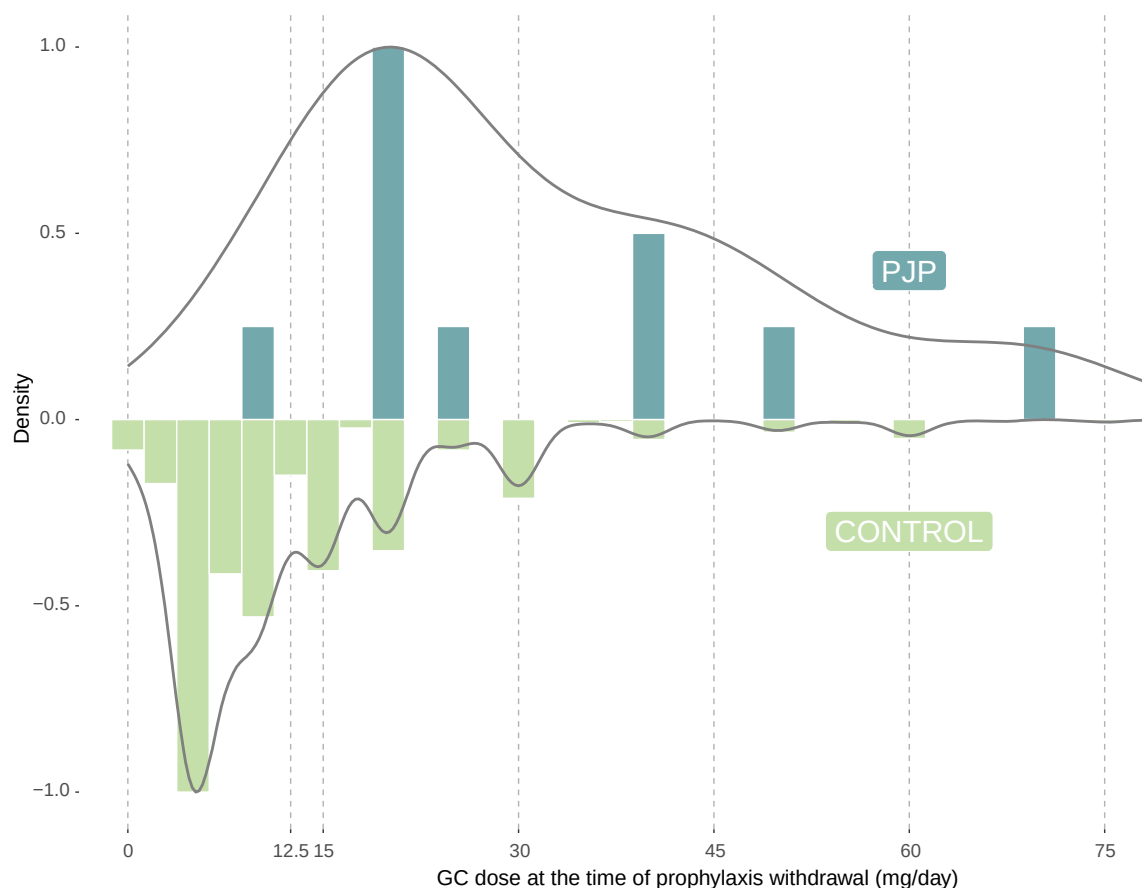


**Figure 1.** Density plot showing the dose of GC at the time of trimethoprim-sulfamethoxazole discontinuation in the PJP and the control group. GC, glucocorticoid; PJP, *Pneumocystis jirovecii* pneumonia. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.43167/abstract>.

**Table 2.** Clinical factors associated with PJP despite primary prophylaxis\*

|                                                | Univariable         |         | Multivariable <sup>a</sup> |         |
|------------------------------------------------|---------------------|---------|----------------------------|---------|
|                                                | HR (95% CI)         | P value | HR (95% CI)                | P value |
| Age                                            | 1.08 (1.03–1.14)    | <0.01   | 1.06 (1.00–1.13)           | 0.04    |
| Sex, female                                    | 0.88 (0.25–3.11)    | 0.84    |                            |         |
| eGFR                                           | 0.96 (0.94–0.99)    | <0.01   |                            |         |
| Azotemia <sup>b</sup>                          | 20.01 (2.53–157.93) | <0.01   | 9.73 (1.17–80.74)          | 0.04    |
| Hemodialysis                                   | 6.77 (1.91–23.99)   | <0.01   |                            |         |
| Lymphopenia <sup>c</sup>                       | 7.97 (2.06–30.82)   | <0.01   | 5.79 (1.44–23.28)          | 0.01    |
| Cancer                                         | 0.70 (0.09–5.50)    | 0.73    |                            |         |
| Diabetes mellitus                              | 3.70 (1.05–13.12)   | 0.04    | 1.29 (0.32–5.11)           | 0.72    |
| Heart failure                                  | 3.90 (0.83–18.35)   | 0.09    | 3.57 (0.68–18.73)          | 0.13    |
| COPD                                           | 1.83 (0.39–8.62)    | 0.44    |                            |         |
| Interstitial lung disease                      | 1.88 (0.53–6.68)    | 0.33    |                            |         |
| SLE                                            | 0.40 (0.05–3.19)    | 0.39    |                            |         |
| AAV                                            | 4.75 (1.37–16.41)   | 0.01    |                            |         |
| IIM                                            | 0.59 (0.07–4.64)    | 0.62    |                            |         |
| RA                                             | 3.75 (0.47–29.57)   | 0.21    |                            |         |
| Behçet's disease                               | 3.54 (0.45–27.95)   | 0.23    |                            |         |
| Polymyalgia rheumatica                         | 11.18 (1.42–88.31)  | 0.02    | 9.29 (0.89–97.35)          | 0.06    |
| Rituximab                                      | 6.75 (1.96–23.33)   | <0.01   | 5.53 (1.37–22.29)          | 0.02    |
| Cyclophosphamide                               | 1.74 (0.45–6.72)    | 0.42    |                            |         |
| Mycophenolate mofetil                          | 1.08 (0.14–8.52)    | 0.94    |                            |         |
| Azathioprine                                   | 1.16 (0.15–9.12)    | 0.89    |                            |         |
| JAK inhibitor                                  | 15.99 (0.12–124.16) | 0.18    |                            |         |
| Tocilizumab                                    | 2.81 (0.02–21.75)   | 0.54    |                            |         |
| Combination non-GC immunosuppressive treatment | 2.56 (0.32–20.21)   | 0.37    |                            |         |
| Baseline GC dose                               | 1.01 (0.99–1.03)    | 0.44    |                            |         |
| Baseline GC pulse                              | 3.71 (0.96–14.36)   | 0.06    |                            |         |

\* AAV, antineutrophil cytoplasmic antibody-associated vasculitis; CI, confidence interval; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; GC, glucocorticoid; HR, hazard ratio; IIM, idiopathic inflammatory myopathies; PJP, *Pneumocystis jirovecii* pneumonia; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

<sup>a</sup> Least absolute shrinkage and selection operator analysis was used to select clinical variables that constitute the optimal predictive model.

<sup>b</sup> Defined as a glomerular filtration rate below 60 mL/minute.

<sup>c</sup> Defined as <800 lymphocytes/ $\mu$ L per microliter.

adjusted (adjusted HR, 13.84; 95% CI, 1.71–111.80;  $P = 0.014$ ) (Table 3).

**Safety of prophylactic TMP-SMX.** There were 63 AEs that occurred in 54 episodes (4.2%): 50 in the control group and 4 in the PJP group, with an incidence of 5.1 per 100 PY (95% CI, 3.9–6.5). The most frequent AEs were liver enzyme abnormalities ( $n = 13$ , 20.6%), rashes ( $n = 11$ , 17.5%), thrombocytopenia ( $n = 10$ , 15.9%), fever ( $n = 7$ , 11.1%), and renal dysfunction ( $n = 6$ , 9.5%) (Table 4). Most AEs were mild to moderate in severity and did not require additional interventions. The causality of each TMP-SMX-related AE was assessed, with 10 cases (15.9%) rated as “probable,” 37 (58.7%) rated as “possible,” and 16 (25.4%) rated as “unlikely.” However, in most cases ( $n = 60$ , 95.2%), the TMP-SMX treatment was discontinued after the occurrence of AEs.

During the observation period, there were four cases of severe AEs, including two cases of pancytopenia and two cases of Stevens-Johnson syndrome (SJS). Two SJS cases were deemed to have probable causality, whereas the remaining cases

had possible causality. All patients with SJS recovered after discontinuation of TMP-SMX.

**Sensitivity analysis.** Multiple sensitivity analyses were performed. Initially, we replicated the analysis after excluding individuals who had been diagnosed with cancer within the five years before the baseline date ( $n = 193$ ). This analysis led to the same result that a GC dose greater than 12.5 mg was significantly associated with the occurrence of PJP (adjusted HR, 15.10; 95% CI, 1.88–120.95) (Supplementary Table 2). Secondly, we replaced covariable “azotemia” with “eGFR” and conducted a repeated analysis, which also showed the consistent increased risk of PJP with the GC >12.5 mg at the end of prophylaxis (adjusted HR, 14.09; 95% CI, 1.75–113.56) (Supplementary Table 3). Third, multivariable Cox regression was fit on the imputed data sets obtained after multiple imputation. The pooled results indicated that the effect of GC >12.5 mg of prednisone equivalent at the end of prophylaxis was consistent with the findings from the original analysis (adjusted HR, 13.84; 95% CI, 1.70–112.69). Lastly, the RSF analysis was conducted to identify the variables of

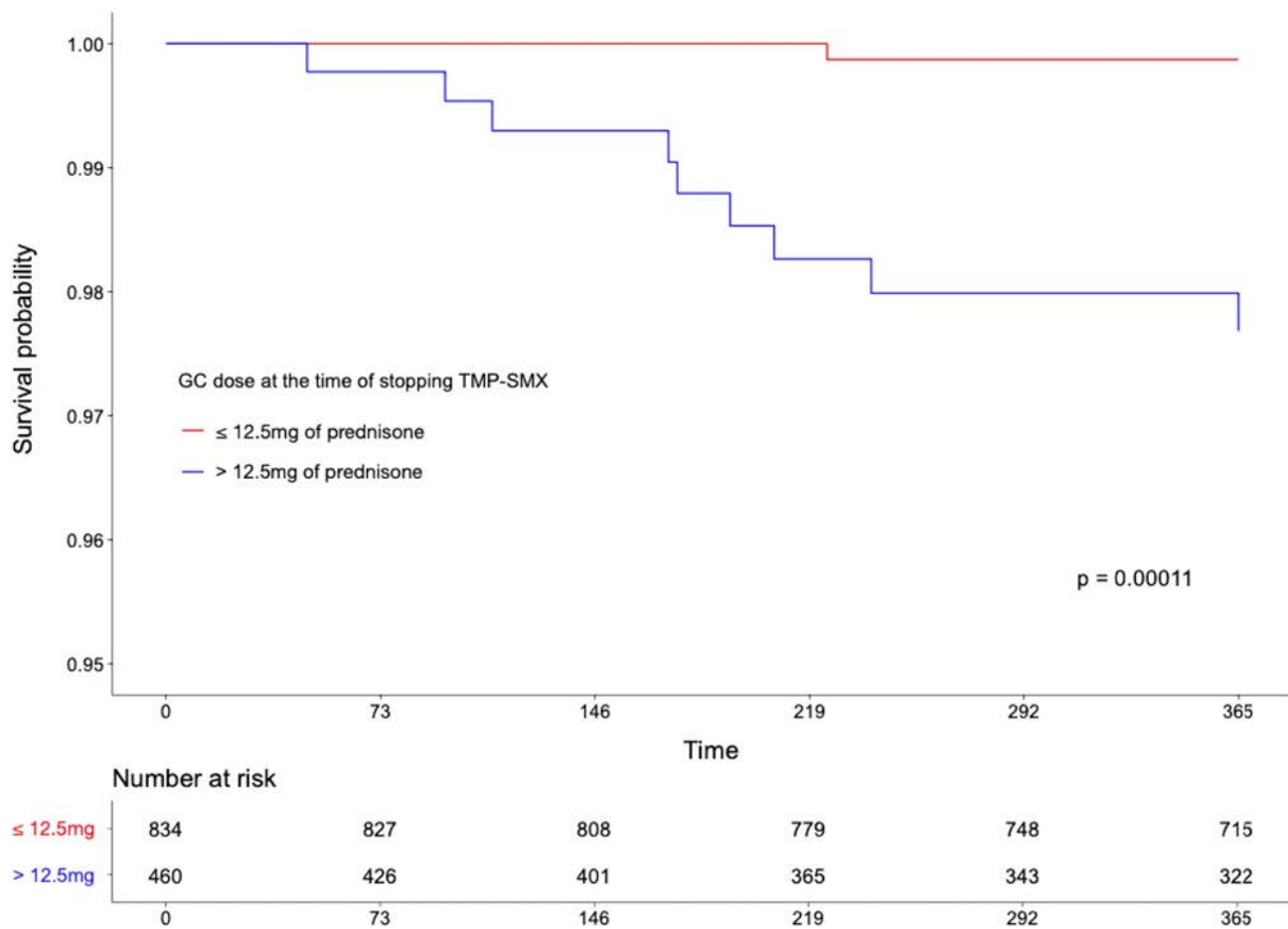


**Figure 2.** Kaplan-Meier curve indicating the *Pneumocystis jirovecii* pneumonia incidence stratified by the dose of GC at the time of discontinuing TMP-SMX. GC, glucocorticoid; TMP-SMX, trimethoprim-sulfamethoxazole.

importance that were associated with PJP development according to the VIMP method. The importance of variables was ranked in the order of the GC dose at the end of prophylaxis, lymphopenia, rituximab, eGFR, and age (Supplementary Figure 3).

## DISCUSSION

Although the effectiveness of PJP prophylaxis in patients with AILDs is widely recognized, there is no clear consensus on the optimal regimen, particularly regarding the duration of

maintenance. Although previous studies have demonstrated the excellent prophylactic efficacy of TMP-SMX in reducing the incidence of PJP, its use carries a potential risk of various AEs. Therefore, establishing appropriate criteria for discontinuing prophylaxis could enhance patient outcomes while improving safety. To the best of our knowledge, this is the first large-scale study to investigate the potential risk factors associated with PJP despite prophylaxis, which may provide crucial evidence for establishing an optimal prophylaxis protocol.

This study showed that the one-year incidence of PJP among patients receiving prophylactic TMP-SMX was low, reinforcing previous reports that TMP-SMX has high prophylactic efficacy against PJP.<sup>9</sup> Furthermore, there was only one case of breakthrough PJP during TMP-SMX prophylaxis. Given the high effectiveness of TMP-SMX in PJP prophylaxis, further research is needed to address next steps, such as identifying specific risk groups and determining the optimal duration for maintaining prophylaxis, to ensure effective prevention.

As in line with previous studies, risk factors such as lymphopenia, impaired renal function, age, and rituximab use were

**Table 3.** Effect of GC dose at the end of prophylaxis on 1-year *Pneumocystis jirovecii* pneumonia incidence\*

|                | Univariable,<br>HR (95% CI) | Multivariable,<br>adjusted HR <sup>a</sup> (95% CI) |
|----------------|-----------------------------|-----------------------------------------------------|
| GC >12.5 mg    | 18.55 (2.35–146.42)         | 13.84 (1.71–111.80)                                 |
| P value for HR | 0.0056                      | 0.0137                                              |

\* CI, confidence interval; eGFR, estimated glomerular filtration rate; GC, glucocorticoid; HR, hazard ratio.

<sup>a</sup> Adjusted for clinical factors with relevant association ( $P < 0.05$ ) in multivariable analysis (age, azotemia defined as eGFR below 60 mL/minute, lymphopenia, rituximab).

**Table 4.** Adverse events that occurred during the prophylactic TMP-SMX administration\*

|                          | Causality, n (%) |            |            | Total AEs  |
|--------------------------|------------------|------------|------------|------------|
|                          | Probable         | Possible   | Unlikely   |            |
| Total AEs                | 10 (100.0)       | 37 (100.0) | 16 (100.0) | 63 (100.0) |
| Mild to moderate AEs     | 8 (80.0)         | 36 (97.3)  | 15 (93.8)  | 59 (93.6)  |
| AST or ALT increased     | 1 (10.0)         | 10 (27.0)  | 2 (12.5)   | 13 (20.6)  |
| Rash                     | 3 (30.0)         | 5 (13.5)   | 3 (18.8)   | 11 (17.5)  |
| Thrombocytopenia         | 0                | 6 (16.2)   | 4 (25.0)   | 10 (15.9)  |
| Leukopenia               | 0                | 2 (5.4)    | 2 (12.5)   | 4 (6.3)    |
| Fever                    | 1 (10.0)         | 4 (10.8)   | 2 (12.5)   | 7 (11.1)   |
| Renal dysfunction        | 2 (20.0)         | 3 (8.1)    | 1 (6.3)    | 6 (9.5)    |
| Pneumonitis              | 1 (10.0)         | 0          | 0          | 1 (1.6)    |
| Headache                 | 0                | 2 (5.4)    | 0          | 2 (3.2)    |
| Allergic reaction        | 0                | 1 (2.7)    | 0          | 1 (1.6)    |
| Nausea                   | 0                | 1 (2.7)    | 1 (6.3)    | 2 (3.2)    |
| Tremor                   | 0                | 1 (2.7)    | 0          | 1 (1.6)    |
| Cognitive disturbance    | 0                | 1 (2.7)    | 0          | 1 (1.6)    |
| Severe AE                | 2 (20.0)         | 1 (2.7)    | 1 (6.3)    | 4 (6.3)    |
| Pancytopenia             | 0                | 1 (2.7)    | 1 (6.3)    | 2 (3.2)    |
| Stevens-Johnson syndrome | 2 (20.0)         | 0          | 0          | 2 (3.2)    |

\* No AEs were evaluated as definite in causality assessment. AE, adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TMP-SMX, trimethoprim-sulfamethoxazole.

significantly associated with PJP.<sup>11,13,18,23,27–29</sup> Our earlier research demonstrated that TMP-SMX prophylaxis reduces the incidence of PJP and related mortality in patients treated with rituximab.<sup>30</sup> An intriguing finding in this study was that among the 16 patients who developed PJP despite prophylaxis, 15 experienced the onset of PJP merely a few months following the cessation of prophylactic TMP-SMX.

In this context, it is noteworthy that the PJP group in our study had a shorter prophylaxis duration than the control group, leading to the discontinuation of prophylaxis while the patients were still receiving relatively higher doses of GCs. Previous studies on PJP in patients with AILD showed that most PJP cases occurred when patients received >15–20 mg/day of prednisone or its equivalents.<sup>11,12,31</sup> Expanding upon this finding, we demonstrated that the risk of PJP persists even with prophylaxis, unless it is continued until GC doses are tapered to ≤12.5 mg/day of prednisone or its equivalent. In fact, patients who stopped prophylactic TMP-SMX at a dose of concomitant GC higher than the threshold showed an approximately 10-times-higher risk of PJP than other patients. This result suggests that tapering the GC dose by 12.5 mg/day or less can be a relevant threshold for considering the withdrawal of prophylactic TMP-SMX.

However, approximately 40% of the study population discontinued TMP-SMX before the threshold was reached. This result could be attributed to various factors, including safety concerns associated with TMP-SMX. Despite the well-established prophylactic efficacy of TMP-SMX, there are concerns regarding adverse drug reactions (ADRs) ranging from severe conditions such as SJS and toxic epidermal necrolysis to minor reactions such as gastrointestinal symptoms, rash, increased serum creatinine, and elevated liver enzymes.<sup>32–35</sup> The study by Nettleton

et al calculated the risk of possible AE associated to PJP prophylaxis and reported that leukopenia (HR, 3.1), rash (HR, 1.9), and nephropathy (HR, 2.6) were higher among those who received prophylaxis as opposed those who did not.<sup>36</sup> However, most of these studies did not thoroughly assess the causality of AEs, leaving the true incidence of ADRs directly related to prophylactic TMP-SMX debatable. It should be noted that AEs commonly linked to TMP-SMX may also stem from underlying diseases and concomitant treatment. This aligns with our finding that only 4.2% of prophylactic episodes were suspected to be AEs. Among these AEs, 10 (15.9%) were identified as true ADR, and only two cases showed serious AEs that required intervention other than stopping TMP-SMX, in which all patients recovered after proper management. Notably, a previous cohort study that included patients treated with rituximab and prophylactic TMP-SMX showed that, among 2,113 AEs, only 92 (4.4%) had definite or probable causality related to TMP-SMX. Of these, only 10 severe ADRs required any intervention.<sup>30</sup> Taken together, these findings suggest that the potential risks associated with the prolonged use of prophylactic TMP-SMX may be less significant than those previously reported.

The study has several limitations that should be addressed. First, due to the small number of PJP cases in this study, including even a few covariates in the multivariable model could potentially result in model overfitting. Although variable selection using LASSO and RSF analysis helped reduce this potential bias, the results of this study should be validated in future larger-scale studies. Second, the prophylactic effect of TMP-SMX may vary among individual AILDs. However, this study could not thoroughly address this variability due to the limited number of patients in each specific disease subgroup. Furthermore, the risk of PJP is

not driven by a single factor alone but rather by a complex interplay of multiple interacting factors. Although this study suggests that GC dose could serve as a relevant and easily applicable threshold for considering prophylaxis withdrawal, the decision should be further guided by more patient-specific factors, which need to be further investigated in future studies. Third, because this study retrospectively collected data from medical records, it is possible that some potential AEs related to TMP-SMX were missed in our analysis. Finally, the results of this study may have been affected by unmeasured factors, an inherent limitation of observational studies. An RCT would provide a clear understanding of this issue. However, considering the extremely low incidence of PJP in the study population, the sample size required to test the hypothesis with an adequate statistical power would not be feasible. As an alternative, further comparative observational studies may offer a viable option for generating additional evidence.

In conclusion, discontinuation of TMP-SMX during GC treatment at doses >12.5 mg prednisone equivalent significantly increased the risk of PJP. This suggests that tapering the GC to 12.5 mg/day may be a reasonable consideration when withdrawing PJP prophylaxis. Although these results need to be confirmed in future studies, they may assist physicians in determining the appropriate timing for discontinuing PJP prophylaxis in patients with AIDs.

## AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Park confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

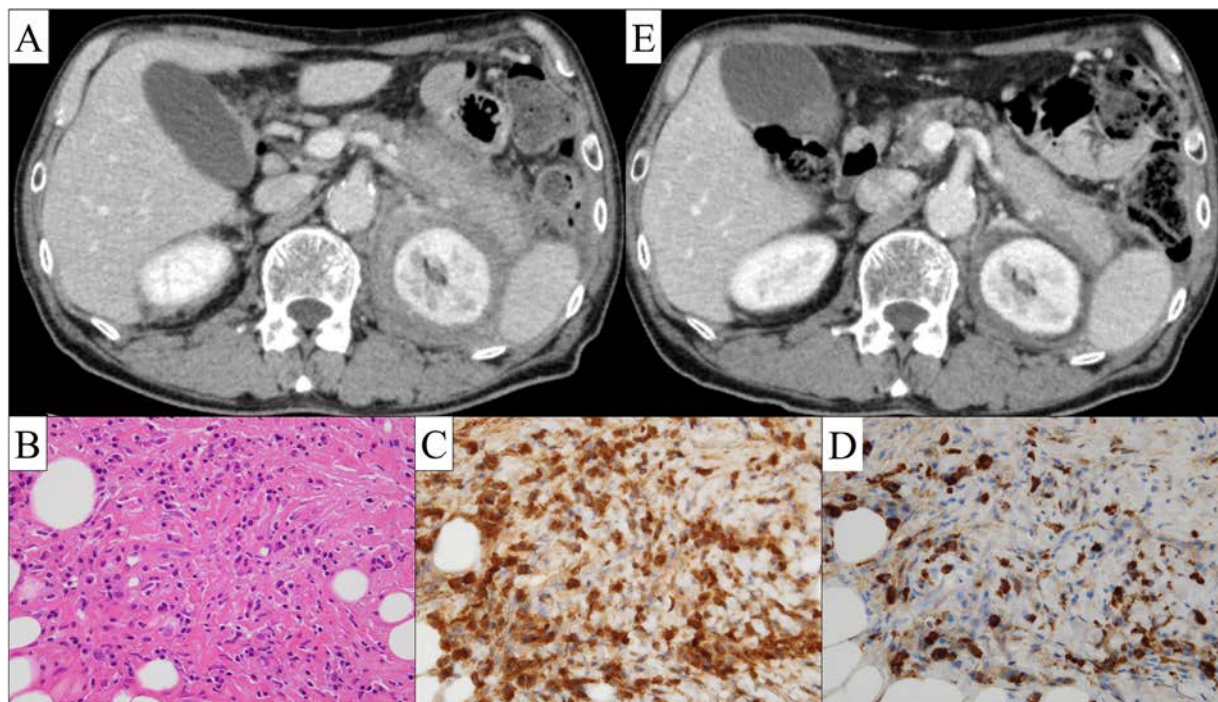
## REFERENCES

- Catherinot E, Lanternier F, Bougnoux ME, et al. *Pneumocystis jirovecii* pneumonia. *Infect Dis Clin North Am* 2010;24(1):107–138.
- Follansbee SE, Busch DF, Wofsy CB, et al. An outbreak of *Pneumocystis carinii* pneumonia in homosexual men. *Ann Intern Med* 1982; 96(6 Pt 1):705–713.
- Kolbrink B, Scheikholeslami-Sabzewari J, Borzikowsky C, et al. Evolving epidemiology of *Pneumocystis pneumonia*: findings from a longitudinal population-based study and a retrospective multi-center study in Germany. *Lancet Reg Health Eur* 2022;18:100400.
- Sepkowitz KA. Opportunistic infections in patients with and patients without acquired immunodeficiency syndrome. *Clin Infect Dis* 2002; 34(8):1098–1107.
- Rego de Figueiredo I, Vieira Alves R, Drummond Borges D, et al. *Pneumocystis pneumonia*: a comparison study between HIV and non-HIV immunocompromised patients. *Pulmonology* 2019;25(5): 271–274.
- Wang Y, Huang X, Sun T, et al. Non-HIV-infected patients with *Pneumocystis pneumonia* in the intensive care unit: A bicentric, retrospective study focused on predictive factors of in-hospital mortality. *Clin Respir J* 2022;16(2):152–161.
- Fischl MA, Dickinson GM, La Voie L. Safety and efficacy of sulfamethoxazole and trimethoprim chemoprophylaxis for *Pneumocystis carinii* pneumonia in AIDS. *JAMA* 1988;259(8):1185–1189.
- Hughes WT, Kuhn S, Chaudhary S, et al. Successful chemoprophylaxis for *Pneumocystis carinii* pneumonitis. *N Engl J Med* 1977; 297(26):1419–1426.
- Stern A, Green H, Paul M, et al. Prophylaxis for *Pneumocystis pneumonia* (PCP) in non-HIV immunocompromised patients. *Cochrane Database Syst Rev* 2014;2014(10):CD005590.
- Honda N, Tagashira Y, Kawai S, et al. Reduction of *Pneumocystis jirovecii* pneumonia and bloodstream infections by trimethoprim-sulfamethoxazole prophylaxis in patients with rheumatic diseases. *Scand J Rheumatol* 2021;50(5):365–371.
- Park JW, Curtis JR, Kim MJ, et al. *Pneumocystis pneumonia* in patients with rheumatic diseases receiving prolonged, non-high-dose steroids-clinical implication of primary prophylaxis using trimethoprim-sulfamethoxazole. *Arthritis Res Ther* 2019;21(1):207.
- Park JW, Curtis JR, Moon J, et al. Prophylactic effect of trimethoprim-sulfamethoxazole for *Pneumocystis pneumonia* in patients with rheumatic diseases exposed to prolonged high-dose glucocorticoids. *Ann Rheum Dis* 2018;77(5):644–649.
- Vananuvat P, Suwannalai P, Sungkanuparph S, et al. Primary prophylaxis for *Pneumocystis jirovecii* pneumonia in patients with connective tissue diseases. *Semin Arthritis Rheum* 2011;41(3):497–502.
- Fragoulis GE, Nikiphorou E, Dey M, et al. 2022 EULAR recommendations for screening and prophylaxis of chronic and opportunistic infections in adults with autoimmune inflammatory rheumatic diseases. *Ann Rheum Dis* 2023;82(6):742–753.
- Fukasawa T, Urushihara H, Takahashi H, et al. Risk of Stevens-Johnson syndrome and toxic epidermal necrolysis associated with antibiotic use: a case-crossover study. *J Allergy Clin Immunol Pract* 2023;11(11):3463–3472.
- Ho JM, Juurlink DN. Considerations when prescribing trimethoprim-sulfamethoxazole. *CMAJ* 2011;183(16):1851–1858.
- Mendel A, Behloul H, Vinet E, et al. Trimethoprim sulfamethoxazole prophylaxis and serious infections in granulomatosis with polyangiitis treated with rituximab. *Rheumatology (Oxford)* 2025;64(4):2041–2049.
- Ogawa J, Harigai M, Nagasaka K, et al. Prediction of and prophylaxis against *Pneumocystis pneumonia* in patients with connective tissue diseases undergoing medium- or high-dose corticosteroid therapy. *Mod Rheumatol* 2005;15(2):91–96.
- Clinicalinfo.HIV.gov. Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV. Accessed February 2, 2025. <https://clinicalinfo.hiv.gov/en/guidelines/adult-and-adolescent-opportunistic-infection>
- Baulier G, Issa N, Gabriel F, et al. Guidelines for prophylaxis of *Pneumocystis pneumonia* cannot rely solely on CD4-cell count in autoimmune and inflammatory diseases. *Clin Exp Rheumatol* 2018;36(3): 490–493.
- Gordon SM, LaRosa SP, Kalmadi S, et al. Should prophylaxis for *Pneumocystis carinii* pneumonia in solid organ transplant recipients ever be discontinued? *Clin Infect Dis* 1999;28(2):240–246.
- Zhou S, Aitken SL. Prophylaxis against *Pneumocystis jirovecii* pneumonia in adults. *JAMA* 2023;330(2):182–183.
- Sowden E, Carmichael AJ. Autoimmune inflammatory disorders, systemic corticosteroids and *Pneumocystis pneumonia*: a strategy for prevention. *BMC Infect Dis* 2004;4(1):42.
- National Cancer Institute. CTCAE Version 5.0. January 3, 2018. [https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE\\_5.0/](https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_5.0/)

25. Edwards IR, Aronson JK. Adverse drug reactions: definitions, diagnosis, and management. *Lancet* 2000;356(9237):1255–1259.
26. Hothorn T, Zeileis A. Generalized maximally selected statistics. *Biometrics* 2008;64(4):1263–1269.
27. Ye WL, Tang N, Wen YB, et al. Underlying renal insufficiency: the pivotal risk factor for *Pneumocystis jirovecii* pneumonia in immunosuppressed patients with non-transplant glomerular disease. *Int Urol Nephrol* 2016;48(11):1863–1871.
28. Mulpuru S, Knoll G, Weir C, et al. *Pneumocystis pneumonia* outbreak among renal transplant recipients at a North American transplant center: risk factors and implications for infection control. *Am J Infect Control* 2016;44(4):425–431.
29. Ghembaza A, Vautier M, Cacoub P, et al. Risk factors and prevention of *Pneumocystis jirovecii* pneumonia in patients with autoimmune and inflammatory diseases. *Chest* 2020;158(6):2323–2332.
30. Park JW, Curtis JR, Jun KI, et al. Primary prophylaxis for *Pneumocystis jirovecii* pneumonia in patients receiving rituximab. *Chest* 2022; 161(5):1201–1210.
31. Yale SH, Limper AH. *Pneumocystis carinii* pneumonia in patients without acquired immunodeficiency syndrome: associated illness and prior corticosteroid therapy. *Mayo Clin Proc* 1996;71(1):5–13.
32. Utsunomiya M, Dobashi H, Odani T, et al. An open-label, randomized controlled trial of sulfamethoxazole-trimethoprim for *Pneumocystis* prophylaxis: results of 52-week follow-up. *Rheumatol Adv Pract* 2020;4(2):rkaa029.
33. Li R, Tang Z, Liu F, et al. Efficacy and safety of trimethoprim-sulfamethoxazole for the prevention of *Pneumocystis pneumonia* in human immunodeficiency virus-negative immunodeficient patients: a systematic review and meta-analysis. *PLoS One* 2021;16(3): e0248524.
34. Hashimoto M, Hiraiwa M, Uchitani K, et al. Sulfamethoxazole-trimethoprim for *Pneumocystis pneumonia* prophylaxis, causes of discontinuation and thrombocytopenia observed during administration: a single-center retrospective study. *J Infect Chemother* 2024; 30(2):141–146.
35. Kokubu H, Kato T, Nishikawa J, et al. Adverse effects of trimethoprim-sulfamethoxazole for the prophylaxis of *Pneumocystis pneumonia* in dermatology. *J Dermatol* 2021;48(4):542–546.
36. Nettleton E, Sattui SE, Wallace Z, et al. Incidence of *Pneumocystis jirovecii* pneumonia in patients with ANCA-associated vasculitis initiating therapy with rituximab or cyclophosphamide. *Arthritis Care Res (Hoboken)* 2024;76(2):288–294.

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### Clinical Images: Hairy kidney in IgG4-related disease



The patient, a 71-year-old man, presented with a one-month history of abdominal pain. Physical examination revealed slight tenderness of the left upper quadrant of the abdomen. Laboratory findings demonstrated elevated immunoglobulin (Ig) G4 (473 mg/dL) and amylase (201 U/L) levels; however, urinalysis, kidney function tests, and complement levels were normal. Moreover, blood cultures were negative, and autoantibodies were not detected. Contrast-enhanced computed tomography (CT) revealed an enlarged pancreas with a capsule-like rim, left-predominant lesions of perirenal homogeneous thickening (A), mild pericardial effusion, and circumferential infiltration of the ascending up to the descending aorta and iliac arteries. Histological examination of the perinephric soft tissues showed lymphoplasmacytic infiltration in the dense fibrosis region surrounding the fatty tissue (B) with an IgG4/IgG ratio of >50% (C and D) and >90 IgG4-positive cells/high-power field (D) without multinucleated giant cells and foamy histiocytes. Thus, we diagnosed IgG4-related disease (IgG4-RD), which mimics Erdheim-Chester disease (ECD). Three months after the initiation of prednisolone (40 mg/day), perirenal infiltration and pancreatic enlargement on CT imaging were ameliorated (E). Kidney lesions in IgG4-RD usually manifest as multiple low-density areas, bilateral diffuse enlargement, diffuse patchy involvement, rim-like lesions, or thickening of the renal pelvis.<sup>1</sup> They rarely involve perirenal fat lesions indicating hairy kidney, which is pathognomonic for ECD, a rare histiocytosis that affects multiple organs.<sup>2</sup> IgG4-RD and ECD can share several radiological features in the retroperitoneum and cardiovascular systems. Moreover, prominent IgG4 responses may be linked to the pathogenesis of ECD.<sup>3</sup> In contrast, pancreatic involvement is unusual in ECD. Thus, this case highlights the importance of comprehensive evaluation in challenging cases to differentiate IgG4-RD from ECD to guide treatment strategies. Written informed consent for publication was obtained from the patient.

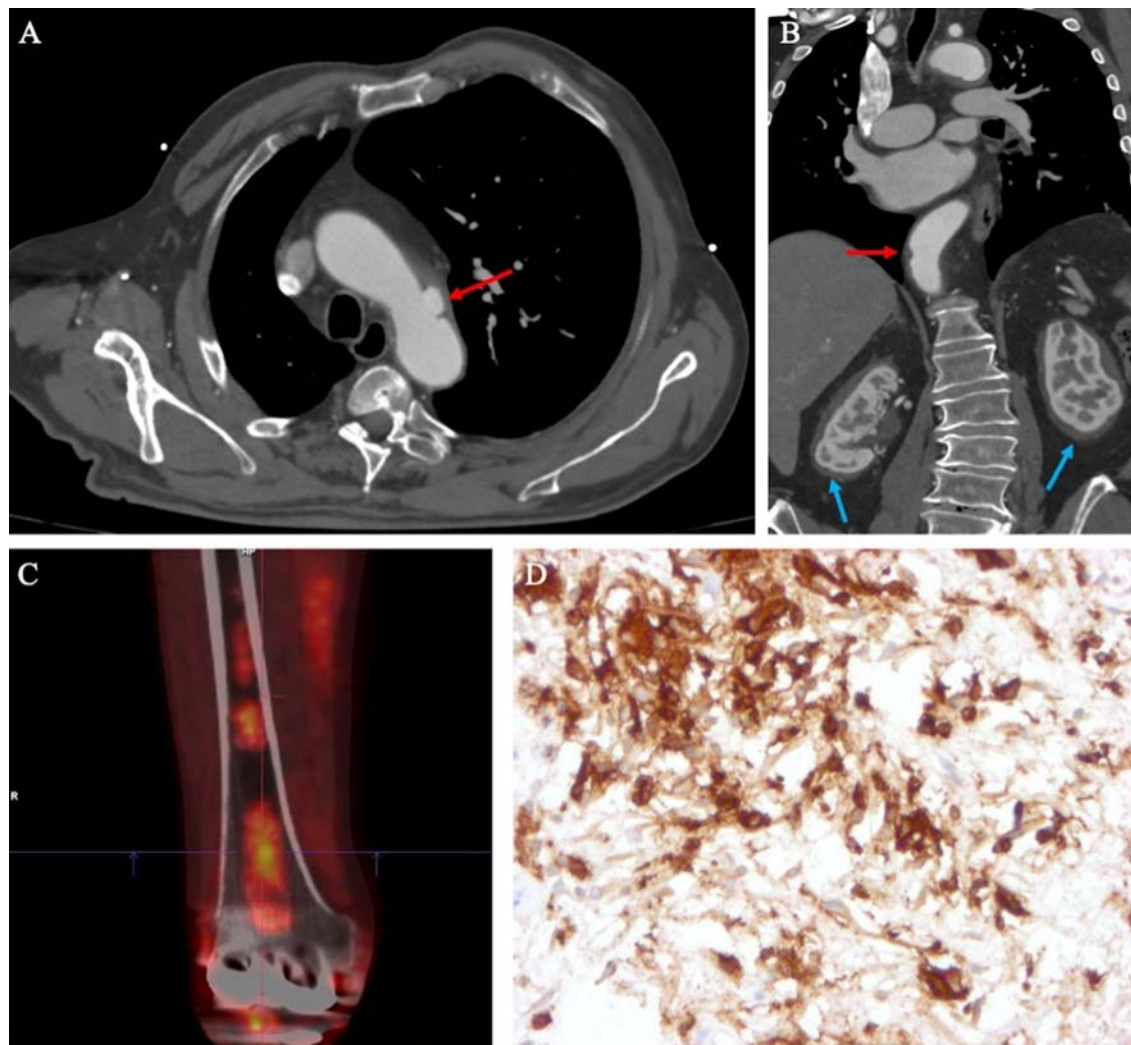
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1. Kawano M, Saeki T, Nakashima H. IgG4-related kidney disease and retroperitoneal fibrosis: an update. *Mod Rheumatol* 2019;29:231–239.
2. Triffo WJ, Dyer RB. The “hairy kidney” sign. *Abdominal Radiol* (NY) 2017;42:979–980.
3. Gianfreda D, Musetti C, Nicastro M, et al. Erdheim-Chester disease as a mimic of IgG4-related disease: a case report and a review of a single-center cohort. *Medicine* (Baltimore) 2016;95:e3625.

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**Clinical Images: Penetrating aortic ulceration as an initial presentation of Erdheim-Chester disease**

The patient, an 88-year-old man with a history of hypertension, was referred to the clinic after a computed tomography (CT) scan revealed a penetrating aortic ulcer during evaluation for intermittent chest pain. At the time of examination, he was asymptomatic, and his physical examination was unremarkable.

Laboratory tests demonstrated normal blood cell counts, no gammopathy, and unremarkable liver and renal function. Markers of inflammation, including C-reactive protein and fibrinogen, were within normal limits. Serologic tests for infectious diseases (HIV, syphilis, and viral hepatitis) were negative, as was screening for autoimmune-related diseases.

CT imaging confirmed the penetrating aortic ulcer (A; red arrow) and revealed vascular sheathing of the aorta with bilateral perirenal infiltrates (B; blue arrows). Doppler ultrasonography identified partial stenosis of the celiac trunk and the right renal artery. Given the high suspicion of malignancy, bone marrow aspiration was performed, showing no morphologic abnormalities. Next generation sequencing revealed mutations in *TET2* and *SF3B1*.

An 18-fluorodeoxyglucose positron emission tomography scan demonstrated hypermetabolic lesions in the long bones, consistent with osteosclerosis (C). Biopsy of the perirenal infiltrates showed fibrosis with a CD68+ and CD1a- histiocyte infiltrate (D), confirming Erdheim-Chester disease (ECD).<sup>1</sup> Molecular analysis did not detect mutations in the mitogen-activated pathway genes, including the absence of the *BRAF*<sup>V600E</sup> mutation. Because of significant vascular involvement, the patient was treated with the MEK inhibitor

cobimetinib.<sup>2</sup> Three months after initiating the kinase inhibitor, the patient developed severe lower limb edema and rhabdomyolysis, necessitating treatment withdrawal. Following these adverse events, the patient and their family declined further evaluation. Although this limited our ability to confirm the resolution of the ulcer with treatment, the absence of significant atherosclerosis remains a strong argument for the role of ECD in the genesis of the lesion.

*Author disclosures and graphical abstract are available at <https://onlinelibrary.wiley.com/doi/10.1002/art.43156>.*

1. Haroche J, Cohen Aubart F, Amoura Z. Erdheim-Chester disease. *Blood* 2020;135(16):1311–1318.
2. Goyal G, Heaney ML, Collin M, et al. Erdheim-Chester disease: consensus recommendations for the evaluation, diagnosis, and treatment in the molecular era. *Blood* 2020;135(22):1929–1945.

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## LETTER

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### Effect of the methotrexate discontinuation for 1 versus 2 weeks on vaccine response to seasonal influenza vaccine in rheumatoid arthritis: a noninferiority randomized controlled trial

To the Editor:

Patients with rheumatoid arthritis (RA) receiving methotrexate (MTX) have impaired vaccine responses (1). We showed previously that temporary discontinuation of MTX for 2 or 4 weeks after seasonal influenza vaccination can improve humoral vaccine response (2,3). To further minimize treatment interruption with MTX, we investigated whether holding MTX for 1 week after seasonal influenza vaccination is noninferior to holding it for 2 weeks. An interim analysis including the primary endpoints (ie, satisfactory vaccine response at 4 weeks after vaccination) of the study was published recently (4). Herein, we report vaccine responses and safety through the conclusion of the study.

In this open-label, prospective, two-arm, parallel-group, multicenter, randomized controlled noninferiority intervention trial, 184 patients with RA were randomized into 2 groups: the

1-week hold group (n = 92) and the 2-week hold group (n = 92) (Supplementary Figure S1, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42644>). Vaccine responses and safety were evaluated at baseline, 4 weeks, and 16 weeks after seasonal influenza vaccination. No modifications were made to the study protocol since the publication of the interim analysis (Supplementary Methods, available at <https://onlinelibrary.wiley.com/doi/10.1002/art.42644>) (4).

The proportion of satisfactory vaccine responses was comparable between the 1-week and 2-week hold groups at both 4 weeks (68.9% versus 75.0%,  $P = 0.364$ ; difference 6.1%, 95% confidence interval [95% CI]: −7.1 to 19.3) and 16 weeks (78.4% versus 79.1%,  $P = 0.915$ ; difference 0.6%, 95% CI: −11.5 to 12.8) with the difference not exceeding the predefined noninferiority margin of −20% points (Figure 1). The antibody titers against all 4 strains increased rapidly by 4 weeks and remained elevated above the protective level throughout 16 weeks after vaccination (Supplementary Table S1, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42644>). Sensitivity analyses

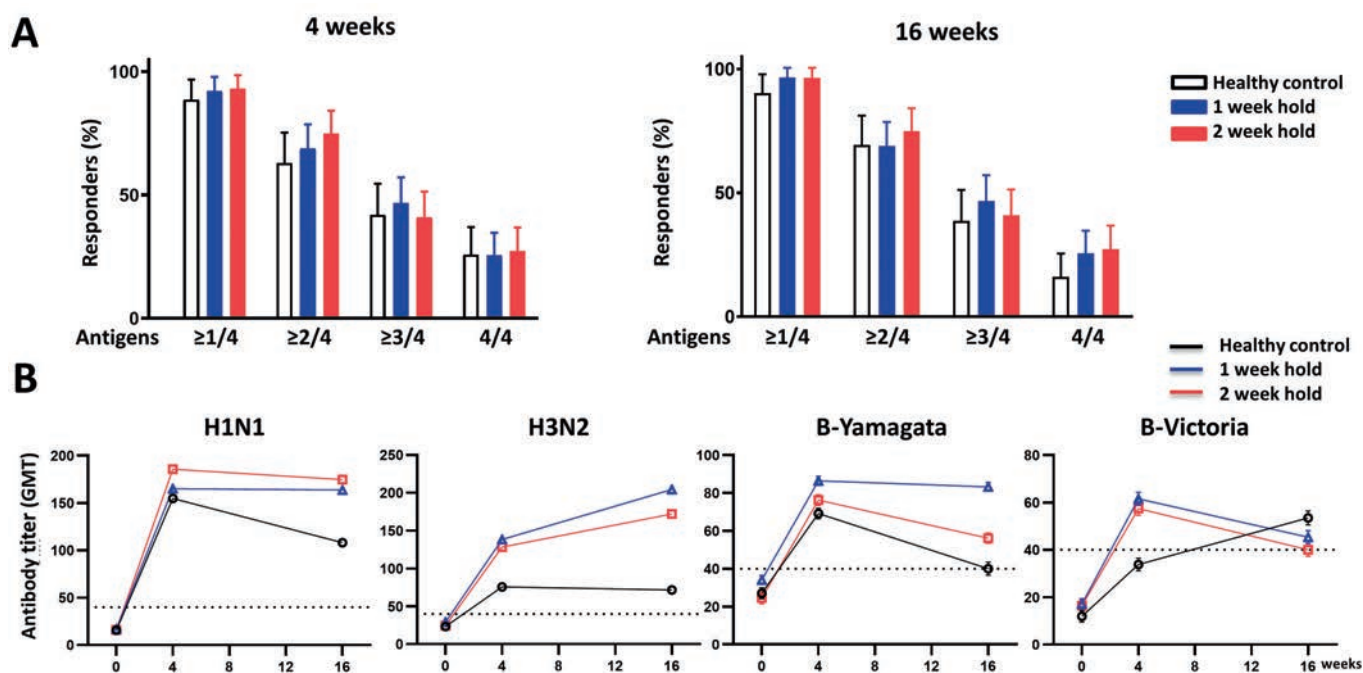


**Figure 1.** Vaccine response at 4 and 16 weeks (A) The percentage of patients achieving a satisfactory vaccine response, defined as  $\geq 4$ -fold increase in hemagglutination inhibition (HI) antibody titer 4 weeks and 16 weeks after vaccination against one or more, two or more vaccine, three or more, and four vaccine strains. Healthy control was included as a reference. The error bars represent the 95% confidence interval. The 1 week and 2 week MTX hold groups were compared by a chi-square test. Both groups did not differ in regards to vaccine response. (B) Antibody titer against each vaccine strains over time. The dotted line presents titer 1:40.

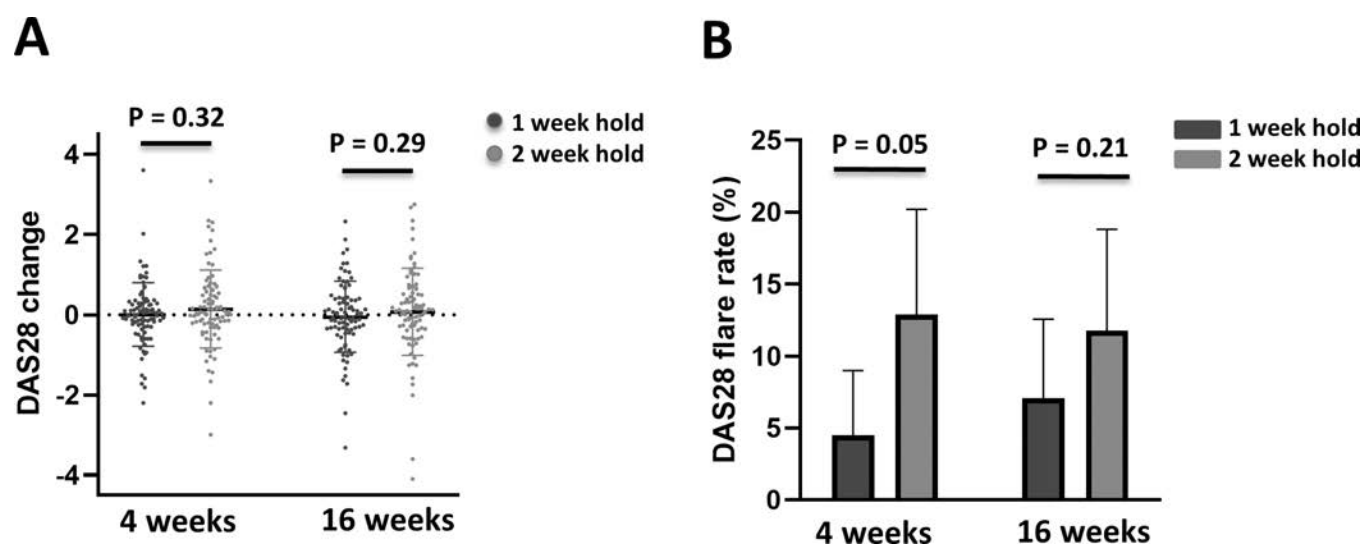


**Figure 2.** Disease activity at 4 weeks and 16 weeks. (A) DAS28 change from baseline. (B) Rate of RA disease flare. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.42644/abstract>.

using the per-protocol population showed similar findings (Supplementary Figure 2). In a subgroup analysis, no factor, including MTX dose, was found to favor either the 1-week or 2-week MTX hold (Supplementary Figure 3, available at <https://onlinelibrary.wiley.com/doi/10.1002/art.42644>).

The change in disease activity score-C-reactive protein from baseline did not differ between the 1-week hold and the 2-week hold group at 4 weeks ( $0.0 \pm 0.7$  versus  $0.1 \pm 0.8$ ,  $P = 0.365$ ) and 16 weeks ( $-0.1 \pm 0.9$  versus  $0.1 \pm 1.1$ ,  $P = 0.415$ ) (Figure 2). The flare rate did not differ between the 1-week hold group and the 2-week hold group at weeks 4 (4.5% versus 12.9%,  $P = 0.050$ ) and at 16 weeks (7.1% versus 11.8%,  $P = 0.293$ ). The vaccine was well tolerated. None of the patients developed influenza-like illness.

This study showed clearly that skipping MTX for 1 week is just as effective as skipping it for 2 weeks in producing a sustained (ie, for up to 16 weeks after vaccination) satisfactory vaccine response to seasonal influenza vaccination in patients with RA taking a stable dose of MTX without worsening disease activity (5). Therefore, patients should skip MTX for 1 or 2 weeks after vaccination. This simple and safe vaccination strategy can be easily implemented in routine clinical practice and help ensure adequate vaccination coverage in clinical practice without uncertainty about the safety and efficacy of vaccines in this patient population (6).

## ACKNOWLEDGMENTS

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decision. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit the study for publication. Dr. Lee has acted as a consultant to Pfizer and received research grants from Green Cross Corp. and Hanmi Pharmaceutical.

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1. Friedman MA, Curtis JR, Winthrop KL. Impact of disease-modifying antirheumatic drugs on vaccine immunogenicity in patients with inflammatory rheumatic and musculoskeletal diseases. *Ann Rheum Dis* 2021;80(10):1255–65.
2. Park JK, Lee YJ, Shin K, 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.
3. Park JK, Lee MA, Lee EY, 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.
4. Park JK, Lee YJ, Shin K, et al. A multicenter, prospective, randomized, parallel-group trial on the effects of temporary methotrexate discontinuation for one week versus two weeks on seasonal influenza vaccination in patients with rheumatoid arthritis. *Arthritis Rheumatol* 2023;75(2):171–7.
5. Park JK, Kim MJ, Choi Y, et al. Effect of short-term methotrexate discontinuation on rheumatoid arthritis disease activity: post-hoc analysis of two randomized trials. *Clin Rheumatol* 2020;39(2):375–9.
6. Seo P, Winthrop K, Sawalha AH, et al. Physician perspectives on vaccination in patients with autoimmune inflammatory rheumatic diseases: an international survey. *J Rheumatol*. 2023;50(2):246–51.

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### **Nociplastic pain: the wolf in sheep's clothing. Comment on the article by Sunzini et al**

*To the Editor:*

The recent article in *Arthritis & Rheumatology*, “The Biology of Pain: Through the Rheumatology Lens” by Sunzini et al, was largely focused on “nociplastic pain,” which seems to occur in many rheumatological conditions, including rheumatoid arthritis (RA), systemic lupus erythematosus, Sjögren's syndrome, osteoarthritis, back pain, etc (1). According to the authors, many patients with these conditions have pain out of proportion to detectable tissue changes. This phenomenon is well known and has previously been described as “fibromyalgianess” (2). Clinical criteria and a grading system for nociplastic pain were published by the International Association for the Study of Pain in 2021, and nociplastic pain was defined as “pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain” (3). The criteria for nociplastic pain are descriptive and similar to the 2016 Fibromyalgia (FM) Criteria, requiring a duration of >3 months and the presence of sleep abnormalities, fatigue, and cognitive difficulties (4). Similar to fibromyalgianess, nociplastic pain is a continuum ranging from mild to severe symptoms, and the most severe forms fulfill the classification criteria for FM. The authors speculate that nociplastic pain depends on altered nervous system responsiveness similar to that proposed in FM and other chronic pain syndromes. However, the proposed mechanisms of nociplastic pain are not based on empiric evidence but on assumptions of central nervous system

dysfunction. The authors provided some support for this hypothesis in patients with RA in whom nonspecific structural, biochemical, and functional brain abnormalities have been reported (5,6). We are concerned that the hypothesis of nociplastic pain mechanisms in patients with RA and other inflammatory joint diseases may result in the application of pharmacological therapies used for chronic pain syndromes such as FM. It is important to point out that pharmacological therapies for FM pain have been only marginally effective (7), and their usefulness for nociplastic RA pain will require careful study and cannot be assumed. Changing the term fibromyalgianess to nociplastic pain is just changing the wolf's clothing.

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1. Sunzini F, Schrepf A, Clauw DJ, et al. The biology of pain: through the rheumatology lens. *Arthritis Rheumatol* 2023;75(5):650–60.
2. Wolfe F. Fibromyalgianess. *Arthritis Rheum* 2009;61(6):715–6.
3. Kosek E, Clauw D, Nijs J, et al. Chronic nociplastic pain affecting the musculoskeletal system: clinical criteria and grading system. *Pain* 2021;162(11):2629–34.
4. Wolfe F, Walitt BT, Rasker JJ, et al. The Polysymptomatic Distress Scale is simple, useful, and effective in clinical care and clinical and epidemiology studies. *J Rheumatol* 2016;43(2):454.
5. Heisler AC, Song J, Muhammad LN, et al. Association of dysregulated central pain processing and response to disease-modifying antirheumatic drug therapy in rheumatoid arthritis. *Arthritis Rheumatol* 2020;72(12):2017–24.
6. Kaplan CM, Schrepf A, Ichesco E, et al. Association of inflammation with pronociceptive brain connections in rheumatoid arthritis patients with concomitant fibromyalgia. *Arthritis Rheumatol* 2020;72(1):41–6.
7. Moore RA, Fisher E, Häuser W, et al. Pharmacological therapies for fibromyalgia (fibromyalgia syndrome) in adults - an overview of Cochrane Reviews. *Cochrane Database Syst Rev* 2021;2021(8):CD013151.

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### **A randomized, double-blind, sham-controlled, clinical trial of auricular vagus nerve stimulation for the treatment of active rheumatoid arthritis: comment on the article by Baker et al**

*To the Editor:*

The use of bioelectronic devices to diagnose, monitor, and treat human disease continues to grow. We read the study of transcutaneous auricular vagus nerve stimulation (taVNS) in patients with rheumatoid arthritis by Baker et al<sup>1</sup> with much interest. The authors should be commended for conducting a

well-designed, sham-controlled study of 113 patients with active RA. Subjects received 15 minutes of stimulation (taVNS or sham) applied to the cymba concha, an area exclusively innervated by the auricular branch of the vagus nerve, daily for 12 weeks. This is the largest and longest sham controlled trial of taVNS. The study observed no significant differences in clinical response between participants receiving true and participants receiving sham stimulation. However, this finding may not be unexpected. The stimulation was applied at 20 kHz. This is approximately 1,000 times greater than the stimulation frequencies used in multiple other studies in which taVNS has been shown to be clinically effective. These include trials in long COVID, tinnitus, systemic lupus erythematosus (SLE), migraine headaches, erosive hand osteoarthritis, pediatric kidney disease, and pediatric inflammatory bowel disease.<sup>2–9</sup> Kilohertz frequency waveforms are used to block, not stimulate, conduction of neural activity.<sup>10–14</sup> Thus, it is not clear why these blocking signals were used in a study that purported to assess the effects of stimulating the auricular branch of the vagus nerve. This is a major departure from the prior, smaller studies that showed clinical benefit. The lack of an observed clinical benefit in this study does highlight other unknowns still existing in this emerging field. There remain open questions about optimum stimulation logistics, device setting, and therapy duration as well as the mechanisms by which taVNS exerts its effects. The focus now should be on designing studies to address these questions.

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1. Baker MC, Kavanagh S, Cohen S, et al. A randomized, double-blind, sham-controlled, clinical trial of auricular vagus nerve stimulation for the treatment of active rheumatoid arthritis. *Arthritis Rheumatol* 2023;75(12):2107–2115.
2. Badran BW, Huffman SM, Dancy M, et al. A pilot randomized controlled trial of supervised, at-home, self-administered transcutaneous auricular vagus nerve stimulation (taVNS) to manage long COVID symptoms. *Bioelectron Med* 2022;8(1):13.
3. Verbanck P, Clarinval AM, Burton F, et al. Transcutaneous auricular vagus nerve stimulation (tvns) can reverse the manifestations of the Long-COVID Syndrome: a pilot study. *Front Neurol Neurosci Res* 2021;2:1–13.
4. Chen S, Du M, Wang Y, et al. State of the art: non-invasive electrical stimulation for the treatment of chronic tinnitus. *Ther Adv Chronic Dis* 2023;14:20406223221148061.

5. Aranow C, Atish-Fregoso Y, Lesser M, et al. Transcutaneous auricular vagus nerve stimulation reduces pain and fatigue in patients with systemic lupus erythematosus: a randomised, double-blind, sham-controlled pilot trial. *Ann Rheum Dis* 2021;80(2):203–208.
6. Straube A, Ellrich J, Eren O, et al. Treatment of chronic migraine with transcutaneous stimulation of the auricular branch of the vagal nerve (auricular t-VNS): a randomized, monocentric clinical trial. *J Headache Pain* 2015;16:543.
7. Courties A, Deprouw C, Maheu E, et al. Effect of transcutaneous vagus nerve stimulation in erosive hand osteoarthritis: results from a pilot trial. *J Clin Med* 2022;11(4):1087.
8. Merchant K, Zanos S, Datta-Chaudhuri T, et al. Transcutaneous auricular vagus nerve stimulation (taVNS) for the treatment of pediatric nephrotic syndrome: a pilot study. *Bioelectron Med* 2022;8(1):1.
9. Sahn B, Pascuma K, Tracey K, et al. P072 non-invasive vagal nerve stimulation to treat crohn disease and ulcerative colitis in children and young adults: a proof-of-concept clinical trial. *Am J Gastroenterol* 2021;116(Suppl 1):S19.
10. Patel YA, Butera RJ. Differential fiber-specific block of nerve conduction in mammalian peripheral nerves using kilohertz electrical stimulation. *J Neurophysiol* 2015;113(10):3923–3929.
11. Bhadra N, Kilgore KL. High-frequency electrical conduction block of mammalian peripheral motor nerve. *Muscle Nerve* 2005;32(6):782–790.
12. Ling D, Luo J, Wang M, et al. Kilohertz high-frequency alternating current blocks nerve conduction without causing nerve damage in rats. *Ann Transl Med* 2019;7(22):661.
13. Avendano-Coy J, Serrano-Munoz D, Taylor J, et al. Peripheral nerve conduction block by high-frequency alternating currents: a systematic review. *IEEE Trans Neural Syst Rehabil Eng* 2018;26(6):1131–1140.
14. Alvarez DM, Serrano-Munoz D, Fernandez-Perez JJ, et al. Effect of percutaneous electrical stimulation with high-frequency alternating currents at 30 kHz on the sensory-motor system. *Front Neurosci* 2023;17:1048986.

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## Reply

*To the Editor:*

We thank Dr Aranow and colleagues for their interest in our study of transcutaneous auricular vagus nerve stimulation (taVNS) for the treatment of rheumatoid arthritis (RA).<sup>1</sup> We appreciate their comments about high-frequency (HF) nerve stimulation and the concern that HF stimulation may result in nerve block. Prior studies using animal models and computational modeling have demonstrated that directly contacting the nerve and stimulating at a high intensity are both critical for achieving block.<sup>2–4</sup> This is further supported by human studies using transcutaneous peripheral nerve stimulation, in which stimulation with substantially higher amplitudes than what was used in our study was needed to achieve block.<sup>5–7</sup> Furthermore, when HF stimulation results in nerve block, an “onset response” precedes the block, inducing activation and resulting in symptoms due to transient, but strong, neural activity.<sup>3</sup> In our study, we used a taVNS device that delivered 20 kHz frequency at low amplitudes without direct contact with the vagus nerve and without inducing an onset response. Lastly, in functional magnetic resonance imaging studies using

low amplitude, HF taVNS, healthy volunteers experienced changes in brain activity consistent with vagus nerve stimulation without side effects or signs of block.<sup>8,9</sup>

More importantly, our initial open-label pilot study in patients with RA using the same stimulation parameters demonstrated efficacy, and the device was well received by patients without any stimulation side effects.<sup>10</sup> When the same device and stimulation parameters were studied in the current randomized, double-blinded, sham-controlled trial, the results were negative. Given this notable discordance, we hope our study highlights the need for larger, controlled trials of taVNS, and we agree that future research should address how stimulation parameters such as frequency and duration impact the response.

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2. Bhadra N, Kilgore KL. High-frequency electrical conduction block of mammalian peripheral motor nerve. *Muscle Nerve* 2005;32(6):782–790.
3. Kilgore KL, Bhadra N. Reversible nerve conduction block using kilohertz frequency alternating current. *Neuromodulation* 2014;17(3):242–255.
4. Lempka SF, McIntyre CC, Kilgore KL, et al. Computational analysis of kilohertz frequency spinal cord stimulation for chronic pain management. *Anesthesiology* 2015;122(6):1362–1376.
5. Kim Y, Cho HJ, Park HS. Technical development of transcutaneous electrical nerve inhibition using medium-frequency alternating current. *J Neuroeng Rehabil* 2018;15(1):80.
6. Serrano-Muñoz D, Avendaño-Coy J, Simón-Martínez C, et al. Effect of high-frequency alternating current transcutaneous stimulation over muscle strength: a controlled pilot study. *J Neuroeng Rehabil* 2018;15(1):103.
7. Serrano-Muñoz D, Avendaño-Coy J, Simón-Martínez C, et al. 20-kHz alternating current stimulation: effects on motor and somatosensory thresholds. *J Neuroeng Rehabil* 2020;17(1):22.
8. Mao Y, Chen C, Falahpour M, et al. Effects of sub-threshold transcutaneous auricular vagus nerve stimulation on cingulate cortex and insula resting-state functional connectivity. *Front Hum Neurosci* 2022;16:862443.
9. Chen C, Chen C, Falahpour M, et al. Effects of sub-threshold transcutaneous auricular vagus nerve stimulation on cerebral blood flow. *Sci Rep* 2021;11(1):24018.
10. Marsal S, Corominas H, de Agustín JJ, et al. Non-invasive vagus nerve stimulation for rheumatoid arthritis: a proof-of-concept study. *Lancet Rheumatol* 2021;3(4):E262–E269.

DOI 10.1002/art.42751

### Genetic data do not provide evidence that lower sclerostin is associated with increased risk of atherosclerosis: comment on the article by Zheng et al

To the Editor:

Zheng et al reported an association between lower sclerostin and atherosclerosis and its risk factors using Mendelian randomization (MR).<sup>1</sup> Of particular interest is the MR analysis using five genetic variants in *cis* of *SOST* (the gene encoding sclerostin) that are associated with circulating sclerostin, as this may mimic the direct effects of therapeutic sclerostin inhibition.<sup>2</sup> The *cis+trans* MR analysis presented is less relevant in this regard given the potential for horizontal pleiotropic effects to be introduced through the inclusion of other genes.<sup>2</sup>

Following the initial online publication in *Arthritis & Rheumatology*, the authors have rectified the inflated *cis* MR results they originally reported.<sup>3</sup> However, the article's title and conclusions have not been amended to reflect these revised results. This is difficult to justify because none of the revised results from the *cis* MR analysis are significant using the authors' original multiple testing corrected *P* threshold of 0.00515 ( $\min(P) = 0.030$ , 15 phenotypes), which they appropriately applied in the initial publication but not after updating their analysis. Multiple testing corrections are essential when performing many tests to control false positive rates, particularly when analyzing genetic data due to its inherent multiplicity.<sup>4</sup> The authors' decision not to correct for multiple testing in the final publication is hard to defend, especially as they highlight its absence as a limitation of their study. Further, the way triangulation was used in relation to myocardial infarction is insufficient given the inconsistent findings from clinical trials for cardiovascular events.<sup>5</sup>

We performed additional genetic analyses using the same available datasets (or equivalent) as the authors. Outside of the known genetic signal with some lipid measures in the *SOST* region, attributed to the nearby *CD300LG* gene,<sup>6</sup> there was no evidence that this region is associated with any of the other phenotypes. For instance, the associations with myocardial infarction resemble statistical noise as opposed to signal (Figure 1A and B). After accounting for the effects of *CD300LG* in the associated lipids, the individual associations of the five sclerostin-associated variants with these phenotypes were in-line with what might be expected by chance (two associations with  $P < 0.05$  from 58 tests,  $\min(P) = 0.036$ ). These variants were also not associated with type 2 diabetes, coronary artery disease, and stroke outcomes (individually or combined) in recent large genetic studies ( $P > 0.05$ ).<sup>7–9</sup> Furthermore, a *cis* MR analysis using a less correlated variant set showed no evidence of association with the atherosclerosis-related diseases and risk factors tested (Figure 1C and D,  $P > 0.05$ ).

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More importantly, our initial open-label pilot study in patients with RA using the same stimulation parameters demonstrated efficacy, and the device was well received by patients without any stimulation side effects.<sup>10</sup> When the same device and stimulation parameters were studied in the current randomized, double-blinded, sham-controlled trial, the results were negative. Given this notable discordance, we hope our study highlights the need for larger, controlled trials of taVNS, and we agree that future research should address how stimulation parameters such as frequency and duration impact the response.

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We performed additional genetic analyses using the same available datasets (or equivalent) as the authors. Outside of the known genetic signal with some lipid measures in the *SOST* region, attributed to the nearby *CD300LG* gene,<sup>6</sup> there was no evidence that this region is associated with any of the other phenotypes. For instance, the associations with myocardial infarction resemble statistical noise as opposed to signal (Figure 1A and B). After accounting for the effects of *CD300LG* in the associated lipids, the individual associations of the five sclerostin-associated variants with these phenotypes were in-line with what might be expected by chance (two associations with  $P < 0.05$  from 58 tests,  $\min(P) = 0.036$ ). These variants were also not associated with type 2 diabetes, coronary artery disease, and stroke outcomes (individually or combined) in recent large genetic studies ( $P > 0.05$ ).<sup>7–9</sup> Furthermore, a *cis* MR analysis using a less correlated variant set showed no evidence of association with the atherosclerosis-related diseases and risk factors tested (Figure 1C and D,  $P > 0.05$ ).

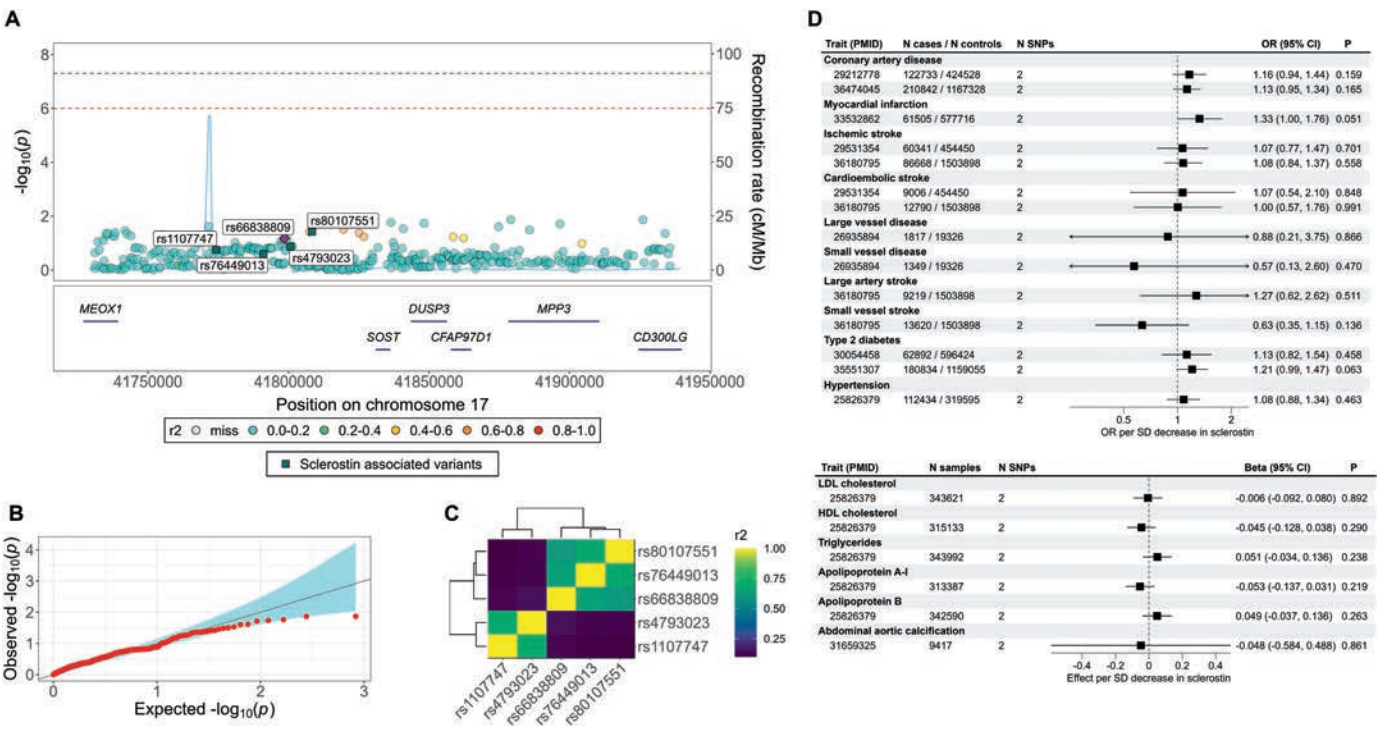


**Figure 1.** Genetic associations of myocardial infarction (MI) in the SOST region and a *cis* Mendelian randomization (MR) analysis including one sclerostin-associated variant from each correlation cluster. **A** Regional association plot of the  $-\log_{10}(P)$  of MI for the SOST region ( $\pm 100\text{KB}$  either side of SOST), the red and green dashed lines are the  $P$  thresholds  $1 \times 10^{-6}$  (regional-wide significance threshold) and  $5 \times 10^{-8}$  (genome-wide significance threshold), respectively. **B** Quantile-quantile plot for the  $-\log_{10}(P)$  of MI from the SOST region, the blue shaded area represents the 95% confidence interval. **C** Linkage disequilibrium plot of the correlations between the five *cis* sclerostin-associated variants (rs66838809, rs1107747, rs4793023, rs80107551, and rs76449013). **D** Forest plot of the *cis* MR analysis using the two variants most associated with sclerostin with  $r^2 < 0.2$  (rs66838809 and rs1107747, or a proxy variant when unavailable), Bonferroni corrected  $P$  threshold = 0.00515. The associations of rs1107747 and rs4793023 with HDL cholesterol and triglycerides have been adjusted for rs72836567 (a variant associated with expression of CD300LG).<sup>3</sup> Since the per SD sclerostin MR associations have been extrapolated from small per allele effects with the outcomes (i.e., odds ratios close to 1 and effect sizes close to 0), caution is required when interpreting these odds ratios and effect sizes. The colocalization results are available in Supplementary Table 10,<sup>1</sup> the probability of colocalization is depicted as PP.H4.abf. Note: the ischemic and cardioembolic stroke results from METASTROKE were replaced with those from MEGASTROKE; the UK Biobank hypertension results from OpenGWAS were replaced with those from Pan-UKBB; and the coronary artery calcification results were not available via application at the time of this analysis.<sup>1</sup> Analyses using sclerostin-associated variants from UK Biobank also yielded similar results (data shown in code book). The code book used to perform these analyses is available at [https://jrs95.github.io/sclerostin\\_pqtl\\_mr](https://jrs95.github.io/sclerostin_pqtl_mr). CI, confidence interval; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OR, odds ratio; Prob., probability; SD, standard deviation; SNP, single-nucleotide polymorphism.

It is recommended that *cis* MR studies are accompanied by colocalization analyses to assess genetic signal overlap to avoid false positive findings like those from candidate gene studies.<sup>10</sup> These analyses were performed by the authors (but not discussed) and indicate that an overlapping genetic signal between circulating sclerostin and these phenotypes is unlikely in the SOST region (*Prob.* < 2.5%, Supplementary Table 10).<sup>1</sup> In summary, contrary to the authors' claims, these genetic data do not provide evidence that lower levels of sclerostin increase the risk of atherosclerosis and its risk factors.

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**ROLE OF THE STUDY SPONSOR**

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1. Zheng J, Wheeler E, Pietzner M, et al. Lowering of circulating sclerostin may increase risk of atherosclerosis and its risk factors: evidence from a genome-wide association meta-analysis followed by Mendelian randomization. *Arthritis Rheumatol* 2023;75:1781–1792.
2. Schmidt AF, Finan C, Gordillo-Maranon M, et al. Genetic drug target validation using Mendelian randomisation. *Nat Commun* 2020;11:3255.
3. Zheng J, Wheeler E, Pietzner M, et al. Lowering of circulating sclerostin may increase risk of atherosclerosis and its risk factors: evidence from a genome-wide association meta-analysis followed by Mendelian randomization. *medRxiv* Posted online June 16, 2022. <https://doi.org/10.1101/2022.06.13.22275915>.
4. Panagiotou OA, Ioannidis JP; Genome-Wide Significance Project. What should the genome-wide significance threshold be? Empirical replication of borderline genetic associations. *Int J Epidemiol* 2012; 41:273–286.
5. Holm H, Sulem P, Tragante V, et al. Comment on “Evaluating the cardiovascular safety of sclerostin inhibition using evidence from meta-analysis of clinical trials and human genetics”. *Sci Transl Med* 2021; 13:eabe8497.
6. Surakka I, Horikoshi M, Magi R, et al. The impact of low-frequency and rare variants on lipid levels. *Nat Genet* 2015;47:589–597.
7. Aragam KG, Jiang T, Goel A, et al. Discovery and systematic characterization of risk variants and genes for coronary artery disease in over a million participants. *Nat Genet* 2022;54:1803–1815.
8. Mahajan A, Spracklen CN, Zhang W, et al. Multi-ancestry genetic study of type 2 diabetes highlights the power of diverse populations for discovery and translation. *Nat Genet* 2022;54:560–572.
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## Reply

To the Editor:

Staley et al point out that, in our recent article,<sup>1</sup> the strength of evidence for the causal effect of sclerostin lowering on increased myocardial infarction (MI) risk we reported was relatively weak and below the Bonferroni adjusted evidence threshold, raising the possibility of a spurious finding. However, absence of evidence does not imply evidence of absence. Contrary to their assertion, triangulation of evidence from other sources points to a similar conclusion. In terms of experimental data, up-regulation of sclerostin inhibited

the development of atherosclerosis in a mouse model,<sup>2</sup> and *SOST* knockout mice developed more extensive vascular calcification.<sup>3</sup> Furthermore, two previous randomized controlled trials (RCTs) identified an increased risk of cardiovascular disease (CVD) events following romosozumab,<sup>4,5</sup> and a meta-analysis of five trials found evidence of increased risk of a composite CVD outcome.<sup>6</sup> However, such trials suggest that any increased MI risk from sclerostin lowering is relatively modest and substantially smaller than the gain in bone mineral density (BMD) underpinning its therapeutic action. This has implications for the statistical power of Mendelian randomization (MR) analyses, which is further compounded by the fact that common *SOST* variants only have modest effects on circulating sclerostin (no genome-wide significant signals in the *SOST* gene encoding sclerostin were identified in our initial genome-wide association study (GWAS) based on approximately 10,000 individuals<sup>7</sup>).

Data from SomaLogic and O-link proteomic platforms, which both include sclerostin, have recently been made publicly available from DeCode<sup>8</sup> and UK Biobank,<sup>9</sup> respectively, providing an opportunity to repeat our analyses based on 100,482 individuals, giving considerably greater statistical power ( $N_{\text{Zheng}} = 31,491$ ;  $N_{\text{UK\_Biobank}} = 33,628$ ; and  $N_{\text{DeCode}} = 35,361$ ). A large-scale analysis combining these additional data with those in our recent article<sup>1</sup> identified two conditionally independent signals in the *cis* *SOST* region. The top hit rs66838809 was the same single-nucleotide polymorphism (SNP) as we reported in our largest published GWAS,<sup>1</sup> and this showed a stronger signal in the updated meta-analysis (Table 1). The secondary hit rs1107748 also showed robust association with sclerostin ( $P = 4.9 \times 10^{-20}$ ; Table 1) and is in perfect linkage disequilibrium (LD) with one of five *cis*-protein quantitative trait loci (pQTL) instruments (rs1107747) we used in our paper.<sup>1</sup> Using these two SNPs as instruments, we conducted an MR analysis of protein levels of sclerostin on 15 atherosclerosis-related outcomes by applying generalized inverse variance weighted, which accounts for weak LD between the two instruments (LD  $r^2 = 0.13$  in the 1000 Genome Europeans). We found robust MR evidence that lower sclerostin levels increase the risk of coronary artery disease<sup>10</sup> (CAD; odds ratio 1.50, 95% confidence interval [CI] 1.15–1.93,  $P = 0.002$ ) (Figure 1A). The CAD result withstands the Bonferroni corrected threshold ( $P = 0.0052$ ) after accounting for correlations among the 15 outcomes. CAD is closely related to MI but more

**Table 1.** Genetic signals of the two conditional independent SNPs associated with plasma protein levels of sclerostin in the *cis*-*SOST* region\*

| Phenotype  | SNP                    | Effect allele | Other allele | Effect allele freq | Beta   | SE    | P value               | N       | Note            |
|------------|------------------------|---------------|--------------|--------------------|--------|-------|-----------------------|---------|-----------------|
| Sclerostin | rs66838809             | A             | G            | 0.087              | −0.080 | 0.008 | $1.7 \times 10^{-24}$ | 100,482 | first signal    |
| Sclerostin | rs1107748              | T             | C            | 0.615              | 0.041  | 0.004 | $4.9 \times 10^{-20}$ | 100,482 | second signal   |
| Sclerostin | rs9910625 <sup>a</sup> | T             | C            | 0.352              | −0.042 | 0.005 | $7.0 \times 10^{-20}$ | 100,482 | second LD proxy |

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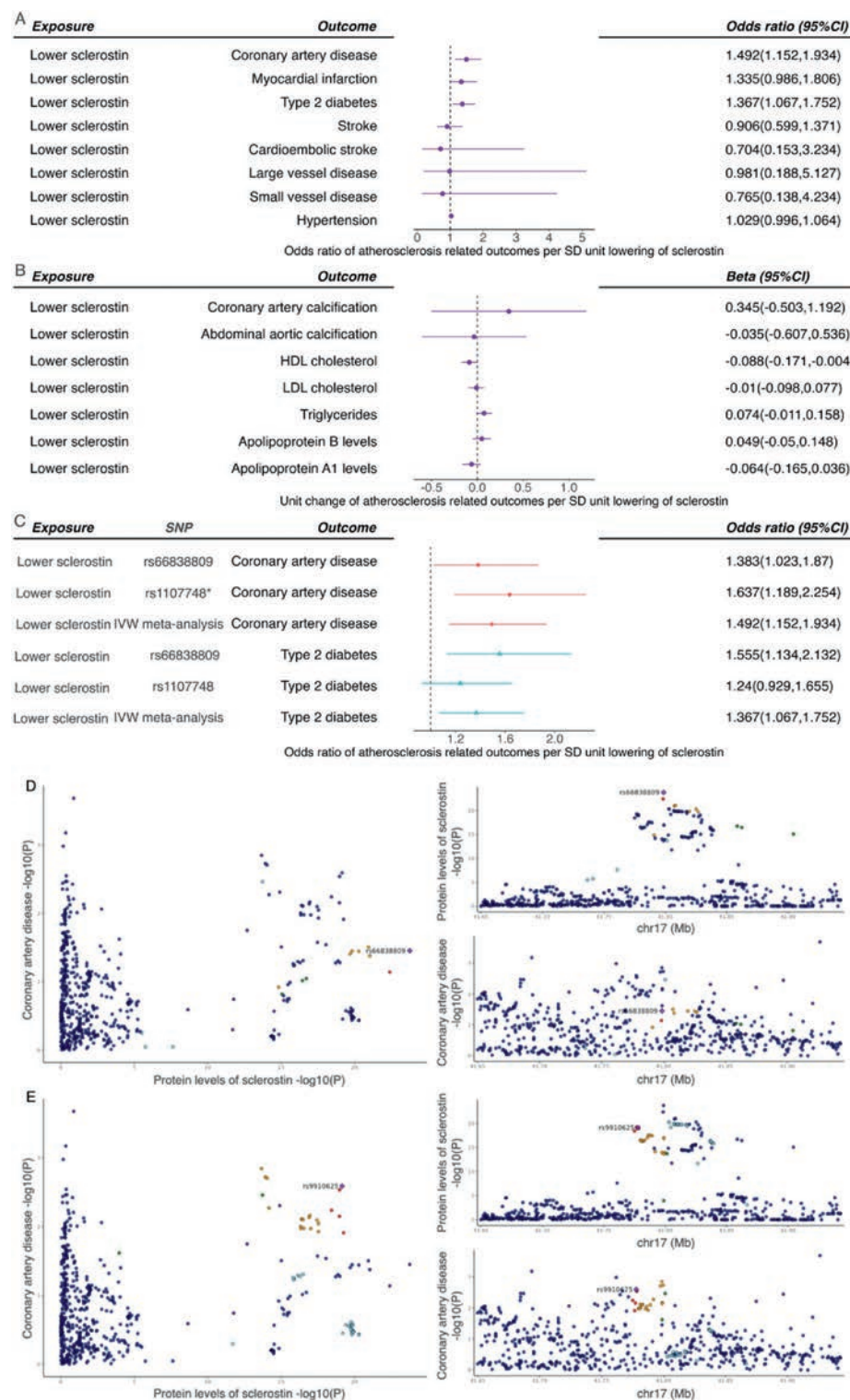


**Figure 1.** Effect of lower sclerostin on 15 atherosclerosis related outcomes. (A) Effect of lower sclerostin on disease outcomes; (B) effect of lower sclerostin on continuous outcomes; (C) single SNP effect of lower sclerostin on coronary artery disease and type 2 diabetes. \*rs1107748 was proxied by rs9910625 (LD  $r^2 = 0.9$ ) due to missing SNPs in the CAD GWAS. (D) Locus comparison plot of sclerostin versus CAD for the top hit rs66838809 in the SOST region. (E) Locus comparison plot of sclerostin versus CAD for the secondary hit rs9910625 in the SOST region. The three subplots for panels D and E are  $-\log_{10}(P)$  value of sclerostin versus the outcome, as well as the regional plot of sclerostin and outcomes in the *cis*-SOST region. CAD, coronary artery disease; CI, confidence interval; GWAS, genome-wide association study; HDL, high-density lipoprotein; IVW, inverse variance weighted; LD, linkage disequilibrium; LDL, low-density lipoprotein; SNP, single-nucleotide polymorphism.

inclusive because it also includes individuals undergoing related procedures such as angioplasty. In addition, marginal predicted effects of lower sclerostin levels on type 2 diabetes mellitus<sup>11</sup> and high-density lipoprotein cholesterol<sup>12</sup> were observed (Figure 1B). The point estimate for an effect on myocardial infarction<sup>13</sup> was very similar to that previously reported<sup>1</sup>; however, in this instance, the lower 95% CI included unity (Figure 1A).

In terms of why a robust effect of sclerostin on CAD was now evident, the new secondary pQTL signal, rs1107748, showed a higher point estimate for an effect on CAD risk compared with the top hit (Figure 1C). The locus plot for CAD associations in the *SOST* region was also consistent with two distinct *cis*-pQTL signals for sclerostin, with the secondary pQTL signal associated with a higher CAD risk estimate compared with the top pQTL (Figure 1D and 1E). The evidence provided by colocalization remains weak, however, with SharePro<sup>14</sup> (an approach that can be applied when a region contains multiple signals) yielding a 13.2% probability that the second sclerostin signal co-localizes with CAD. Power for this test in the context of the modest effect size compared to that on BMD is low, however.

In summary, these additional analyses identified multiple *cis*-pQTL signals in the *SOST* region, which together show a clear albeit weak effect of sclerostin lowering on CAD risk. Taken together with evidence from other domains, these findings run contrary to the assertion by Staley et al that this region has “no” association with any other phenotypes beyond BMD. In terms of the clinical significance of our findings, more data are required as to the propensity of sclerostin inhibition to increase cardiovascular risk, including findings from RCTs with other sclerostin inhibitors.

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1. Zheng J, Wheeler E, Pietzner M, et al. Lowering of circulating sclerostin may increase risk of atherosclerosis and its risk factors: evidence from a genome-wide association meta-analysis followed by Mendelian randomization. *Arthritis Rheumatol* 2023;75(10):1781–1792.
2. Krishna SM, Seto SW, Jose RJ, et al. Wnt signaling pathway inhibitor sclerostin inhibits angiotensin II-induced aortic aneurysm and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2017;37(3):553–566.
3. De Mare A, Opdebeeck B, Neven E, et al. Sclerostin protects against vascular calcification development in mice. *J Bone Miner Res* 2022;37(4):687–699.
4. Lewiecki EM, Blicharski T, Goemaere S, et al. A phase III randomized placebo-controlled trial to evaluate efficacy and safety of romosozumab in men with osteoporosis. *J Clin Endocrinol Metab* 2018;103(9):3183–3193.
5. Saag KG, Petersen J, Brandi ML, et al. Romosozumab or alendronate for fracture prevention in women with osteoporosis. *N Engl J Med* 2017;377(15):1417–1427.
6. Lv F, Cai X, Yang W, et al. Denosumab or romosozumab therapy and risk of cardiovascular events in patients with primary osteoporosis: systematic review and meta-analysis. *Bone* 2020;130:115121.
7. Zheng J, Maerz W, Gergei I, et al. Mendelian randomization analysis reveals a causal influence of circulating sclerostin levels on bone mineral density and fractures. *J Bone Miner Res* 2019;34(10):1824–1836.
8. Ferkingstad E, Sulem P, Atlason BA, et al. Large-scale integration of the plasma proteome with genetics and disease. *Nat Genet* 2021;53(12):1712–1721.
9. Sun BB, Chiou J, Traylor M, et al. Plasma proteomic associations with genetics and health in the UK Biobank. *Nature* 2023;622(7982):329–338.
10. Aragam KG, Jiang T, Goel A, et al. Discovery and systematic characterization of risk variants and genes for coronary artery disease in over a million participants. *Nat Genet* 2022;54(12):1803–1815.
11. Xue A, Wu Y, Zhu Z, et al. Genome-wide association analyses identify 143 risk variants and putative regulatory mechanisms for type 2 diabetes. *Nat Commun* 2018;9(1):2941.
12. Sakaue S, Kanai M, Tanigawa Y, et al. A cross-population atlas of genetic associations for 220 human phenotypes. *Nat Genet* 2021;53(10):1415–1424.
13. Hartiala JA, Han Y, Jia Q, et al. Genome-wide analysis identifies novel susceptibility loci for myocardial infarction. *Eur Heart J* 2021;42(9):919–933.
14. Zhang W, Lu T, Sladek R, et al. SharePro: an accurate and efficient genetic colocalization method accounting for multiple causal signals. *bioRxiv* Published online July 26, 2023. <https://doi.org/10.1101/2023.07.24.550431>.

DOI 10.1002/art.43131

**Systemic lupus erythematosus and atherosclerosis progression risk: comment on the article by Papazoglou et al.**

*To the Editor:*

We have read the article published by Papazoglou et al<sup>1</sup> with great interest. The authors found that patients with systemic lupus erythematosus (SLE) had a 2.3-fold higher 10-year atherosclerosis progression risk compared to healthy controls.

Lupus nephritis manifests in approximately 30% to 60% of all patients with SLE during their disease course.<sup>2</sup> The presence of microalbuminuria, resulting from glomerular damage, is a hallmark feature of lupus nephritis.<sup>2</sup> The presence of microalbuminuria has been linked to an increased risk of cardiovascular (CV) events owing to its potential to contribute to the development of atherosclerosis by causing endothelial dysfunction. Therefore, it would be better if the microalbuminuria levels of the patients in the present study had also been evaluated.

Clonal hematopoiesis of indeterminate potential (CHIP) refers to the clonal expansion of hematopoietic cells driven by somatic mutations in genes commonly associated with hematologic malignancies.<sup>3</sup> CHIP has been observed in patients with SLE, although its prevalence and clinical significance in this population are still unclear.<sup>4</sup> CHIP has been strongly linked to increased CV events in many studies.<sup>3,4</sup> Some CHIP mutations have been shown to promote a proinflammatory phenotype in clonal hematopoietic cells. The presence of mutated hematopoietic stem and progenitor cells results in accelerated production of myeloid cells, which in turn secrete elevated levels of inflammatory cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and tumor necrosis factor  $\alpha$ .<sup>3</sup> The development and progression of atherosclerosis is significantly influenced by chronic inflammation. In addition, this inflammatory environment contributes to the destabilization of atherosclerotic plaques and increases the risk of rupture and acute CV events, such as myocardial infarction or stroke. A seminal study published in 2017 demonstrated that individuals with CHIP had a two-fold increase in the risk of coronary artery disease and ischemic stroke compared to those without CHIP, independent of traditional cardiovascular risk factors.<sup>5</sup> Despite the challenges in evaluating CHIP, it is crucial to acknowledge that CHIP may also contribute to elevated CV risk in patients with SLE. A multitude of factors associated with the incidence of CV events in patients with SLE have been identified. Consequently, these factors should be considered when interpreting the study's findings.

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1. Papazoglou N, Sfikakis PP, Tektonidou MG. Atherosclerotic plaque progression and incident cardiovascular events in a 10-year prospective study of patients with systemic lupus erythematosus: the impact of persistent cardiovascular risk factor target attainment and sustained DORIS remission. *Arthritis Rheumatol* 2025;77(6):716–726. <https://doi.org/10.1002/art.43097>
2. Anders HJ, Saxena R, Zhao MH, et al. Lupus nephritis. *Nat Rev Dis Primers* 2020;6:7.
3. Marnell CS, Bick A, Natarajan P. Clonal hematopoiesis of indeterminate potential (CHIP): linking somatic mutations, hematopoiesis, chronic inflammation and cardiovascular disease. *J Mol Cell Cardiol* 2021; 161:98–105.
4. David C, Duployez N, Eloy P, et al. Clonal haematopoiesis of indeterminate potential and cardiovascular events in systemic lupus erythematosus (HEMATOPLUS study). *Rheumatology (Oxford)* 2022;61(11): 4355–4363.
5. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N Engl J Med* 2017;377(2): 111–121.

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**Reply**

*To the Editor:*

We read with interest the comments by Drs. Sener YZ and Sener S on our article entitled “Atherosclerotic plaque progression and incident cardiovascular events in a 10-year prospective study of patients with systemic lupus erythematosus: the impact of persistent cardiovascular risk factor target attainment and sustained DORIS remission.”<sup>1</sup> The authors reported that because microalbuminuria is a key feature of lupus nephritis (LN) and has been associated with cardiovascular disease (CVD) events in the general population, it should have been evaluated in our 10-year plaque progression and incident CVD events study.<sup>1</sup> Indeed, microalbuminuria is recognized as a predictor of CVD in adults, particularly among patients with diabetes, essential hypertension, and renal impairment.<sup>2</sup> However, in systemic lupus erythematosus (SLE), “total” proteinuria (protein level >500 mg/24 hours) rather than microalbuminuria (albumin level 30–300 mg/24 hours) is a hallmark manifestation of LN and is the primary laboratory test for LN diagnosis, as well as a major predictor of renal outcomes.<sup>3</sup> Thus, our study did not test microalbuminuria for LN diagnosis or assessment of its outcomes, or for CVD risk assessment, given that previous evidence identified LN history and associated chronic kidney disease, but not microalbuminuria, as CVD risk factors in SLE.<sup>4</sup> We tested both LN diagnosis and estimated glomerular filtration rate as potential predictors, but we have not observed any association with atherosclerotic plaque progression in the 10-year follow-up study<sup>1</sup> or in our previous 3- and 7-year follow-up studies.<sup>5</sup> Also, no association between LN and incident CVD events was found, but the small number of events developed over 10 years may limit the power of this analysis.

The authors also commented on the potential impact of clonal hematopoiesis of indeterminate potential (CHIP) on the increased CVD risk in patients with SLE. We agree that CVD pathogenesis in SLE is multifactorial, and several factors that have not previously been extensively examined may play a role. Drivers of vascular inflammation and damage in SLE include B and T cells, monocytes, proinflammatory cytokines, chemokines, type 1 interferon, platelets, neutrophils, and oxidized low-density lipoprotein.<sup>6</sup> Interestingly, clonal hematopoiesis has been associated with an enhanced interleukin-1 $\beta$  (IL-1 $\beta$ ) expression and atherosclerosis in animal models, and recent studies have shown the contribution of CHIP driver genes DNMT3A, TET2, ASXL1, and JAK2 in murine models of atherosclerosis.<sup>7</sup> Evidence has demonstrated the role of TET2 mutations and IL-1 $\beta$  production in atherosclerosis in vivo, and of JAK2<sup>V617F</sup> mutations in neutrophil extracellular trap formation,<sup>7</sup> a component of innate immunity that has also been linked to atherothrombosis in SLE.<sup>6</sup>

The presence of CHIP in SLE has been investigated by one study,<sup>8</sup> which also examined its association with CVD. The study reported a 10.7% CHIP prevalence in patients with SLE but failed to detect an association with incident CVD events. Notably, the prevalence of CHIP increased with age (1.8% in 20–29-year-old, 6.8% in 30–39-year-old, 11.1% in 40–49-year-old, and 26.3% in 50–59-year-old subgroups). Age is the strongest risk factor for CHIP,<sup>9</sup> as well as for atherosclerotic CVD. CHIP probably represents a novel aging-related CVD risk factor in the general population, but its role in CVD risk in SLE remains uncertain.

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1. Papazoglou N, Sfikakis PP, Tektonidou MG. Atherosclerotic plaque progression and incident cardiovascular events in a 10-year prospective study of patients with systemic lupus erythematosus: the impact of persistent cardiovascular risk factor target attainment and sustained DORIS remission. *Arthritis Rheumatol* 2025;77(6):716–726. <https://doi.org/10.1002/art.43097>
2. Barzilay JL, Farag YMK, Durthaler J. Albuminuria: an underappreciated risk factor for cardiovascular disease. *J Am Heart Assoc* 2024;13(2):e030131.
3. Kapsia E, Marinaki S, Michelakis I, et al. Predictors of early response, flares, and long-term adverse renal outcomes in proliferative lupus nephritis: a 100-month median follow-up of an inception cohort. *J Clin Med* 2022;11(17):5017.
4. Drosos GC, Vedder D, Houben E, et al. EULAR recommendations for cardiovascular risk management in rheumatic and musculoskeletal diseases, including systemic lupus erythematosus and antiphospholipid syndrome. *Ann Rheum Dis* 2022;81(6):768–779.
5. Papazoglou N, Krawvariti E, Konstantonis G, et al. The impact of traditional cardiovascular risk factor control on 7-year follow-up atherosclerosis progression in systemic lupus erythematosus. *Rheumatology (Oxford)* 2024;63(1):50–57.

6. Ambler WG, Kaplan MJ. Vascular damage in systemic lupus erythematosus. *Nat Rev Nephrol* 2024;20(4):251–265.
7. Khetarpal SA, Qamar A, Bick AG, et al. Clonal hematopoiesis of indeterminate potential reshapes age-related CVD: JACC review topic of the week. *J Am Coll Cardiol* 2019;74(4):578–586.
8. David C, Duployez N, Eloy P, et al. Clonal haematopoiesis of indeterminate potential and cardiovascular events in systemic lupus erythematosus (HEMATOPLUS study). *Rheumatology (Oxford)* 2022; 61(11):4355–4363.
9. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 2014; 371(26):2488–2498.

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### Hydroxychloroquine dose after hospitalization for systemic lupus erythematosus: comment on the article by Nestor et al

To the Editor:

Nestor et al<sup>1</sup> found in a case-crossover study that the use of lower doses of hydroxychloroquine in patients with systemic lupus erythematosus (SLE) is associated with a higher likelihood of hospitalizations due to SLE.<sup>1</sup> In the study, they used case periods that ended with hospitalizations (index event) and control periods that did not end with hospitalizations, which were allowed to be before or after the index event. Although the periods before the index event were prioritized for control period selection, it is conceivable that for an SLE-related hospitalization, the patient would more likely be discharged with a similar or higher dose of hydroxychloroquine rather than a lower dose compared to the period immediately before the hospitalization unless the use of the drug is impossible due to compelling reasons such as intolerance or adverse reactions.

Case-crossover design is a very good method to study acute events related to short-term exposures,<sup>2</sup> but the validity of the findings with the use of postindex control periods in a case-crossover study relies on the assumption that the index event does not affect subsequent exposure. Given our above-mentioned concern about the hydroxychloroquine dosing after hospitalization, it is not *prima facie* obvious that this assumption should hold in the study by Nestor et al.<sup>1</sup> It is therefore essential that the authors report how many of these control periods were before and how many were after the index hospitalizations and what the average hydroxychloroquine doses in these periods were. A sensitivity analysis excluding the postindex control periods would alleviate our concerns altogether.

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1. Nestor J, Choi H, Mancini C, et al. Hydroxychloroquine dose and hospitalizations for active lupus. *Arthritis Rheumatol* 2024;76(10): 1512–1517.

2. Lewer D, Petersen I, Maclure M. The case-crossover design for studying sudden events. *BMJ Med* 2022;1(1):e000214.

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Reply

To the Editor:

We appreciate the comments from Sulu and Tascilar in response to our article “Hydroxychloroquine dose and hospitalizations for active lupus.”<sup>1</sup> They raised the issue of whether bias could be introduced into a case-crossover study if an earlier event (case period) influenced the exposure in a subsequent control period. Given this possibility, we had designed our case-crossover study to prioritize the inclusion of control periods (ie, six-month periods in which a patient received hydroxychloroquine [HCQ] with no hospitalization for active systemic lupus erythematosus [SLE]) before case periods (the six-month period before a hospitalization for active SLE). However, it was not standard practice to increase the HCQ dose in response to hospitalizations for active lupus during the study period, January 2011 to December 2021,<sup>2,3</sup> and we did not anticipate a major impact on HCQ dosage following hospitalization events.

As per the letter authors’ suggestions, we have further analyzed the 266 control periods, 77 of which occurred after

a case period (28.9%). We found that the mean (±SD) HCQ dosage was similar in control periods that occurred before case periods (337.8 [±85.2 mg/day]) and after case periods (340.3 [±77.8 mg/day]). Furthermore, we conducted a sensitivity analysis in which we excluded control periods that occurred after case periods, and the results were similar to those of the primary analysis (Table 1). After exclusion of the control periods after hospitalizations, the use of lower weight-based HCQ dose, ≤5 mg/kg/day, was still associated with an increased risk of hospitalizations for active SLE compared with >5 mg/kg/day HCQ dose (adjusted hazard ratio 3.38, 95% confidence interval [CI] 1.01–11.27). For the comparison of non–weight-based HCQ dose, the use of <400 mg/day versus 400 mg/day was notable for an unadjusted odds ratio (OR) of 3.32 (95% CI 1.11–9.97), but the adjusted OR of 2.94 (95% CI 0.93–2.25) was no longer significant (*P* = 0.07). HCQ dose of <300 mg/day remained associated with an increased risk of hospitalizations for active SLE compared with the use of 400 mg/day (adjusted OR 3.54, 95% CI 1.09–11.50). These results are very similar to those we reported in our manuscript.<sup>1</sup> This sensitivity analysis also addresses the possibility that patient adherence to HCQ was better following hospitalization, regardless of dosage. In summary, the inclusion or exclusion of control periods following hospitalization for active lupus did not change our overall conclusion that lower HCQ doses are associated with increased hospitalizations for active lupus, and there remains a need to reevaluate current HCQ dosage guidelines to address the balance of minimizing potential toxicity and maximizing patient benefit.

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**Table 1.** Association between hydroxychloroquine dosage and hospitalizations for active SLE in a patient-crossover study (sensitivity analysis limited to control periods preceding patient periods)\*

| Hydroxychloroquine dose | Hospitalizations/patient periods, n | Control periods, n <sup>a</sup> | Unadjusted OR (95% CI) | Adjusted OR (95% CI) <sup>b</sup> |
|-------------------------|-------------------------------------|---------------------------------|------------------------|-----------------------------------|
| Overall                 | 137                                 | 189                             | –                      | –                                 |
| Weight-based dosage     |                                     |                                 |                        |                                   |
| High dose, >5 mg/kg/d   | 39                                  | 56                              | 1.00 (ref)             | 1.00 (ref)                        |
| Low dose, ≤5 mg/kg/d    | 98                                  | 133                             | 3.72 (1.15–11.99)      | 3.38 (1.01–11.27)                 |
| 4–5 mg/kg/d             | 44                                  | 73                              | 3.33 (0.67–16.42)      | 2.63 (0.52–13.15)                 |
| <4 mg/kg/d              | 54                                  | 60                              | 3.82 (1.15–12.62)      | 3.63 (1.05–12.57)                 |
| Non-weight-based dosage |                                     |                                 |                        |                                   |
| High dose, 400 mg/d     | 64                                  | 111                             | 1.00 (ref)             | 1.00 (ref)                        |
| Low dose, <400 mg/d     | 73                                  | 78                              | 3.32 (1.11–9.97)       | 2.94 (0.93–9.25)                  |
| 300 to <400 mg/d        | 26                                  | 41                              | 1.59 (0.36–6.93)       | 1.30 (0.27–6.14)                  |
| <300 mg/d               | 47                                  | 37                              | 3.88 (1.27–11.92)      | 3.54 (1.09–11.50)                 |

\* CI confidence interval; OR, odds ratio; ref, reference; SLE, systemic lupus erythematosus.

<sup>a</sup> Only control periods that occurred before the patient period were included in this analysis.

<sup>b</sup> Analyses were completed using conditional logistic regression matched within a patient and adjusted for clinical Systemic Lupus Erythematosus Disease Activity Index, glucocorticoid use, SLE immunosuppressant use, and estimated glomerular filtration rate. These variables were assessed at the most recent rheumatology visit before each patient or control period.

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1. Nestor J, Choi H, Mancini C, et al. Hydroxychloroquine dose and hospitalizations for active lupus. *Arthritis Rheumatol* 2024;76(10):1512–1517.
2. Lee SJ, Silverman E, Bargman JM. The role of antimalarial agents in the treatment of SLE and lupus nephritis. *Nat Rev Nephrol* 2011;7(12):718–729.
3. Fanouriakis A, Kostopoulou M, Alunno A, et al. 2019 update of the EULAR recommendations for the management of systemic lupus erythematosus. *Ann Rheum Dis* 2019;78:736–745.